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CAIO MATHEUS SANTOS DA SILVA CALADO

TRATAMENTO NEONATAL COM RESVERATROL EM RATOS SUBMETIDOS À
PARALISIA CEREBRAL: repercussões comportamentais e no hipocampo

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2024

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Dissertação entregue e apresentada ao Programa de Pós-graduação em Neuropsiquiatria e Ciências do Comportamento, do Centro de Ciências Médicas da Universidade Federal de Pernambuco, como requisito para obtenção do título de mestre em Neuropsiquiatria e Ciências do Comportamento.

Área de concentração: Neurociências.

Orientadora: Prof.^a Dr.^a. Ana Elisa Toscano.

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“A coragem nem sempre ruge! Às vezes, coragem é
a pequena voz no final do dia que diz: tentarei de
novo amanhã.”

– Mary Anne Radmacher

RESUMO

A paralisia cerebral é uma condição do neurodesenvolvimento caracterizada por uma lesão prematura no cérebro com repercussões na postura e no movimento. Além dos prejuízos motores, nota-se uma série de alterações inespecíficas que comprometem a memória e aprendizagem. O resveratrol, polifenol, apresenta uma série de propriedades antiinflamatórias e antioxidantes, além de promover um aumento da neuroplasticidade, repercutindo em melhoras em aspectos motores, cognitivos e emocionais em lesões cerebrais. Por essa razão, esse estudo teve como objetivo investigar os efeitos do tratamento neonatal com resveratrol sobre a memória episódica, a neuroinflamação e a proliferação celular no hipocampo de ratos submetidos à PC. O projeto teve seu início após aprovação no comitê de Ética da Universidade Federal de Pernambuco (CEUA- 0082/2022). Foram utilizados 60 filhotes ratos machos Wistar que foram divididos em grupos experimentais de acordo com o modelo da PC e da manipulação farmacológica. O modelo da PC associou à anóxia perinatal (P0-P1) e a restrição sensório-motora das patas posteriores (P2-P28). Os animais foram tratados com resveratrol (10mg/kg, 0,1 ml/100g) ou salina (P3-P21) por via intraperitoneal. Os grupos foram formados aleatoriamente, sendo eles: CS (n=15), CR (n=15), PCS (n=15) e PCR (n=15). Após o término do tratamento, foram avaliados nos testes de reconhecimento do novo objeto (TRO), Labirinto em T, Caixa Claro-Escuro, Labirinto Elevado em Cruz (LEC) e foram eutanasiados no P29 para avaliação da expressão gênica e imuno-histoquímica para células Iba+ e BrdU+ do hipocampo. Em comparação com os animais do grupo de CS, os animais com PCS demonstraram um desempenho inferior nas tarefas de memória, demonstrando uma redução no índice de discriminação no TRO no P22 ($p<0,0001$) e no P27 ($p<0,0001$), e uma redução da alternância no labirinto em T ($p<0,01$). Além disso, os animais apresentaram traços comportamentais semelhantes à ansiedade através de um aumento no tempo de permanência no lado escuro da Caixa Claro Escuro no P22 ($p<0,0001$) e no P29 ($p= 0,0117$), e um aumento no tempo de permanência nos braços fechados do LEC ($P<0,001$) no P28. Apesar disso, o tratamento com o resveratrol demonstrou ser capaz de atenuar esses danos, promovendo no grupo PCR um aumento no do índice de discriminação no TRO no P22 ($p<0,001$) e no P27 ($p<0,0001$), e um aumento da alternância no labirinto em T ($p<0,001$), melhorando a formação e recuperação de memórias episódicas de curto e longo prazo. Em adição a isso, os animais do grupo PCR demonstraram uma melhora no comportamento emocional, através de um aumento no tempo de permanência no lado claro da Caixa Claro-Escuro no P22 ($p<0,0001$) e P29 ($p<0,0001$), como também um aumento no tempo de permanência nos braços abertos do LEC no P28. Os animais do grupo PCR apresentaram uma redução na expressão gênica da IL-6 ($p= 0,0175$) e

TNF- α ($p=0,0007$) e um aumento nos níveis de CREB-1 ($p=0,0020$). Os animais do grupo PCS apresentaram um aumento do número de micróglias ativadas (Iba+) no DG ($p<0,0001$), CA1 ($p<0,0001$) e CA3 ($p<0,0001$) e uma redução da proliferação celular (BrdU+) no DG ($p<0,0001$), CA1 ($p<0,0001$) e CA3 ($p<0,0001$), enquanto os animais PCR apresentaram uma redução na porcentagem de micróglias ativadas no DG ($p<0,0001$), CA1 ($p=0,0014$) e CA3 ($p<0,0001$) e um aumento na proliferação celular no CA1 ($p=0,0003$) e CA3 ($p=0,0008$). Esses resultados demonstram efeitos promissores do resveratrol na paralisia cerebral reduzindo prejuízos na formação de memórias episódicas de reconhecimento de objetos e localização espacial e as alterações emocionais através da redução da neuroinflamação e do aumento de respostas neuroplásticas através do aumento de CREB-1 e da proliferação celular no hipocampo.

Palavras-chaves: Ansiedade; aprendizagem; lesão cerebral; memória episódica; pericrítico; polifenóis; resveratrol.

ABSTRACT

Cerebral palsy is a neurodevelopmental condition characterized by premature damage to the brain with repercussions on posture and movement. In addition to motor impairments, a series of non-specific alterations compromise memory and learning. Resveratrol, a polyphenol, has anti-inflammatory and antioxidant properties, promoting an increase in neuroplasticity, resulting in improvements in motor, cognitive and emotional aspects in brain injuries. For this reason, this study aimed to investigate the effects of neonatal treatment with resveratrol on episodic memory, neuroinflammation and cell proliferation in the hippocampus of rats subjected to CP. The project began after approval by the Ethics Committee of the Federal University of Pernambuco (CEUA- 0082/2022). A total of 60 male Wistar rats pups were divided into experimental groups according to the CP model and pharmacological manipulation. The CP model was associated with perinatal anoxia (P0-P1) and sensory-motor restriction of the hind limbs (P2-P28). Animals were treated with resveratrol (10mg/kg, 0.1 ml/100g) or saline (P3-P21). The groups were randomized as follows: CS (n=15), CR (n=15), CPS (n=15) and CPR (n=15). After completion of treatment, the animals were evaluated in the novel object recognition (NORT), T-Maze, Light-Dark Box (LDB), Elevated Plus Maze (EPM) tests and were euthanized at P29 for evaluation of gene expression and immunohistochemistry for Iba+ and BrdU+ cells of the hippocampus. Compared to animals in the CS group, CPS animals showed a lower performance in memory tasks, demonstrating a reduction in the discrimination index in the NORT at P22 ($p<0.0001$) and P27 ($p<0.0001$), and a reduction of alternation in the T-maze ($p<0.01$). In addition, the animals showed anxiety-like behavior traits through an increase in time spent on the dark side of the DLB on P22 ($p<0.0001$) and P29 ($p=0.0117$) and an increase in time spent in the closed arms of the EPM on P28 ($p<0.001$). Despite this, resveratrol treatment was demonstrated to be able to attenuate these damages, promoting in the CPR group an increase in the discrimination index in the NORT at P22 ($p<0.001$) and P27 ($p<0.0001$), and an increase in alternation in the T-maze ($p<0.001$), improving the formation and retrieval of short- and long-term episodic memories. Additionally, animals in the CPR group showed an improvement in emotional behavior, through an increase in time spent on the light side of the LDB on P22 ($p<0.0001$) and P29 ($p<0.0001$), as well as an increase in time spent in the open arms of the EPM on P28. Animals in the CPR group showed a reduction in IL-6 ($p=0.0175$) and TNF- α ($p=0.0007$) gene expression and an increase in CREB-1 levels ($p=0.0020$). Animals in the CPS group showed an increase in the number of activated microglia (Iba+) in DG ($p<0.0001$), CA1 ($p<0.0001$) and CA3 ($p<0.0001$) and a reduction in cell

proliferation (BrdU+) in DG ($p < 0.0001$), CA1 ($p < 0.0001$) and CA3 ($p < 0.0001$), while CPR animals showed a reduction in the percentage of activated microglia in DG ($p < 0.0001$), CA1 ($p = 0.0014$) and CA3 ($p < 0.0001$) and an increase in cell proliferation in CA1 ($p = 0.0003$) and CA3 ($p = 0.0008$). These results demonstrate promising effects of resveratrol in cerebral palsy by reducing impairments in the formation of episodic memories of object recognition and spatial localization and emotional disturbances through reduced neuroinflammation and increased neuroplastic responses through increased CREB-1 and cell proliferation in the hippocampus.

Keywords: Anxiety; brain injury; critical period; behavior; episodic memory; learning; polyphenols; resveratrol.

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LISTA DE ABREVIATURAS

BDNF- Fator Neurotrófico Derivado do Cérebro (*Brain derived neurotrophic factor*).

BrdU- Bromodeoxiuridina.

CA1- Cornu Ammonis 1 do hipocampo.

CA2- Cornu Ammonis 2 do hipocampo.

CA3- Cornu Ammonis 3 do hipocampo.

CB1- receptores astrocitários no hipocampo.

ChAT- Choline acetyltransferase gene.

CR- Controle resveratrol.

CREB- *cAMP-responsive element binding protein*.

CS- Controle salina.

DG- Giro denteado do hipocampo.

IL-1 β - Interleucina-1 beta.

IL-6- Interleucina 6.

I.P- intraperitoneal.

LEC- Labirinto Elevado em Cruz.

LTP- Potenciação de longo prazo.

NORT- Teste do Reconhecimento do novo objeto (*Novel recognition object test*).

P- Dia de vida pós natal.

PC- Paralisia cerebral.

PCR- Paralisia cerebral submetida ao tratamento com resveratrol.

PCS- Paralisia cerebral submetida ao tratamento com salina.

PI3K-Akt- Fosfatidilinositol 3-quinase/Akt.

Resv- Resveratrol.

RSM- Restrição sensório motora.

SIRT1- Sirtuina 1.

SN- Sistema nervoso.

SNC- Sistema nervoso central.

SNP- Sistema nervoso periférico.

TNF- α - Fator de necrose tumoral-alfa.

TRO- Teste de reconhecimento do novo objeto.

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1. INTRODUÇÃO

Durante os primeiros anos de vida de um organismo, ainda sob a proteção materna, seja durante a fase pregressa ao nascimento, denominada gestação, ou na fase posterior ao nascimento, denominada lactação, ocorrem processos metabólicos, fisiológicos cruciais e únicos que, por sua vez, fazem com que esse período temporal -gestação e lactação- seja crítico para o estabelecimento de diversas funções dos seres vivos, sobretudo nos Mamíferos (Rice; Barone, 2000; Suzuki, 2018; West-Eberhard, 2005). Apesar de diversas estruturas surgirem ainda durante a vida intrauterina, o sistema nervoso dos mamíferos possui componentes que são desenvolvidos após o nascimento (Rice; Barone, 2000). À vista disso, os seres vivos são seres gregários e dependem do ambiente, em suas mais diversas facetas e conceitos para seu desenvolvimento, e é através do processo de interação que diversos fenômenos e processos são desenvolvidos e aperfeiçoados, inclusive os de aprendizagem e sociabilidade (De Felice et al., 2023; Ilyka; Johnson; Lloyd-Fox, 2021).

Por essa razão, sabe-se que as experiências ambientais durante o período perinatal, uma fase composta pela gestação e lactação, impactam diretamente no curso do desenvolvimento de um organismo, podendo trazer consequências fenotípicas precoces e/ou tardias (Margolis; Gabard-Durnam, 2024). Por definição, o fenótipo de um indivíduo é o resultado da interação entre o genótipo, a herança genética de um organismo, e o ambiente, todos os estímulos físicos e sociais que interagem com o organismo (Overskeid, 2018). Tais modificações fenotípicas têm como base processos epigenéticos que são essenciais para a seleção natural e evolução das espécies (Skinner, 2015). As modificações epigenéticas envolve processos como a metilação do DNA, modificação de histonas e a regulação de RNAs (Bianco-Miotto et al., 2017). As alterações epigenéticas podem modificar as funções cerebrais (Ochi; Dwivedi, 2023) que, por consequência, pode impactar nos processos de aprendizagem (Macbeth et al., 2023) e no surgimento de determinadas condições (Suzuki, 2018).

Nessa perspectiva, as variações ambientais na vida precoce podem levar às mudanças fenotípicas com repercussões permanentes no metabolismo, estrutura e função dos sistemas fisiológicos (Hales; Barker, 1992), isso porque o período perinatal é uma fase extremamente sensível e responsiva, modificando sua forma e função de acordo com o tipo de estimulação ao qual o organismo é exposto (Gluckman; Hanson; Beedle, 2007; Ismail; Fatemi; Johnston, 2017). A exposição a condições ambientais inadequadas e desafiadoras para o desenvolvimento infantil podem levar ao surgimento de determinadas condições clínicas, como a paralisia

cerebral (PC), visto que as taxas são mais altas em países de baixa renda e em situações de subdesenvolvimento (Mcintyre et al., 2022). A PC é uma condição em que o neurodesenvolvimento é afetado por lesão cerebral precoce e não progressiva (Fragopoulou et al., 2019) com repercussões na postura e no movimento (Graham et al., 2016). Dentre os eventos ambientais experienciados de forma precoce, relacionam-se com a patogênese da PC: o parto prematuro, infecções, inflamação e hipóxia-isquemia durante a gravidez ou logo após o nascimento (MCINTYRE et al., 2013). A PC é considerada uma das principais causas de deficiência física e intelectual na infância, garantindo-a o título de uma mais desafiadora desordem neurológica surgida na infância (Korzeniewski et al., 2018).

Apesar de existir um componente genético presente na patogênese da PC (Pham et al., 2020), o modelo de compreensão multifatorial é mais plausível devido à ausência de padrões claros de herança monogênica em sua etiologia (FAHEY et al., 2017). Posto isso, sabe-se que o surgimento da PC envolve interação entre fatores ambientais e genéticos (TOLLANES et al., 2014). Apesar de existir uma complexidade de componentes na sua patogênese, dados recentes apontam que a injúria precoce promove um desequilíbrio químico nos níveis antioxidantes e pró oxidantes em regiões corticais (Souza et al., 2023a; Visco et al., 2022b) que, por sua vez, pode levar a uma cascata de reações aumentando os níveis de citocinas pró inflamatórias (Gouveia et al., 2023) associadas a um aumento da ativação das micróglias e uma redução da proliferação celular em regiões como o hipocampo, cerebelo e hipotálamo (Calado et al., 2023b; Costa-de-Santana et al., 2023; Pereira et al., 2024). Essa cascata de reações contribui para prejuízos comportamentais, afetando negativamente a memória episódica, o comportamento locomotor e alimentar dos animais (Calado et al., 2023b; Costa-de-Santana et al., 2023; Pereira et al., 2024), de forma similar aos prejuízos clínicos observados em crianças com PC (De Freitas Feldberg et al., 2021; Van Rooijen; Verhoeven; Steenbergen, 2016).

Embora haja um comprometimento severo das habilidades motoras (Halma et al., 2020), devido à danos em componentes do sistema musculoesquelético, como a atrofia muscular, alterações no padrão da marcha e uma redução da locomoção e da coordenação motora (Da Silva Souza et al., 2023b; Pereira et al., 2021, 2024), nota-se também uma alta prevalência de problemas cognitivos e comportamentais que interferem na aprendizagem, sendo observados em aproximadamente 40% das crianças com PC (Himmelmann et al., 2006; Van Rooijen; Verhoeven; Steenbergen, 2016). Os prejuízos na formação de novas memórias e o comprometimento dos movimentos motores finos estão relacionados com o baixo desempenho escolar dessas crianças (Van Rooijen; Verhoeven; Steenbergen, 2016). Assim. O

comprometimento intelectual está presente em vários casos de crianças diagnosticadas com PC, manifestando-se de diferentes formas, como atrasos nas habilidades escolares através de prejuízos nos sistemas executivos (DE FREITAS FELDBERG et al., 2021; PEREIRA et al., 2018).

Estudos pré-clínicos sugerem que a injúria precoce é capaz de prejudicar a aprendizagem através da redução da consolidação, armazenamento e evocação de memórias, como também através do aumento de comportamentos similares à ansiedade em condições similares à PC (Granja et al., 2021; Soares et al., 2013a). Em modelos experimentais de PC, foi constatado que o hipocampo é uma região cerebral bastante afetado, observando um aumento do estresse oxidativo e da neuroinflamação (Calado et al., 2023a; Gouveia et al., 2023; Visco et al., 2021). No entanto, algumas regiões do hipocampo parecem ser mais afetadas do que outras, achados elucidam que *cornu ammonis* 1 (CA1) e 3 (CA3) e giro denteado (DG) são mais danificados pela privação de oxigênio, ecoando em prejuízos comportamentais significativos (Matsuda et al., 2021; Takada et al., 2016). Assim, a microarquitetura do hipocampo é prejudicada de modo geral após a injúria cerebral.

Frente a esses desafios, diversas intervenções são estudadas com o propósito de reduzir as sequelas e promover uma melhor qualidade de vida. Dentre essas intervenções, os polifenóis ocupam um lugar de destaque devido aos seus efeitos promissores em diversas doenças neurológicas e metabólicas (Cory et al., 2018; Manach et al., 2004). Os polifenóis são compostos encontrados em plantas e alimentos (CORY et al., 2018; MANACH et al., 2004). Dentre os polifenóis, destaca-se o resveratrol, um composto do grupo estilbenóides que pode ser encontrado em uvas, amoras, vinho tinto e que possui capacidades anti-inflamatórias, antioxidantes e neuroprotetora (Rebas et al., 2020). Por esse motivo, apresenta-se como um forte agente neuroprotetor e modulador de processos comportamentais como a aprendizagem e o humor. Todavia, muito ainda permanece obscuro sobre a atuação do resveratrol na memória e aprendizagem, mas sabe-se que ele pode reduzir efeitos deletérios no hipocampo e, por consequência, promove efeitos benéficos na aprendizagem (Khanna et al., 2020). Com base nesses postulados, levanta-se a hipótese de que a memória episódica e o comportamento emocional são prejudicados no modelo experimental da PC e o tratamento no período neonatal com o resveratrol é capaz de atenuar esses danos através da redução da neuroinflamação e aumento da proliferação celular no hipocampo.

2. REVISÃO DA LITERATURA

2.1 PLASTICIDADE FENOTÍPICA E DO DESENVOLVIMENTO

O fenótipo de um indivíduo representa a manifestação resultante da interação entre seus genes e o ambiente circundante (West-Eberhard, 2005). Nos mamíferos, o estabelecimento do fenótipo é particularmente sensível a determinados estímulos ambientais durante períodos específicos do desenvolvimento, como a fase perinatal (Barker et al., 1990). Nessa perspectiva, ainda sob a proteção materna, seja durante a vida intrauterina ou durante a lactação, ocorrem fenômenos e processos únicos para o estabelecimento do fenótipo em suas diversas facetas (Rice; Barone, 2000; Suzuki, 2018; West-Eberhard, 2005). Essa sensibilidade e responsividade ao ambiente faz com os primeiros anos de vida seja considerado uma janela crítica para o desenvolvimento de diversas funções (Knight; Sorensen, 2001; Rice; Barone, 2000). Por essa razão, alterações ambientais desafiadoras, como a má nutrição ou exposição a insultos cerebrais, podem eliciar alterações metabólicas, estruturais e funcionais que podem favorecer a gênese de determinadas doenças ou condições desafiadoras na infância ou até em outros estágios da vida (Lacerda et al., 2017; Silva et al., 2016; Suzuki, 2018; Toscano et al., 2008).

Sob esse ponto de vista, os estímulos ambientais experienciados de forma precoce podem induzir respostas fenotípicas duradouras, ecoando em modificações no metabolismo, morfologia e função do organismo (Hales; Barker, 1992). Essa sensibilidade e responsividade pode ser compreendida através do fenômeno da plasticidade do desenvolvimento e plasticidade fenotípica (Bateson et al., 2004; Hanson; Gluckman, 2014), na qual refere-se a capacidade do organismo de responder ao ambiente, de forma adaptativa ou não, através de modificações em sua forma, estado ou padrão de funcionamento (Ghalambor et al., 2007; Gluckman; Hanson; Buklijas, 2010) (figura 1).

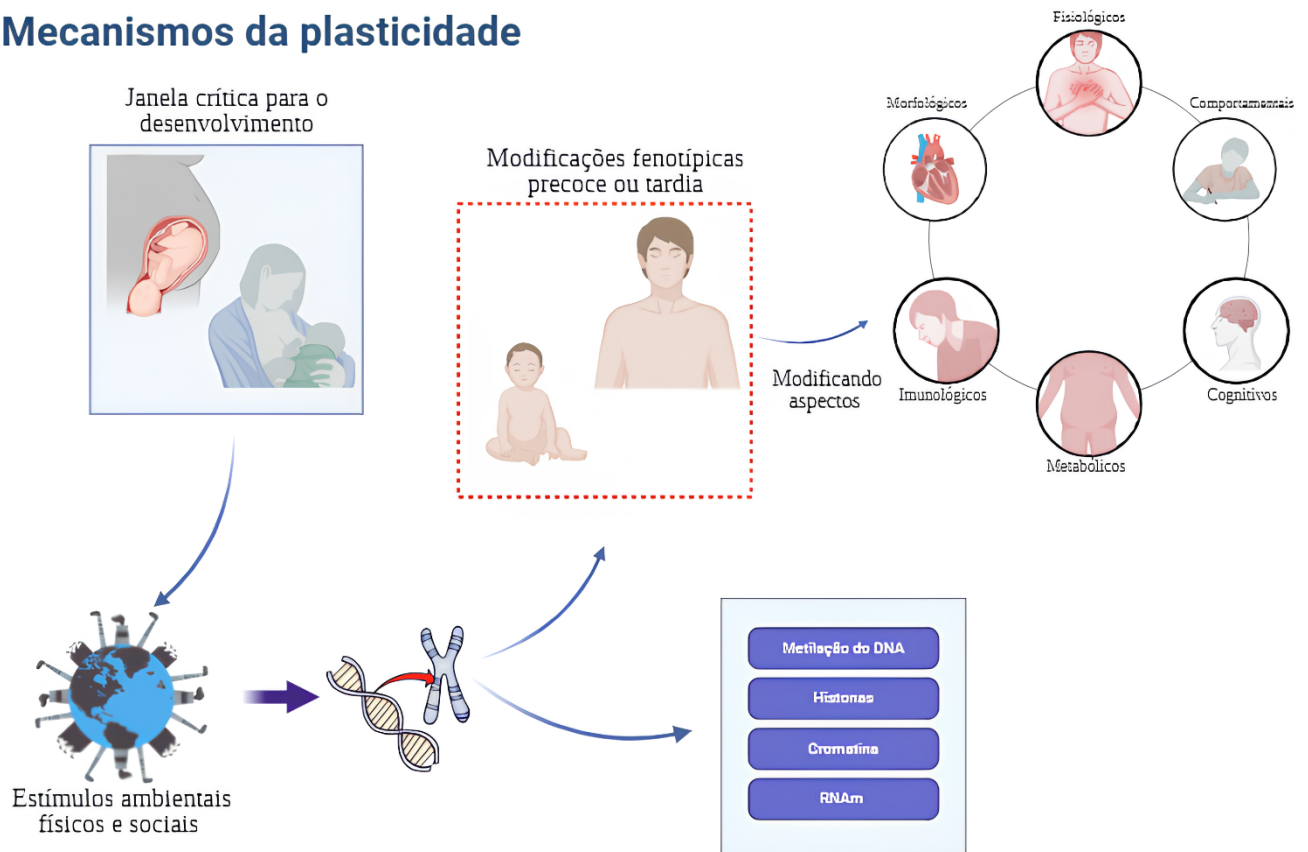
Em termos conceituais, a plasticidade fenotípica é um fenômeno caracterizado como a capacidade de um organismo emitir diferentes fenótipos, como respostas adaptativas ou não, quando exposto a diferentes estímulos ambientais (Kelly; Panhuis; Stoeckl, 2012), sendo a epigenética a base para a compreensão de tais modificações. Essas respostas desempenham também um papel crucial na seleção e evolução das espécies (Skinner, 2015). Inicialmente, acreditava-se que a base molecular para as variações fenotípicas estava nos processos de mutações nas estruturas do ácido desoxirribonucleico (DNA) (LALAND et al., 2014), mas, Lamarck propôs alterações nessa compreensão, advogando em prol de que o ambiente promove alterações fenotípicas atreladas ao processo evolutivo (Skinner, 2015). Assim, os mecanismos epigenéticos são atuantes no processo de variação fenotípica, através de fenômenos como a

metilação do próprio DNA, modificações das histonas, alteração na estrutura da cromatina e no RNA não codificante seletivo (ncRNA) (Skinner, 2014a, 2014b). Tais modificações podem tornar-se programadas e serem herdadas ao longo das gerações, promovendo a herança epigenética transgeracional da variação fenotípica (Skinner, 2014a, 2014b), evidencial assim o papel da plasticidade na sobrevivência, seleção e evolução das espécies (Fusco; Minelli, 2010; West-Eberhard, 2005).

O papel da plasticidade na evolução foi fortalecido através da elucidação de três mecanismos ao longo dos estudos no campo da plasticidade fenotípica (Sommer, 2020). O primeiro, diz que as novas características eliciadas têm sua gênese iniciada a partir da propriedade responsiva do organismo ao ambiente (Levis; Pfennig; Levis, 2019). Assim, a mudança ocorre através da capacidade plástica preexistente no organismo e, posteriormente, é modulada e lapidada pelo processo de seleção para transformação em um fenótipo adaptativo (Levis; Pfennig, 2019). O segundo mecanismo diz que essas respostas fenotípicas requerem a reprogramação das estruturas gênicas, na qual repercute em alterações metabólicas, estruturais e funcionais no organismo (Sommer, 2020). Essa reprogramação ocorre a partir de mudanças na expressão de determinados genes (Sommer, 2020). O último mecanismo diz que a plasticidade, e as respostas produzidas e emitidas através dela, tornam-se geneticamente codificadas (Sommer, 2020). Devido a essa codificação, essas modificações podem ser transmitidas e herdadas para as gerações futuras (Kelly et al., 2019).

Figura 1: Esquematização dos mecanismos da plasticidade do desenvolvimento. O período perinatal representa uma janela crítica para o desenvolvimento, no qual promove alterações fenotípicas no organismo através de processos epigenéticos.

Mecanismos da plasticidade



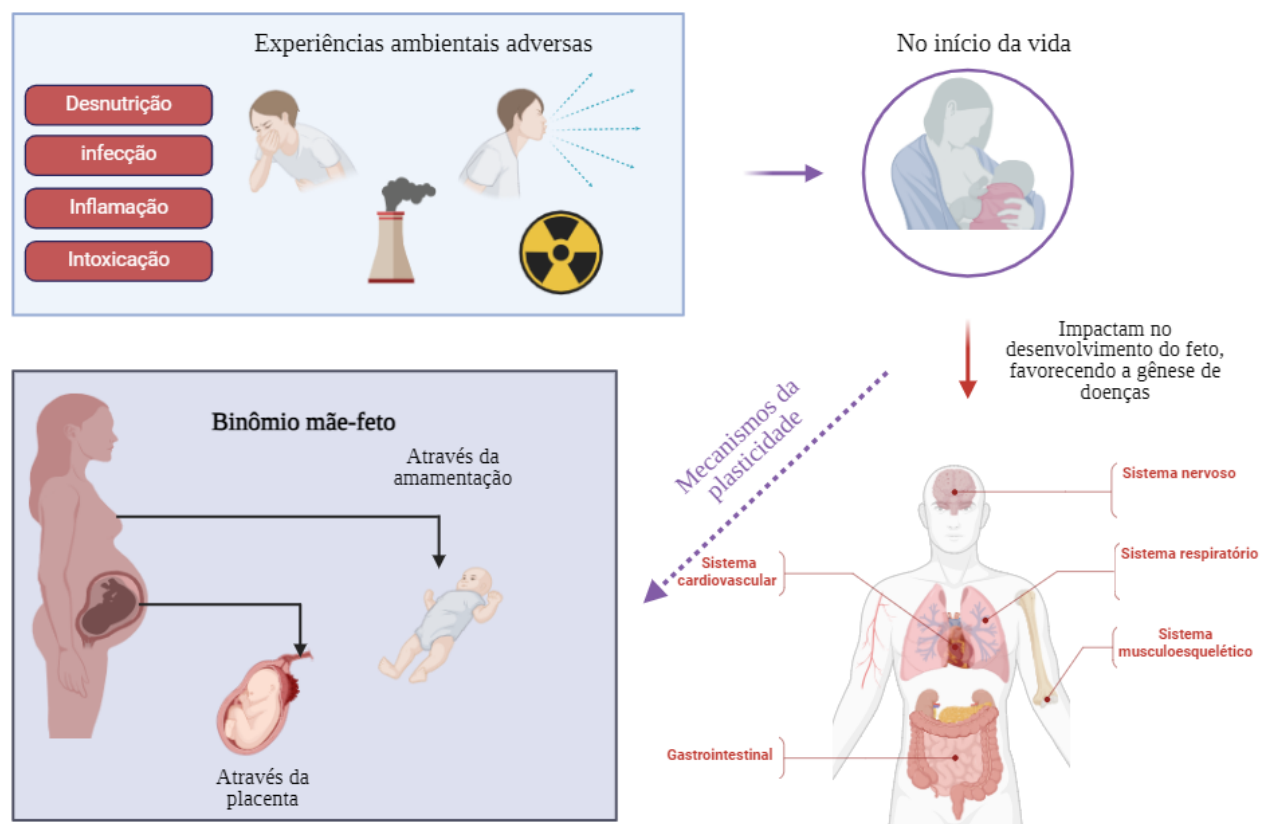
Fonte: Autor (2024).

Todavia, o fenômeno da plasticidade fenotípica, e do surgimento de novos fenótipos, depende da capacidade plástica do organismo, denominada como a plasticidade do desenvolvimento (Sommer, 2020). A plasticidade do desenvolvimento refere-se à capacidade responsiva e adaptativa do organismo que ocorre a partir da interação entre o genótipo e o ambiente, na qual ecoa em mudanças fenotípicas (West-Eberhard, 2005). Assim, as espécies apresentam variações no seu desenvolvimento a partir de processos genéticos e epigenéticos (West-Eberhard, 2005). Norteados por esses postulados, emerge uma área de pesquisa intitulada *Developmental Origins of Health and Disease* (DOHaD) tendo como objetivo a investigação de como os estímulos ambientais no início da vida afetam o organismo, favorecendo o desenvolvimento de doenças que podem repercutir na infância ou em outras fases da vida (Holliday, 1994; Suzuki, 2018) (figura 2).

Os estímulos no início da vida apresentam-se como importantes instrumentos eliciadores de alterações fenotípicas, por ser um período crucial no desenvolvimento e crescimento nos mamíferos (Gluckman; Hanson; Beedle, 2007). Essas alterações no fenótipo podem envolver uma sequência de alterações metabólicas e fisiológicas que podem favorecer o desenvolvimento de doenças (Knight; Sorensen, 2001), podendo causar consequências permanentes no metabolismo, estrutura e função do organismo (Hales, 1997). Estudos com

roedores apontam que alterações no período perinatal podem promover mudanças permanentes no sistema cardiovascular, na qual observa-se uma redução da área e perímetro de células cardíacas (Toscano et al., 2008) e no sistema musculoesquelético, reduzindo a quantidade de fibras musculares (Pereira et al., 2021; Toscano; Manhães-de-Castro; Canon, 2008). Além disso, a exposição de determinados estímulos ambientais durante a gestação pode repercutir em neurodesenvolvimento atípico da prole, na qual ecoa em alterações comportamentais na vida adulta (Vuong et al., 2020).

Figura 2: Esquemática de como as experiências ambientais maternas impactam no desenvolvimento do feto, através da placenta, durante a gestação, ou do aleitamento, durante a amamentação, favorecendo o surgimento de determinadas condições ou doenças em outras fases do desenvolvimento.



Fonte: Autor (2024).

Sob a perspectiva da plasticidade, o organismo pode ser modificado de acordo com a exposição ambiental (Sommer, 2020). No entanto, apesar do organismo emitir respostas que favoreçam a sua adaptação ao ambiente, como visto no processo de seleção das espécies (West-Eberhard, 2005), também existem respostas não adaptativas ou má adaptativas (Ghalambor et al., 2007). Modelos experimentais da PC elucidam mecanismos subjacentes à plasticidade neural desadaptativa, como alterações na representação dos membros inferiores no córtex somatossensorial promovendo anormalidades nos comandos efetores do movimento (Coq et al., 2020, 2016; Delcour et al., 2018). Esses resultados sinalizam o poder significativo que o

ambiente exerce no organismo, podendo alterar o seu processamento e funcionamento (Canu et al., 2012), promovendo uma resposta mal adaptativa.

A cognição também pode ser compreendida por essa mesma lente, na qual estudos apontam que insultos, como hipóxia-isquemia, inflamação e quadro de desnutrição, no período perinatal promovem desordens na memória que podem perdurar na vida adulta (De Souza; Fernandes; Tavares do Carmo, 2011; Fesser et al., 2021; Potter; Rosenkrantz; Fitch, 2018; Presti et al., 2006). Adicionalmente, achados indicam que a lesão cerebral no período crítico promove prejuízos na memória episódica a partir da manifestação de prejuízos visuoespaciais estando correlacionado com o baixo peso dos animais (Delcour et al., 2012). Em contrapartida, o período perinatal também representa uma janela promissora para o tratamento de determinadas sequelas e condições, e a utilização de compostos naturais têm demonstrado como fortes ferramentas terapêuticas (Pontes et al., 2023). Isso porque, ainda sob o amparo materno, pré-natal e pós-natal, que ocorrem processos plásticos, garantido ao organismo a capacidade de moldar-se de acordo com a exposição ambiental, modificando aspectos metabólicos, morfológicos e funcionais (Ismail; Fatemi; Johnston, 2017; Patton; Blundon; Zakharenko, 2019). Essas modificações, por sua vez, ecoam em mudanças no funcionamento de processos cognitivos e comportamentais (Ismail; Fatemi; Johnston, 2017; Patton; Blundon; Zakharenko, 2019). Assim, o período perinatal não é somente sensível ao ambiente no surgimento e agravamento de doenças, mas também no tratamento através dos mecanismos de adaptação da plasticidade (Fusco; Minelli, 2010; Kelly; Panhuis; Stoeck, 2012; Nemoto; Sagawa, 2024). Por essa razão, intervenções no período crítico tem demonstrado resultados promissores no tratamento das sequelas presentes em condições similares a PC (Pontes et al., 2023), e é partindo dessas premissas que o presente trabalho se guia.

2.2 MEMÓRIA

2.2.1 A MEMÓRIA ENQUANTO COMPORTAMENTO

O funcionamento da memória vem sendo estudado ao longo dos anos com o objetivo de compreender as razões dos seres vivos conseguirem recordar de determinadas informações, acontecimentos e eventos, enquanto outras informações são simplesmente esquecidas (Yonelinas et al., 2019). Com os avanços no campo científico, entende-se atualmente que a cognição possui mecanismos específicos a depender da habilidade cognitiva (Posner; Digirolamo, 2000), afastando o estudo da cognição de uma perspectiva mentalista que predominava anteriormente. Apesar da complexidade de identificar correlatos neurais envolvidos em determinados processos cognitivos, os mecanismos de funcionamento da memória foram bem elucidados com os clássicos experimentos do Kandel (Kandel, 2001a).

Apesar dos processos de aprendizagem e memória ocorrerem através da interação entre o organismo e o ambiente, sabe-se que a aquisição, consolidação e evocação de informações aprendidas promove uma cascata de alterações no organismo, tal qual a síntese de novas proteínas, transcrição gênica, crescimento e nascimento de neurônios e a formação de conexões e vias sinápticas que vão atuar nos processos envolvidos na aprendizagem (Kandel, 2001a). Ademais, na mesma perspectiva, também ocorrem modificações sinápticas e morfológicas no processo do esquecimento (Wang et al., 2020).

Os estudos no campo da memória tornam-se imprescindíveis porque a formação e evocação de memórias não só estão intimamente ligadas à sobrevivência das espécies, mas também desempenham um papel crucial na construção da singularidade de um organismo e na definição de uma identidade coletiva. (Tomaz, 1993). A memória é o instrumento que possibilita a formação de identidade e, por consequência, é o que torna cada organismo único e singular (Tomaz, 1993). Um dos aspectos mais característicos da memória é que são constantemente criadas memórias (Bekinschtein et al., 2008). Os mamíferos são seres extremamente sensíveis e vulneráveis ao ambiente em que está/foi exposto, possuindo assim a capacidade de coletar novas informações e armazená-las para produzir mudanças comportamentais (Bekinschtein et al., 2008). Por essa razão, a memória está intimamente ligada à aprendizagem, sendo possível somente distinguir esses processos em termos conceituais.

A aprendizagem pode ser conceituada enquanto o processo pelo qual um organismo adquire informações (Bekinschtein et al., 2008), enquanto a memória pode ser definida como a capacidade do organismo de consolidar e evocar as informações aprendidas (Bekinschtein et al., 2008). Em virtude disso, compreende-se que a memória e aprendizagem são dois processos únicos que operam de forma entrelaçada. A memória e aprendizagem serem processos que ocorrem na interface entre o organismo e o ambiente (figura 3), ou seja, são comportamentos que dependem das contingências impostas pelo ambiente, físico ou social, para que sejam instaurados, mantidos e ampliados (Branch, 1977; Delaney; Austin, 1998).

Devido à complexidade envolvida nos processos de aprendizagem, a memória envolve uma série de comportamentos que podem ser classificados em três tipos principais: i) a codificação; ii) o armazenamento e iii) a evocação das informações (Izquierdo et al., 2013; Xue, 2018). A primeira classe de comportamentos, conhecida como codificação, refere-se à percepção do estímulo sensorial e ao processo pelo qual o organismo transforma essas informações em um formato compreensível (Campos; Barroso; De Lara Menezes, 2010). O armazenamento é definido como a capacidade na qual a informação do estímulo é pareada e associada a outras informações instauradas e armazenadas pelo organismo (Campos; Barroso;

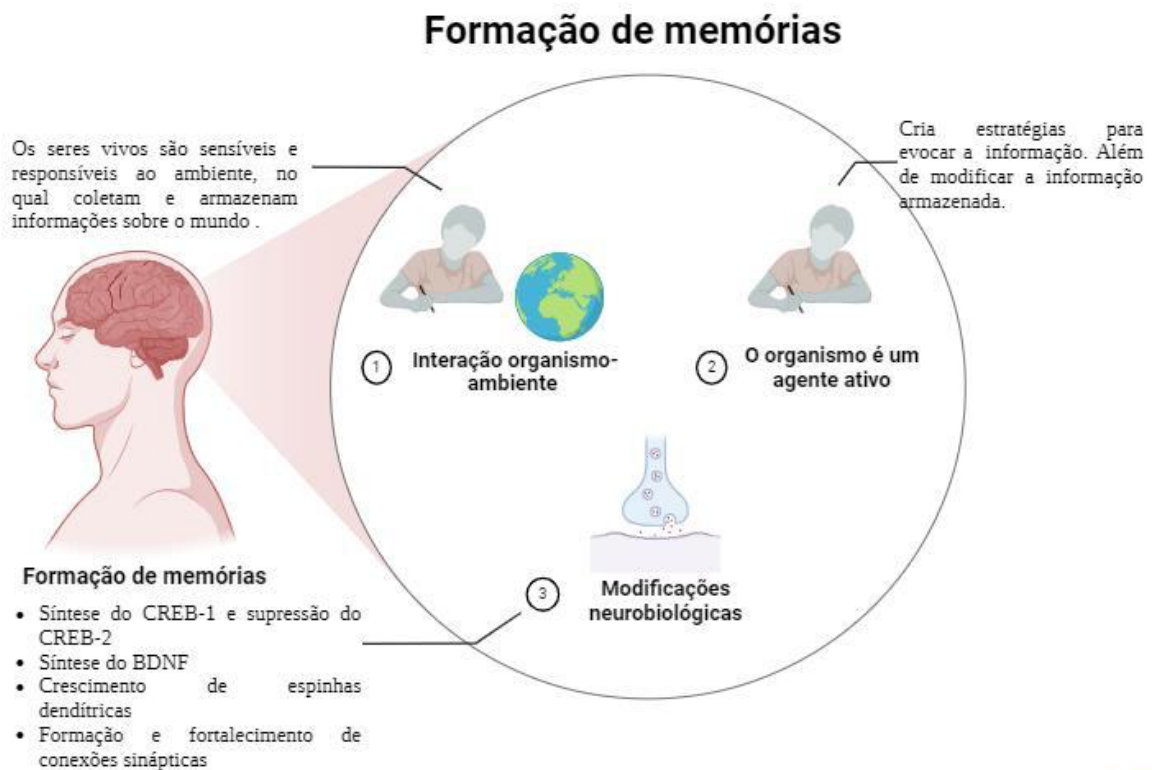
de Lara Menezes, 2010). Em outros termos, é o processo em que o organismo retém a informação sensorial que foi codificada (Sternberg, 2008).

A última classe, chamada de evocação ou recuperação, caracteriza-se pelo processo em que o organismo acessa e recupera as informações previamente codificadas e armazenadas, permitindo sua lembrança ou reconhecimento (Campos; Barroso; De Lara Menezes, 2010). Apesar de parecer um processo linear, os processos de codificação, armazenamento e recuperação podem interagir entre si e um alterar o outro, como também o processo de memorização nem sempre corresponde à realidade física (Sternberg, 2008). Tal fenômeno ocorre porque o organismo seleciona e interpreta a informação tanto no momento de codificar e armazenar, como também no momento de recuperar a informação (Sternberg, 2008). Dessa maneira, as memórias, em grande parte, não correspondem de forma idêntica a informação sensorial que foi captada.

Em uma perspectiva behaviorista, a memória está atrelada a história de aprendizagem do organismo, na qual a evocação da informação está sob controle de determinados estímulos ambientais, bem como as habilidades de armazenar e evocar a informação são mantidas pelas consequências que produzem (Branch, 1977; Delaney; Austin, 1998). Compreende-se assim que a memória é um comportamento que envolve uma série de processos e estratégias para a aquisição e evocação da informação (Palmer, 1989; Shillingsburg et al., 2019). Dessa forma, o organismo é ativo na formação de memórias, através da elaboração de estratégias distintas para o comportamento de lembrar, e tais estratégias constituem um aspecto essencial da memória (Delaney; Austin, 1998)

De acordo das contingências em que a evocação a informação ocorra, pode fazer com que a memória seja classificada com modalidades específicas do comportamento, como um comportamento verbal ou não verbal (Palmer, 1989; Shillingsburg et al., 2019). Dessa maneira, pode ser configurada enquanto um comportamento verbal, em um contexto em que a evocação da informação resulta em um processo de interação com um ouvinte que, por sua vez, produz consequências (Palmer, 1989; Shillingsburg et al., 2019). Comportamentos privados, como de descrever sentimentos e sensações, ou de relatar eventos passados (Shillingsburg et al., 2019) são exemplos de comportamentos que são atrelados à memória e podem ser classificados como comportamentos verbais. Por outro lado, em contextos em que o próprio comportamento de evocar a informação produz consequências reforçadoras, tal comportamento será considerado um comportamento não verbal (Delaney; Austin, 1998; Palmer, 1989).

Figura 3: Esquematização de como ocorrem as memórias. Primeiro, elas são criadas e mantidas através das contingências ambientais, no qual o organismo é ativo no processo de codificação, armazenamento e evocação. Por fim, a formação de memórias envolve mudanças estruturais no organismo.



Fonte: Autor (2024).

2.2.2 TIPOS, CLASSIFICAÇÃO E ESTÁGIOS DA MEMÓRIA

A memória representa uma série de comportamentos distintos, no qual ela pode ser classificada em diferentes tipos de acordo com o tempo de duração ou com o conteúdo da informação armazenada e evocada (Izquierdo et al., 2013). Um dos critérios mais utilizados é o tempo de retenção da informação, no qual possibilita a classificação da memória em dois subgrupos conhecidos como memória de curto prazo e longo prazo (Campos; Barroso; De Lara Menezes, 2010; Rosat, 2006). Apesar de possuírem características próprias, o processamento desses dois tipos de memória ocorre em paralelo e com estruturas neurais similares (Izquierdo et al., 2013). A memória de curto prazo pode ser definida como uma memória que a sua principal característica é que a informação fica retida por um curto período temporal variando de minutos até poucos segundos (Campos; Barroso; De Lara Menezes, 2010). Enquanto a memória de longo prazo pode ser definida como um subtipo de memória com características mais duradouras, na qual a informação é retida desde horas, anos, ou até mesmo durante a vida toda (Campos; Barroso; De Lara Menezes, 2010; Izquierdo et al., 2013). As memórias de curto prazo também podem ser convertidas em memórias de longo prazo a partir de um sistema molecular que envolve a participação de diferentes vias metabólicas, podendo observar a participação do *AMPC response element-binding protein* (CREB) e do Fator Neurotrófico derivado do cérebro (BDNF) (Bekinschtein et al., 2007, 2008).

Por outro lado, utilizando o mesmo parâmetro de tempo de duração da informação, também existe um outro tipo mais recente de memória com um funcionamento específico, conhecida como memória de trabalho, que faz parte de um sistema cognitivo, conhecido como sistema executivo (Campos; Barroso; De Lara Menezes, 2010). Sendo um tipo de memória transitória, semelhante à de curto prazo, na qual retém a informação em intervalos de tempo variáveis dependendo da relevância e utilidade da informação (Campos; Barroso; De Lara Menezes, 2010). Esse tipo de memória atua retendo temporariamente a informação para a execução de algum comportamento mais complexo, tal qual a compreensão da linguagem, resolução de problemas e tomada de decisão (Baddeley., 2012; Cowan, 2010).

A memória não é um processo único com apenas uma função, sabendo-se que os organismos, sejam eles humanos, primatas, roedores e até mesmo insetos, possuem diferentes tipos de memórias com funções distintas (Henke, 2010). Por essa razão, as memórias de longo prazo podem ser divididas em dois grandes grupos: memórias declarativas e memórias não declarativas (Henke, 2010). A memória declarativa pode ser dividida em memórias episódicas ou semânticas, enquanto a memória não declarativa está relacionada com as memórias motoras utilizadas em hábitos e procedimentos (Henke, 2010). Apesar de possuírem características distintas, são memórias que operam em conjunto, a exemplo do comportamento de emitir fala que necessita da evocação de habilidades de compreensão da informação e da articulação motora para execução da fala (Henke, 2010).

Em termos conceituais, a memória semântica pode ser explicada como a memória relacionada com o armazenamento do conhecimento do organismo, retendo e evocando informações sobre pessoas, lugares, eventos e as habilidades associadas ao intelecto (Stabler, 1986). Essa memória é responsável também pela compreensão dos significados das palavras, como substantivos, verbos e adjetivos, além de atuar em processos de nomeação dos estímulos ambientais (Jefferies, 2013; Ralph et al., 2017). É uma memória responsável tanto pelo conhecimento mais básico e elementar de um organismo, quanto pelo conhecimento mais robusto e complexo (Jefferies, 2013; Ralph et al., 2017), sendo crucial no processo de interação, sobrevivência e evolução das espécies.

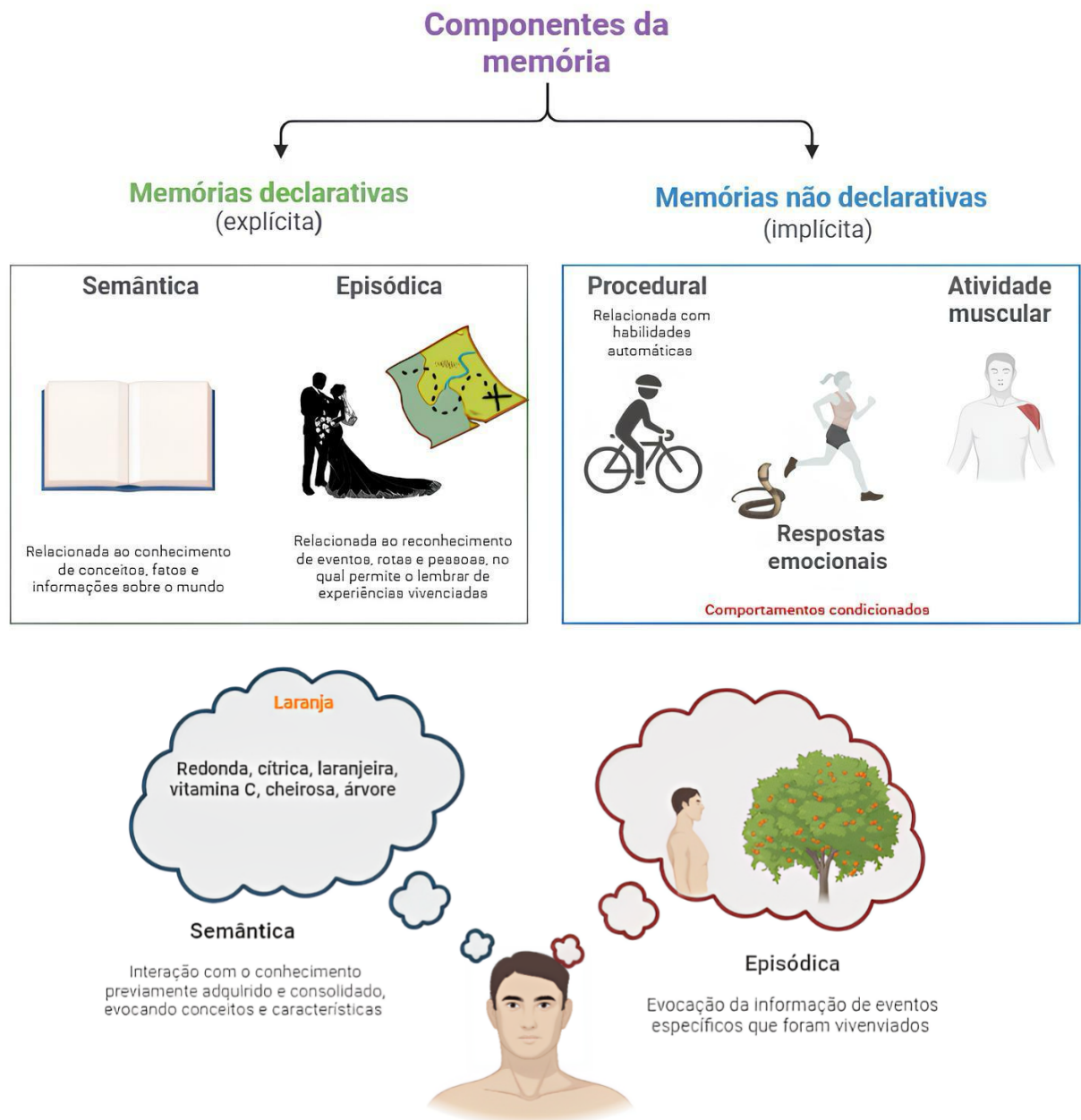
No processo de formação de memórias semânticas ocorrem distintos processos de associação de estímulos, no qual as informações recém codificadas interagem com informações adquiridas e consolidadas anteriormente para que ocorra a aprendizagem de forma mais rápida e efetiva (Jefferies, 2013; Ralph et al., 2017). Nessa etapa de associação de estímulos, ocorrem interações com informações generalistas ou específicas para formar uma informação única como, por exemplo, a integração de informações sensoriais visuais e auditivas para o

agrupamento das informações em uma única classe de memória (Patterson; Nestor; Rogers, 2007; Ralph et al., 2017). Em grande parte, configura-se como um tipo de memória duradoura e cristalizada, mesmo enfrentando declínios no envelhecimento e em doenças neurodegenerativas (Hodges; Patterson, 2007).

O segundo tipo de memórias declarativas, a memória episódica, pode ser descrita como a capacidade de codificação associativa rápida do organismo (Henke, 2010). De modo geral, é uma memória relacionada com os comportamentos de discriminar e recordar de determinados estímulos (figura 4), como pessoas, objetos e eventos, permitindo diferenciá-los de outros estímulos desconhecidos (Xue, 2018). Assim, possuem a função de discriminar uma memória de outra (Bower & Function, 1967. Underwood, 1969). É uma memória frequentemente investigada em modelos animais de aprendizagem, na qual verificou-se redes hipocampais envolvidas no processo de armazenamento e evocação desses estímulos (Eichenbaum, 2017; Eichenbaum et al., 1988; Eichenbaum; Fagan; Cohen, 1986). Curiosamente, observou-se que as redes hipocampais atuam em processos de codificação de informações visuo espaciais e temporais que constituem a memória episódica (Xue, 2018).

Adicionalmente, também existem vias olfativas conectadas ao hipocampo, possibilitando o reconhecimento e discriminação de odores, uma das várias características da memória episódica (Baudry, 2020). As memórias não-declarativas, ou também denominadas memórias implícitas ou de procedimentos, são memórias ligadas aos hábitos e procedimentos condicionados, como andar, escrever e digitar (Izquierdo et al., 2013). Diferente das declarativas, as memórias procedurais não necessitam necessariamente de conexões hipocampais, estando relacionada com o funcionamento de regiões motoras, como o estriado e córtex motor, apesar de haver conexões com o hipocampo (Ackermann; Rasch, 2014).

Figura 4: Esquematização dos subtipos de memória de acordo com o tempo de duração da e conteúdo da informação.



Fonte: Autor (2024).

2.2.3 MECANISMOS ANATÔMICOS, FUNCIONAIS E MOLECULARES DA MEMÓRIA E DO ESQUECIMENTO

Apesar da memória ser um comportamento que ocorre através da interação entre o organismo e o ambiente, estudos recentes apontam para um denso sistema orgânico presente no organismo que funciona durante o processo de formação de memórias, no qual é composto por estruturas e redes neurais que são moduladas por substâncias químicas para diferentes tipos de memória (Izquierdo et al., 2013). Tendo em vista isso, a memória é produzida, instaurada e

mantida pelas contingências ambientais que, em contrapartida, promove ou necessita de determinadas estruturas ou sistemas presentes no organismo para o seu funcionamento pleno.

Tomando isso como premissa básica, estudos encontraram estruturas anatômicas e conexões neurais que atuam no funcionamento da memória de acordo com o seu tipo (Bevilaqua et al., 2008; Izquierdo et al., 2013). Dentre essas estruturas, observa-se a atuação da amígdala na aprendizagem do medo, estando presente em respostas emocionais, e o hipocampo na formação e evocação de memórias declarativas (Bevilaqua et al., 2008; Izquierdo et al., 2013). No mesmo caminho, nota-se a participação de determinadas estruturas nas memórias não declarativas, nas memórias procedurais, detectando uma participação de estruturas subcorticais, como o núcleo caudado e outros núcleos da base do telencéfalo (Izquierdo et al., 2013; Izquierdo 1992). De forma mais recente, encontrou-se um novo tipo de memória relacionada com o funcionamento do córtex pré-frontal e suas conexões, denominada como memória de trabalho (Diamond, 2013; Izquierdo et al., 2013). A memória de trabalho é uma memória com características próprias, na qual mantém a informação por um curto período, mas a utilizada para a execução de comportamentos envolvidos na tomada de decisão (Diamond, 2013; Izquierdo et al., 2013).

O hipocampo é uma estrutura essencial na formação e evocação de memórias declarativas (Bekinschtein et al., 2007, 2008; Izquierdo et al., 2013). A descoberta dos mecanismos moleculares envolvidos na memória somente foi possível devido aos experimentos de Eric Kandel com a *Aplysia californiana* (Faria, 2020). Como resultado desses experimentos, observou-se que a aprendizagem de novos comportamentos resulta em mudanças nas conexões sinápticas (Kandel, 2001b, 2001a). Curiosamente, a aprendizagem de curto prazo e de longo prazo podem causar modificações nas mesmas conexões sinápticas (Kandel, 2001b, 2001a), assim entende-se que a neurobiologia da aprendizagem de curto prazo e longo prazo encontra-se nas sinapses.

Nessa perspectiva, para entender os mecanismos da memória no nível neural e molecular, observou-se que o armazenamento da informação depende da comunicação entre os neurônios pré e pós-sinápticos, na qual essa comunicação é mediada pela liberação do neurotransmissor glutamato durante o potencial de ação (Kandel, 2001), evidenciando os processos iniciais na formação e consolidação da memória. Posteriormente, descobriu-se também que os interneurônios participam desse processo através da liberação da serotonina que, por sua vez, opera intensificando a liberação do glutamato (Kandel, 2001), assim os neurotransmissores glutamato e serotonina atuam diretamente nos processos de aprendizagem.

Subsequentemente, observou-se que outra molécula sinalizadora participa também desse processo, sendo ela o monofosfato cíclico de adenosina (AMPC) (Kandel, 2001).

A serotonina, o glutamato e o AMPC operam como mediadores na comunicação sináptica, na qual produzem um aumento da excitabilidade e, conseqüentemente, um aumento do potencial de ação, através da diminuição das correntes específicas de potássio, liberando um maior influxo de cálcio no terminal pré-sináptico (Kandel, 2001a). Apesar dessas modificações sinápticas, elas não perduram, sendo transitórias como a retenção da informação (Kandel, 2001), sendo esses os mecanismos moleculares presentes na formação de memórias de curto prazo (Kandel, 2001a). A partir dessas observações, na medida em que se notou que as mudanças são transitórias, tal qual a retenção da informação, levantou-se a hipótese de que seriam necessárias mudanças permanentes nas conexões para que a retenção da informação fosse duradoura.

O circuito neural responsável pela memória de longo prazo envolve complexas vias de sinalização bioquímica e molecular de forma similar a memória de curto prazo, observando que o glutamato e a serotonina atuam na facilitação da comunicação sináptica e na consolidação das memórias de curto prazo, e o AMPC como o principal agente regulador da sinalização intracelular (Faria, 2020; Kandel, 2001b, 2001a). Todavia, essas mudanças funcionais promovem somente memórias de curto prazo, sendo necessárias também mudanças morfológicas nos neurônios (Kandel, 2001a). Para que ocorram mudanças duradouras nos neurônios, as proteínas quinases são ativadas por mitógenos (MAPK) e vão ser agentes essenciais na transcrição do CREB e na consolidação da informação de longo prazo (Faria, 2020; Kandel, 2001b, 2001a). Ademais, para que ocorra a consolidação da informação, é necessário que ocorra a transcrição do CREB-1 e, em paralelo, a supressão do CREB-2 (Faria, 2020).

O CREB é um agente que vai atuar regulando a expressão gênica e a síntese de novas proteínas nos neurônios que, por sua vez, permitirão o desenvolvimento de estruturas essenciais na formação de novas conexões sinápticas (Bekinschtein et al., 2007). A partir disso, inicia-se uma cascata de alterações bioquímicas que levam a síntese do BDNF (Bekinschtein et al., 2007; Salimi et al., 2021). Todavia, a transcrição do gene que codifica o BDNF pode ser modulada pelo influxo de cálcio no neurônio, sendo assim um agente atuante na memória de longo prazo (Bekinschtein et al., 2007; Salimi et al., 2021). Esses achados fornecem evidências da presença de uma cascata transcricional que possibilita a formação de estruturas que fazem com que a memória de curto prazo se transforme em uma memória de longo prazo.

Assim, uma importante molécula nesse processo é o BDNF, tendo em vista que ele é fundamental na plasticidade neuronal e no crescimento das espinhas dendríticas em diversas regiões do cérebro, sendo também essencial na formação de novas memórias de longo prazo (Bekinschtein et al., 2007, 2008) (figura 5). Esse processo ocorre devido à sua capacidade de melhorar a sinapse e alterar o potencial de ação do neurônio, facilitando assim consolidação de novas memórias (Kandel, 2001a, 2001b). Estudos com roedores apontam para uma relação com a expressão de BDNF com o armazenamento e evocação de memórias episódicas, na qual atua no comportamento de reconhecer e discriminar estímulos (Gonzalez et al., 2019; Gonzalez; Radiske; Cammarota, 2019). Portanto, compreende-se que a memória de curto prazo e longo prazo podem alterar as mesmas conexões sinápticas, mas diferenciam-se na medida em que na memória de curto prazo são recrutadas proteínas preexistentes no neurônio para a manipulação da informação, enquanto na memória de longo prazo ocorrem processos de síntese e supressão de proteínas específicas.

Outros sistemas de neurotransmissão também participam da consolidação da memória, como acetilcolina (ACh), presente no hipocampo, que possui papel importante nos processos de aprendizagem e memória (Parent; Baxter, 2004). O sistema colinérgico está envolvido na memória de diversas formas, na qual o aumento da expressão gênica do ChAT no hipocampo está relacionado com a melhora da função cerebral (Lee et al., 2021). Em doenças neurodegenerativas, como a Doença de Alzheimer, os níveis de acetilcolina e da ChAT estão reduzidos e associados aos prejuízos cognitivos (Wilcock et al., 1982), indicando possíveis relações entre os níveis da ChAT também com o funcionamento a formação de conexões sinápticas.

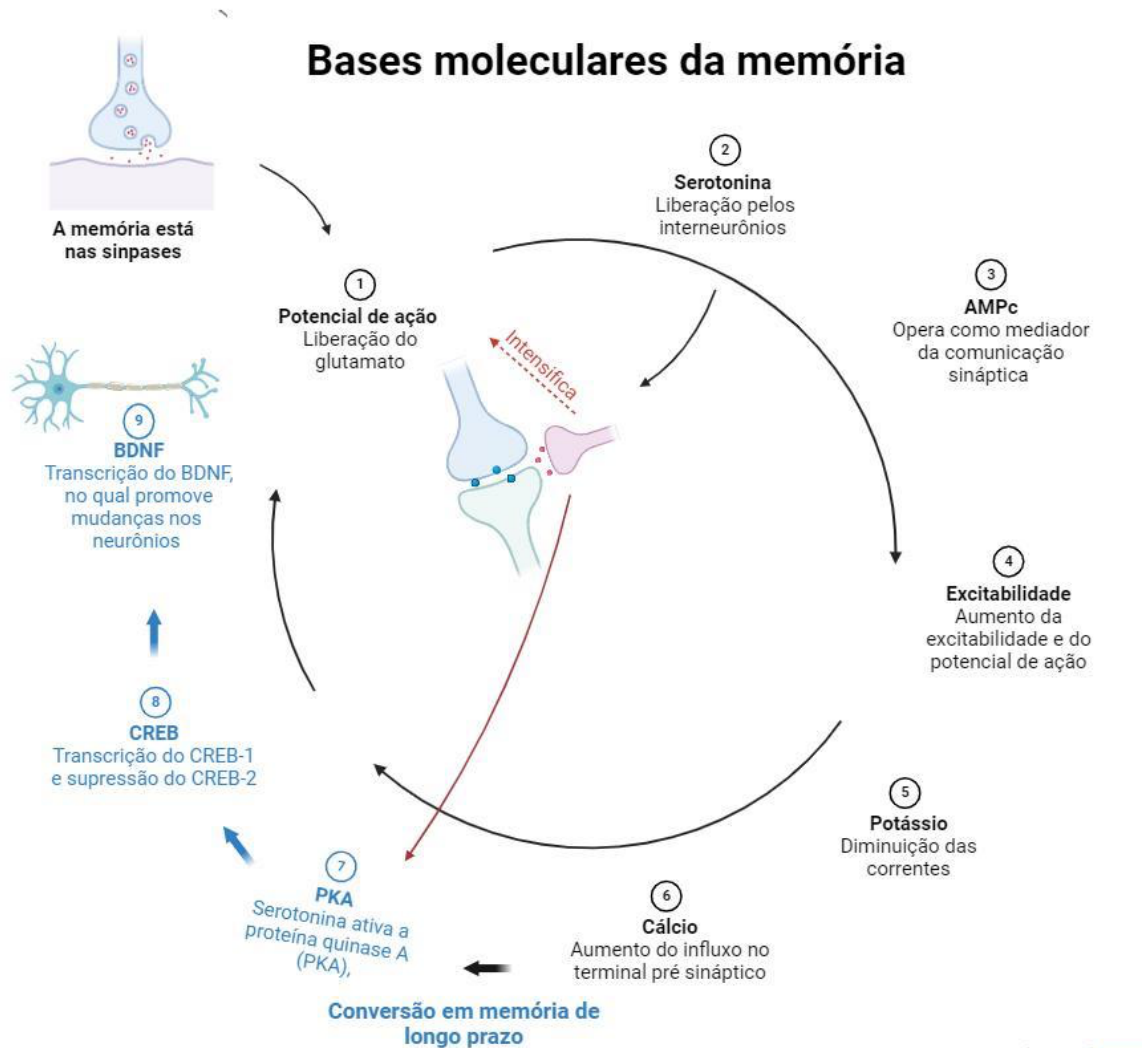
Essas mudanças nas conexões sinápticas foram posteriormente denominadas de engramas, referindo-se às mudanças físicas que ocorrem no cérebro após a aprendizagem, sendo considerado o locus onde habita a informação armazenada (Josselyn; Köhler; Frankland, 2015). Quando a informação sensorial é codificada e armazenada pelo organismo ocorre um aumento da potência sináptica, visto que ocorrem uma cascata de alterações sinápticas no neurônio (Kandel, 2001a, 2001b). Esse aumento da potência sináptica aumenta a probabilidade que o mesmo padrão de atividade neural seja reproduzido, facilitando a recuperação da informação (Josselyn; Köhler; Frankland, 2015), sinalizando que a repetição e o treino facilitam a recuperação da informação posteriormente. Apesar disso, o organismo é um ser ativo na construção e reconstrução de memórias e, por essa razão, os engramas podem ser modificados através de mecanismos epigenéticos, modificando, inclusive, a natureza da informação armazenada (Dudai; Eisenberg, 2004; Josselyn; Köhler; Frankland, 2015).

Por essa razão, mudanças no circuito sináptico podem ocasionar o enfraquecimento ou a perda de conexões sinápticas entre os neurônios que constituem o engrama, levando ao esquecimento de memórias adquiridas (Wang et al., 2020). Apesar da sua importância, pouco se fala sobre o fenômeno do esquecimento, na medida em que qualquer organismo é tudo aquilo que lembra, mas é, sobretudo, tudo aquilo que esqueceu e ficou no passado. O esquecimento é um processo natural que ocorre com qualquer organismo vivo, sendo um importante mecanismo adaptativo e evolutivo porque permite o aprendizado de novas coisas, além de possibilitar o perdoar e o seguir em frente, facilitando a adaptação enquanto espécie (Chowdhury; Dai; Mémoli, 2018).

Devido a sua importância na adaptação e evolução no nível ontogenético e filogenético, o esquecimento vem sendo um tema de pesquisa atual, no qual achados demonstram uma participação significativa das células da micróglia no fenômeno do esquecimento (Wang et al., 2020). A micróglia é uma célula da glia bastante conhecida por realizar funções cruciais no desenvolvimento neural, no qual atua na eliminação de sinapses em excessos através da poda sináptica (Paolicelli et al., 2011; Schafer et al., 2012; Wang et al., 2020), sendo através da eliminação dessas conexões que a micróglia promove o esquecimento. Agora não somente o neurônio pré e pós-sináptico participam dos processos de aprendizagem e esquecimento, mas as células da glia também através dos astrócitos e das micróglia (Frost; Schafer, 2016; Lim et al., 2013; Pascual et al., 2012). Sabe-se que a participação dos astrócitos ocorre em respostas imunes através da ativação das micróglia (Pascual et al., 2012), já as micróglia vão atuar através da eliminação de conexões sinápticas menos ativas e mais fracas (Schafer et al., 2012).

O hipocampo é uma região marcada pela ocorrência de neurogênese, na qual ocasiona em uma necessidade de reorganização sináptica (Toni et al., 2007). Em uma perspectiva adaptativa, em condições basais, a micróglia atua na poda sináptica auxiliando na reorganização sináptica (Wang et al., 2020). Entretanto, um processo de ativação da micróglia em excesso ocorre frente a exposição precoce a insultos, no qual prejudica o funcionamento adequado do hipocampo (Gouveia et al., 2023; Visco et al., 2022b). Isso porque a micróglia é uma das primeiras células a responder a esses insultos precoces gerando o estado de ativação (Gouveia et al., 2023). Se a ativação for em excesso, pode comprometer o metabolismo cerebral e promover um estado de neurotoxicidade (Haukedal et al., 2023), podendo levar a destruição de redes neurais envolvidas em comportamentos essenciais (Wang et al., 2020), no qual pode levar a perdas de memória ou em impedimentos na formação de novas memórias (Calado et al., 2023b).

Figura 5: Processos moleculares envolvidos no processo de formação de memórias de curto e longo prazo.



Fonte: Autor (2024).

2.2.4 MEMÓRIA EM MODELOS EXPERIMENTAIS

A memória é uma habilidade que está presente em diversos organismos, inclusive em roedores, sendo possível detectar o funcionamento da memória episódica e dos seus correlatos neurais de forma similar aos humanos (Templer; Hampton, 2013). Experimentos demonstram a capacidade dos animais de aprender, discriminar e recordar de determinados estímulos (Berger et al., 2019; Diaz-Chávez et al., 2020). Para avaliar esse tipo de memória existem dois tipos de paradigmas: os baseados em treinamento e os livres de treinamento (Chao et al., 2020). Nos testes baseados em treinamento, os animais são treinados a aprender determinadas regras a partir de consequências produzidas após o comportamento, como o reforço positivo, reforço negativo ou punição (Chao et al., 2020; Clayton; Dickinson, 1998). Assim, os animais podem se esquivar de estímulos aversivos ou seguir em direção de estímulos reforçadores de acordo com a história de aprendizagem (Chao et al., 2020).

Por outro lado, os testes livres de treinamento visam reduzir a aplicação do treinamento prévio, e são projetados para atenuar variáveis emocionais e motivacionais no desempenho dos animais, como estímulos punitivos, como dor ou privação de alimentos (Chao et al., 2020). Tais paradigmas utilizam medidas baseadas na natureza inata do animal de se comportar em determinadas contingências ambientais, a exemplo do comportamento de exploração espontânea de objetos novos, sendo um traço comportamental utilizado para avaliar a memória de reconhecimento (Chao et al., 2020). Alguns parâmetros como o tempo de exploração no objeto novo em comparação com o tempo de exploração no objeto familiar são utilizados na avaliação desse tipo de memória, obtendo-se, a partir da diferença entre o tempo de exploração de cada objeto, o índice de discriminação que é utilizado como indicador para avaliar a memória episódica (Calado et al., 2023; Ennaceur & Delacour, 1988; Ennaceur & Meliani, 1992; Lueptow, 2017). Nessa lógica, os animais tendem a explorar mais os objetos novos porque se lembram dos familiares (Berlyne, 1950). Por essa razão, esses paradigmas não necessitam de sucessivas e frequentes sessões de treino ou de estímulos reforçadores ou aversivos para motivar e controlar o comportamento (Lueptow, 2017).

A literatura aponta para paradigmas utilizados para avaliar a memória (figura 6) e a aprendizagem em animais com lesões cerebrais precoces, na qual observa-se a utilização do teste de reconhecimento do novo objeto (Berger et al., 2019) para avaliar a memória episódica de discriminação de objetos. Por outro lado, os testes do Labirinto em Y e Labirinto em T são utilizados para verificar a alternância do animal, ou seja, a capacidade explorar ambientes novos (Kang et al., 2018; Kim et al., 2006). Labirinto Aquático de Morris também é um teste utilizado, no qual se baseia na aprendizagem do animal para escapar do estímulo aversivo que é a água (Chen et al., 2014; Griva et al., 2017; Halis et al., 2019). Por último, observa-se também a utilização do teste da Caixa de Esquiva em Step-Down, no qual tem como premissa a apresentação de estímulos aversivos (choque) para avaliar a capacidade de aprendizagem dos animais (Li et al., 2020).

Figura 6: Principais testes utilizados para avaliar a memória e aprendizagem em roedores. Figura criada no BioRender.



Fonte: Autor (2024).

2.3 ANSIEDADE E CORRELATOS NEURAIS

Atualmente a ansiedade e depressão são problemas de saúde pública, sendo os dois transtornos mentais que mais acometem a população em geral (Demyttenaere et al., 2004). A ansiedade é o segundo transtorno mental que mais acomete crianças e adolescentes (Lawrence et al., 2015). Quando se fala-se em PC, dados apontam que adultos com PC apresentam um risco mais elevado para o desenvolvimento de transtornos mentais como depressão e ansiedade quando comparados a adultos saudáveis (Demyttenaere, 2004). De forma similar, crianças com doenças crônicas, como a PC, apresentam uma maior probabilidade para desenvolverem transtornos mentais (Lavigne e Faier-Routman, 1992). Nessa perspectiva, observa-se em crianças com PC uma série de comportamentos ansiosos (McMahon et al., 2020). Sabe-se que crianças com PC frequentemente sofrem de dor crônica (McKearnan et al., 2004), o que pode contribuir para problemas emocionais e comportamentais como a ansiedade (Yamaguchi; Nicholson Perry; Hines, 2014).

Apesar das controvérsias envolvendo a definição da ansiedade, ela pode ser compreendida enquanto um tipo de manifestação do comportamento emocional, na qual é caracterizada, principalmente, por estados emocionais desagradáveis (Gentil e Neto, 1996). Devido à sua complexidade, a ansiedade pode ocorrer como resposta a distintos estímulos ambientais, mas, frequentemente, ocorre em resposta a estímulos estressores ambientais (Clement, 1998). Embora seja um fenômeno emocional comum durante a vida, presente em diversas culturas desde os primórdios da humanidade, em excesso pode ser patológica, como o

transtorno de ansiedade generalizada, transtorno do pânico, transtorno de ansiedade social ou fobias (Bandelow; Michaelis, 2015), trazendo implicações severas na aprendizagem e socialização.

A ansiedade frequentemente apresenta teor antecipatório, como o medo e/ou preocupação se algo vai acontecer, geralmente relacionada com estímulos aversivos (Fowles, 2000). Por outro lado, em condições basais, o medo é um comportamento essencial na sobrevivência das espécies, na qual apresenta-se como um comportamento essencial em contextos que o organismo necessite de uma defesa contra um perigo potencial (Dunsmoor e Paz, 2015). Assim, a generalização adequada do medo aprendido, através de uma história de reforçamento, permite que o organismo discrimine estímulos semelhantes ao qual foi exposto de forma aversiva e emita comportamentos com a função de se proteger (Dunsmoor e Paz, 2015). Por outro lado, a generalização do medo em excesso, em situações inofensivas, é uma das características desadaptativas e mais memoráveis no transtorno de ansiedade (Dunsmoor e Paz, 2015). O medo em excesso e os quadros ansiosos podem comprometer negativamente a socialização de um organismo de distintas formas, a mais observável e comum, é a introversão (Gray, 1970), que pode levar ao isolamento social.

Nessa perspectiva, compreende-se que a generalização do medo é um dos mecanismos da ansiedade (Dunsmoor e Paz, 2015), causando repercussões severas para o organismo. Apesar da ansiedade estar relacionada com a história de aprendizagem do organismo, a exposição a determinadas contingências ambientais adversas pode comprometer estruturas cerebrais, como a substância cinzenta periaquedutal dorsal do mesencéfalo (Clement, 1998) e o hipocampo ventral (Fanselow; Dong, 2010), no qual dificultam o controle emocional e contribuem para o um estado de ansiedade. Outras estruturas também atuam no controle de respostas emocionais, como a amígdala e no córtex pré-frontal, atuando em processos de aprendizagem e generalização do medo, na qual contribuem para comportamentos ansiogênicos (Dunsmoor e Paz, 2015; Maren et al., 2013; Quirk & Mueller, 2008).

Em termos de classificação, a ansiedade pode ser classificada em dois tipos: ansiedade estado e ansiedade traço (Cavalcanti et al., 2021). A ansiedade-traço refere-se a comportamentos transitórios, compreende-se assim como um comportamento observado somente em contingências ambientais específicas, na qual é possível notar os antecedentes ambientais envolvidos nesses comportamentos (Almeida-Souza; Goes; Teixeira-Silva, 2015). Os comportamentos que compõem a ansiedade traço frequentemente são observados em contextos que envolvem o medo, inquietação, angústia, impaciência ou excitação (Almeida-Souza; Goes; Teixeira-Silva, 2015). Enquanto a ansiedade-estado relaciona-se a comportamentos mais

estáveis e característicos do organismo, sendo esses comportamentos característicos emitidos de forma mais frequente em contextos distintos (Cavalcanti Et Al., 2021; Spielberger, Gorsuch E Lushene, 1970).

Visando avaliar os dois tipos de ansiedade em modelos animais, alguns aparatos foram testados e validados para analisar os traços comportamentais semelhantes à ansiedade. Um dos aparatos mais conhecidos é o Labirinto Elevado em Cruz, o qual foi validado há quase 4 décadas atrás (Pellow et al., 1985). Em termos de estrutura, configura-se enquanto um aparato elevado do chão constituído por uma área central conectada a quatro braços, no qual dois braços são abertos e dois braços são fechados (Pellow et al., 1985). Baseando-se na premissa de que roedores são animais que preferem naturalmente locais escuros e fechados, o aumento do tempo de confinamento nos braços fechados é o principal indicador de comportamentos relacionados à ansiedade (Pellow et al., 1985).

Baseado nessa mesma premissa, outro aparato empregado no estudo do comportamento emocional em roedores é a caixa claro-escuro. Em termos estruturais, esse dispositivo é formado por um compartimento dividido em dois ambientes: um lado iluminado e outro lado escurecido, no qual esses dois espaços são separados por uma divisória que possui apenas uma abertura que permite a transição do animal entre o ambiente claro e o escuro (Bourin; Hascoët, 2003). Esse é um teste baseado em comportamentos naturais dos roedores que promovem conflitos, no qual eles possuem uma tendência natural para explorar ambientes novos, enquanto também possuem uma preferência por permanecer em ambientes escuros (Bourin; Hascoët, 2003). Por essa razão, uma situação na qual ocorre uma redução na quantidade de transições entre os lados da caixa e um aumento no tempo de permanência no lado escuro são traços indicadores de ansiedade (Bourin; Hascoët, 2003).

Apesar da ansiedade configurar-se enquanto uma classe de comportamentos emitidos em determinadas contingências ambientais, sob a perspectiva do Dohad, experiências adversas precoce contribuem de forma negativa no desenvolvimento do organismo, traduzindo-se em uma maior suscetibilidade para o desenvolvimento de desordens emocionais e transtornos mentais mais tardiamente (Delcour Et Al., 2012; Gluckman; Hanson; Beedle, 2007; Macbeth Et Al., 2023; Ochi; Dwivedi, 2023; Roseboom et al., 2011; Wu; de Asis-Cruz; Limperopoulos, 2024; Yu et al., 2020; Zhu et al., 2024). Dentre as experiências adversas, a má nutrição durante a gestação, como a exposição a dietas hiperlipídicas, é uma das variáveis que promovem um aumento em biomarcadores da inflamação e comportamentos similares à ansiedade nos filhotes (Cavalcanti et al., 2021). De forma similar, agressões durante a fase de lactação, como a hipóxia-isquemia, não se limitam somente a impactar a aprendizagem, mas, a priori, programam

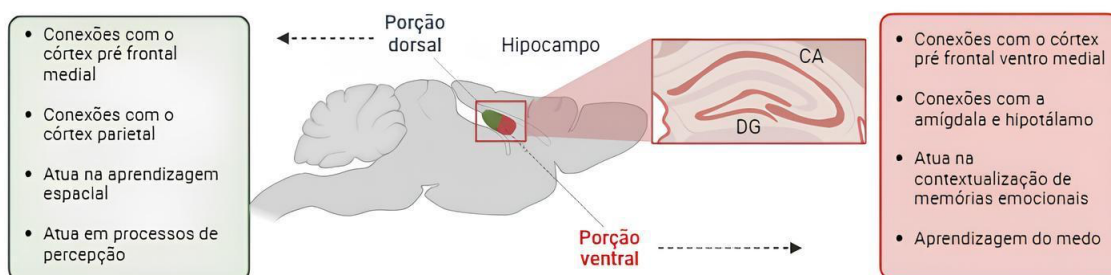
o organismo para responder, a determinados estímulos ambientais, de forma desadaptativa (Soares et al., 2013a), traduzindo-se em um maior tempo de permanência dos braços fechados no LEC, associado a uma redução dos neurônios hipocampais (Soares et al., 2013a).

Curiosamente, o hipocampo e o hipotálamo são regiões que tem o seu desenvolvimento afetado em condições ambientais adversas, observando uma redução na proliferação celular no hipocampo (Visco et al., 2022b) e um aumento da ativação microglial no hipotálamo (Costa-de-Santana et al., 2023), trazendo uma série de perturbações no comportamento, inclusive no comportamento emocional (Soares et al., 2013a). Apesar do hipocampo ser conhecido pela sua relação com a formação de memórias declarativas (Kandel, 2001a), ele é uma estrutura multifacetada, no qual possui diferenças em aspectos da expressão gênica, morfologia, conexões e em funções comportamentais (Chao et al., 2020; Swanson & Cowan, 1977).

Por essa razão, a porção dorsal e ventral são distintas em termos de implicações comportamentais, na qual a porção dorsal está relacionada com a formação de memórias, diferentemente da porção ventral (Moser et al., 1995). Em situações de aprendizagem espacial, nota-se alterações epigenéticas no hipocampo dorsal, sobretudo na porção direita, em roedores (Klur et al., 2009), de forma similar, humanos parecem demonstrarem o mesmo padrão de ativação do hipocampo dorsal direito no momento de evocar informações espaciais (Maguire et al., 1997). Em termos de expressão gênica, na porção dorsal nota-se uma maior expressão de genes presentes em regiões corticais responsáveis por processar informações e com o funcionamento cognitivo (Fanselow; Dong, 2010).

Por outro lado, a porção ventral do hipocampo parece estar implicada com funções emocionais (Fanselow; Dong, 2010), na qual observa-se, em condições de lesões nessa estrutura, um aumento em comportamentos desadaptativos similares à ansiedade (Kjelstrup et al., 2002). Dessa forma, observa-se que a porção ventral possui conexões com regiões como o hipotálamo, amígdala e córtex pré frontal que são estruturas que atuam em comportamentos complexos como o comportamento alimentar, emocional e motivacional (Fanselow; Dong, 2010). Dessa maneira, compreende-se que o hipocampo opera em processos comportamentais distintos a depender da sua região, na qual a porção dorsal opera em processos de aprendizagem, enquanto a porção ventral em processos emocionais.

Figura 7: Divisão anatômica do hipocampo dorsal e ventral e suas implicações comportamentais. Figura criada no BioRender.



Fonte: Autor (2024).

2.4 PARALISIA CEREBRAL E O SEU IMPACTO NA APRENDIZAGEM

Sob a perspectiva da plasticidade do desenvolvimento, compreende-se a influência da exposição ambiental precoce no desenvolvimento, no qual pode levar a variações fenotípicas, em mais amplo conceito, benéficas ou deletérias. Nesse paradigma, destaca-se a influência do ambiente adverso no desenvolvimento cerebral, no qual observa-se em países de baixa renda e em situação de desenvolvimento, maiores taxas de prevalência de desordens do neurodesenvolvimento como a PC (Mcintyre et al., 2013, 2022). Embora haja um componente genético envolvido na patogênese da PC (Pham et al., 2020), a abordagem de compreensão multifatorial é mais convincente, considerando a ausência de padrões claros de herança monogênica em sua etiologia (Fahey et al., 2017). Ancorando-se nessas premissas, entende-se que a patogênese da PC envolve a interação entre fatores ambientais e genéticos (Tollanes et al., 2014).

A partir dessa compreensão, a literatura elenca algumas das condições ambientais adversas que, se experienciadas durante a gestação ou nos primeiros dias de vida, podem levar ao desenvolvimento do fenótipo da PC, na qual sabe-se que, dentre os fatores pós natal, a prematuridade, asfixia durante o parto e baixo peso ao nascer (Bax et al., 2005; Blair; Stanley, 2002; McIntyre et al., 2013, 2022). Em relação aos fatores durante a gestação, destacam-se as múltiplas gravidezes, insuficiência placentária, infecções, inflamações, hipóxia-isquemia (Mcintyre et al., 2013). Assim, de um modo geral, o surgimento da PC está atrelado às condições ambientais insuficientes, observando assim determinantes sociais nesse processo. Nessa conjuntura, a PC enquadra-se enquanto um dos mais desafiadores distúrbios na infância, sendo uma das principais causas de deficiência na infância (Korzeniewski et al., 2018).

Por essa razão, atualmente, dados apontam que a PC afeta cerca de 3,4 por 1.000 nascidos vivos em países de baixa renda e 1,5 por 1.000 nascidos vivos em países de alta renda (Mcintyre et al., 2022). Devido à ausência de estudos epidemiológicos sobre a PC no Brasil, é difícil determinar sua prevalência e incidência com precisão, mas estima-se que a prevalência

de PC em países em desenvolvimento seja de 7 para cada 1.000 nascidos vivos (Brasil, 2013), evidenciando o papel dos determinantes sociais na patogênese da PC. Acredita-se que esse fenômeno ocorra em países em desenvolvimento, como o Brasil, devido à ausência de cuidados obstétricos e neonatais adequados (Vitrikas; Dalton; Breish, 2020).

A PC apresenta-se enquanto uma condição extremamente desafiadora devido à heterogeneidade de sintomas, na qual os sintomas advêm de uma lesão precoce no cérebro impactando negativamente a postura e movimento (Bax et al., 2005; Graham et al., 2016). Apesar de ser uma lesão de cunho não progressiva (Fragopoulou et al., 2019; Graham et al., 2016), as sequelas da PC podem ser modificadas, apresentando uma redução ou exacerbação a depender das condições ambientais, sobretudo, as experienciadas durante o período perinatal (Lacerda et al., 2017; Pereira et al., 2021; Silva et al., 2016; Visco et al., 2023), através dos mecanismos da plasticidade fenotípica presentes na interação mãe e filho.

Nessa perspectiva, nota-se que a lesão cerebral precoce promove uma cascata de alterações no desenvolvimento do organismo. No contexto da PC, observa-se principalmente a manifestação de déficits no desenvolvimento neuromotor, no qual é possível encontrar alterações como espasticidade, discinesia, redução na força muscular, prejuízos na motricidade ampla e fina que, por sua vez, comprometem a marcha (Bearden et al., 2016; Richards; Malouin, 2013). Essa cascata de prejuízos motores também está associada ao baixo desempenho escolar, na medida em que apresentam-se enquanto barreiras significativas na aprendizagem (Van Rooijen; Verhoeven; Steenbergen, 2016). Outras comorbidades e alterações são encontradas frequentemente, tais como convulsões, alterações sensório-perceptuais que contribuem para prejuízos em comportamentos complexos de linguagem e na aprendizagem (Bearden et al., 2016; Shevell; Dagenais; Hall, 2009).

Anteriormente, a PC era classificada de acordo com o grau de comprometimento, sendo eles: i) leve, ii) moderado e iii) grave (Wimalasundera; Stevenson, 2016). Com o passar do tempo e o avanço no campo científico, esse modelo de classificação ficou ultrapassado, no qual foram utilizados critérios mais específicos e adequados para realizar a classificação. Utilizando como critério o comprometimento topográfico, a PC pode ser classificada como diplégica, hemiplégica ou quadriplégica (Bax et al., 2005; Wimalasundera; Stevenson, 2016). O comprometimento do movimento também pode ser utilizado como um fator para a classificação, no qual pode-se encontrar a espasticidade, hipotonia, hipercinesia ou ataxia (Johari et al., 2016; Wimalasundera; Stevenson, 2016).

Apesar dos prejuízos neuromotores serem utilizados em termos de diagnóstico e classificação, a PC acomete outras esferas para além do sistema musculoesquelético, no qual

podem ser encontradas variações de cunho sensório-perceptual que contribuem para o comprometimento de outras habilidades como a memória (Al-Nemr; Abdelazeim, 2018; Hirsh et al., 2010; Pirila et al., 2004; Pueyo et al., 2009; Stadskeiv et al., 2018; White; Christ, 2005). Apesar da dificuldade em estabelecer um perfil neuropsicológico na PC, achados recentes indicam prejuízos na memória de trabalho, associada a perturbações nas ondas alfa e beta em áreas pré-frontais e temporais em adultos com PC (Hoffman et al., 2021). Em consonância com esses dados, crianças com PC apresentam prejuízos intelectuais, traduzindo-se em dificuldades na aprendizagem e alterações nas funções executivas, sendo manifestado de diferentes formas (De Freitas Feldberg et al., 2021; Pereira et al., 2018).

Curiosamente, a memória é um dos comportamentos mais afetados, na qual observa-se prejuízos em domínios distintos, traduzindo-se em prejuízos na memória visuoespacial, na memória de trabalho e em componentes das funções executivas (De Freitas Feldberg et al., 2021; Stadskeiv et al., 2018). Por consequência dessas dificuldades na aprendizagem, nota-se uma alta prevalência de problemas cognitivos em crianças com PC (Himmelman et al., 2006; Van Rooijen; Verhoeven; Steenbergen, 2016). Por outro lado, mesmo com esses achados que tentaram estabelecer um perfil neuropsicológico dos sujeitos acometidos pela PC, esses comprometimentos na aprendizagem são heterogêneos, no qual manifestam-se de formas diferentes dependendo da gravidade e extensão da lesão. Compreende-se dessa forma que diferentes tipos de diagnóstico de PC podem apresentar um perfil neuropsicológico distinto, onde algumas habilidades são mais afetadas que outras (Stadskeiv et al., 2018).

Devido à complexidade da PC e heterogeneidade dos sintomas, diversos modelos animais de estudo surgem com o objetivo de investigar como a lesão compromete o desenvolvimento de diferentes formas (Da Conceição Pereira et al., 2021; Visco et al., 2021). Favoravelmente, os modelos animais permitem analisar as alterações neurobiológicas associadas aos prejuízos comportamentais. Assim, os principais modelos utilizados para mimetizar o fenótipo da PC utilizam a exposição a determinadas injúrias durante o período crítico do desenvolvimento, durante a gestação ou nos primeiros dias de vida (Da Conceição Pereira et al., 2021; Lacerda et al., 2017), e tais modelos afetam de diferentes formas o sistema nervoso (Visco et al., 2021).

Em relação aos insultos, nota-se a utilização de hipóxia-isquemia, inflamação induzida por lipopolissacarídeos (LPS), hemorragia intraventricular, anoxia ou restrição sensório-motora (RSM) para replicar a PC (Da Conceição Pereira et al., 2021). Nessa perspectiva, um dos insultos utilizados é a hipóxia-isquemia nos primeiros dias de vida, sendo considerada uma das mais comuns lesões cerebrais perinatais (Nelson; Chang, 2008). Resultados histopatológicos e

de neuroimagem observaram danos no córtex sensório-motor, no corpo estriado e no hipocampo após a hipóxia-isquemia (Wilson, 2015). Grande parte dos modelos de HI utilizam o dano na primeira semana de vida do animal (Da Conceição Pereira et al., 2021), acredita-se que isso ocorra porque os roedores são animais no qual o desenvolvimento do sistema nervoso nos primeiros 10 dias de vida é equivalente ao desenvolvimento de fetos humanos no terceiro trimestre de gestação (Sengupta, 2013; Wilson, 2015). Por essa razão, o desenvolvimento neuromotor é extremamente vulnerável nessa fase, no qual a imaturidade da barreira hematoencefálica faz com que o dano isquêmico promova lesões mais graves (Muramatsu et al., 1997).

Compreende-se que os eventos isquêmicos precoces podem levar a respostas inflamatórias, bem como a necrose da substância branca em áreas próximas aos ventrículos cerebrais laterais (Folkerth, 2005; Rumpel et al., 1997), devido a vulnerabilidade do cérebro nessa fase do desenvolvimento. Estudos elucidaram, após a privação de oxigênio, um aumento da expressão de enzimas associadas ao estresse oxidativo como caspase-3, óxido nítrico (NO) e TBARS (Azevedo et al., 2020; Chen et al., 2014; Greggio et al., 2011). Esse aumento de espécies reativas de oxigênio pode levar ao estado de estresse oxidativo que pode promover a neuroinflamação e danificar o tecido nervoso (Deb; Sharma; Hassan, 2010; Wu et al., 2021). A neuroinflamação é um dos mecanismos subjacentes à lesão cerebral que está relacionada com a patogênese da PC (Fragopoulou et al., 2019). Tal vulnerabilidade pode ser compreendida pela deficiência relativa da superóxido dismutase, a enzima que neutraliza o superóxido no desenvolvimento da substância branca (Fern; Möller, 2000). Por isso, após o evento isquêmico, nota-se uma cascata de eventos que sinalizam a neuroinflamação, como aumento de citocinas pró inflamatórias, da ativação microglial e destruição dos oligodendrócitos pré-mielinizantes (Wilson, 2015), sendo o estresse oxidativo um dos eventos associados aos distúrbios neuromotores presentes na PC (Da Silva Souza et al., 2023b).

Por outro lado, as experiências adversas enfrentadas durante a gestação podem levar ao desenvolvimento do fenótipo da PC através dos mecanismos de comunicação entre a mãe e o feto, assim desde a década de 90 estuda-se a relação entre a infecções e inflamações com o surgimento da PC (Fern; Möller, 2000). Dados apontam que a exposição a infecções durante a gestação pode repercutir em respostas inflamatórias no feto, no qual pode levar ao parto prematuro e/ou a outras sequelas neurológicas (Hagberg; Mallard, 2005), no qual entende-se a inflamação como um dos principais componentes no surgimento da PC (Fragopoulou et al., 2019). Estudos com endotoxinas bacterianas durante a gestação demonstraram modificações no feto, no qual, a priori, ele parece desenvolver uma suscetibilidade e responsividade a eventos

hipóxicos e isquêmicos, levando a respostas inflamatórias (Fern; Möller, 2000). Em consonância com isso, observa-se um aumento das taxas de PC em bebês descendentes de mães que tiveram infecções perinatais (Ahlin et al., 2013; Bear; Wu, 2016), sugerindo que a exposição a inflamação materna pode deixar o feto mais vulnerável a determinados insultos.

Observa-se uma correspondência entre infecções e inflamações durante a gestação e o surgimento da PC (Schendel; Schuchat; Thorsen, 2002), no qual modelos experimentais utilizam infecções maternas por LPS para mimetizar o fenótipo da PC. Em tais modelos é possível observar um atraso no desenvolvimento dos reflexos neurológicos (Toso et al., 2005), redução da função motora (Zhang et al., 2016) e aumento do comportamento similar a ansiedade (Makinson et al., 2017). Todavia, dados sugerem que a idade gestacional é crítica para as alterações comportamentais (Straley et al., 2017). Apesar disso, a utilização de um único insulto parece *a priori* reproduzir apenas uma parte das sequelas da PC, a depender da modalidade da injúria (Coq et al., 2008). O modelo proposto que associa dois insultos, a anóxia perinatal em conjunto com a RSM das patas posteriores, apresenta uma série de modificações desadaptativas na locomoção, coordenação motora, força muscular, mastigação (DE Albuquerque et al., 2023; Gouveia et al., 2023; Lacerda et al., 2017; Marcuzzo et al., 2010; Pereira et al., 2021; Visco et al., 2022b).

A utilização do duplo insulto -anóxia e RSM- consegue mimetizar de forma mais fiel o fenótipo da PC diplégica espástica (Da Conceição Pereira et al., 2021; Delcour et al., 2018; Pereira et al., 2021; Strata et al., 2004). Assim, a utilização desse modelo vem sendo utilizado para a compreensão das repercussões da PC no desenvolvimento, no qual, em termos de sistema nervoso, observa-se um desequilíbrio químico nos níveis antioxidantes e pró oxidantes em regiões corticais (Da Silva Souza et al., 2023a; Visco et al., 2022b) que, por sua vez, pode levar a uma cascata de reações aumentando os níveis de citocinas pró inflamatórias (Gouveia et al., 2023) associadas a um aumento da ativação das micróglia e uma redução da proliferação celular em regiões como o hipocampo, cerebelo e hipotálamo (Calado et al., 2023b; Costa-de-Santana et al., 2023; Pereira et al., 2024). Essa cascata de reações contribui para prejuízos comportamentais, afetando negativamente o comportamento locomotor, alimentar e a aprendizagem dos animais (Calado et al., 2023b; Costa-de-Santana et al., 2023; Pereira et al., 2024), de forma similar aos prejuízos clínicos observado em crianças com PC (De Freitas Feldberg et al., 2021; Van Rooijen; Verhoeven; Steenbergen, 2016).

2.5 POLIFENÓIS COMO TRATAMENTO PROMISSOR NAS SEQUELAS NEUROLÓGICAS

Devido a série de comprometimentos motores e cognitivos presentes na PC, diversas intervenções são estudadas com o intuito de atenuar os prejuízos funcionais e promover uma melhor qualidade de vida para essas crianças. Dentre as intervenções, destaca-se a utilização dos polifenóis por apresentar resultados promissores em doenças neurológicas (Karalis et al., 2011). Os polifenóis, por definição, são compostos naturais metabólitos encontrados geralmente em plantas e alimentos e possuem em sua estrutura química ao menos um anel aromático ligado a uma hidroxila (Vinson, 2019). Dentre as suas ações, destaca-se efeitos antioxidantes e anti-inflamatórios (Cory et al., 2018; Manach et al., 2004).

Estudos com roedores apontam para efeitos benéficos do tratamento neonatal com polifenóis na PC, reduzindo os danos na locomoção e no sistema nervoso (Visco et al., 2022b). Uma das explicações para isso é que existe uma relação entre atividade metabólica, o consumo alimentar e atividade neuronal, no qual esses compostos penetram a barreira hematoencefálica e promovem uma série de mecanismos plásticos, facilitando assim a adaptação e recuperação a determinados insultos (Pontes et al., 2023). Dentre os vários mecanismos de atuação, sabe-se que o tratamento com os polifenóis age facilitando a produção de BDNF, repercutindo em melhorias na função cerebral (Gomez-Pinilla; Nguyen, 2012).

Apesar dos polifenóis possuírem propriedades antioxidantes, eles também atuam na saúde e homeostase através de mecanismos pró oxidantes e pró apoptóticos, além de atuar na função mitocondrial (Stevens; Revel; Maier, 2018). O resveratrol, um dos polifenóis, apresentou efeitos promissores na modulação da função mitocondrial, apresentando efeitos no complexo mitocondrial I, mas esses efeitos foram observados somente em doses baixas e em camundongos jovens, enquanto em animais idosos observou-se que o tratamento levou a um aumento do estresse oxidativo (Gueguen et al., 2015), apontando mecanismos complexos e ainda desconhecidos da atuação dos polifenóis no organismo.

Os polifenóis lipofílicos podem ser categorizados de acordo com a sua estrutura química, podendo ser fenólicos, flavonoides e não flavonoides (Gomez-Pinilla; Nguyen, 2012). Eles podem ser encontrados na natureza em tons de amarelo, laranja e vermelho, no qual os ácidos fenólicos consistem em uma estrutura que possui um anel fenólico e um ácido carboxílico (Gomez-Pinilla; Nguyen, 2012). Enquanto os flavonoides são compostos contendo cetonas que são compostos por muitos subgrupos, incluindo flavonóides, dihidroflavonóis, flavonas, isoflavonas e flavanonas (Gomez-Pinilla; Nguyen, 2012). Enquanto os não flavonóides têm uma estrutura química diferente dos flavonóides e são comumente encontrados em vinhos tintos (Gomez-Pinilla; Nguyen, 2012).

O grupo dos não flavonóides são formados por três compostos principais: i) curcumina, encontrada em plantas de açafrão, ii) cumarina, encontrada em cereja, morango, damasco, canela e alcaçuz, iii) resveratrol, encontrado em casca de uva, vinho tinto e nozes (Chu, 2014). Sendo compostos utilizados no tratamento de diversas doenças neurológicas e psiquiátricas devido às suas propriedades anti-inflamatórias e antioxidantes (Cory et al., 2018; Manach et al., 2004). Os polifenóis podem atuar na regulação da plasticidade sináptica e, por consequência, promove efeitos terapêuticos em disfunções cerebrais com comprometimentos comportamentais (Wollen, 2010).

Dentre os polifenóis, destaca-se o resveratrol, do grupo estilbenóides, por apresentar efeitos benéficos à saúde, apresentando propriedades anti obesogênica através da inibição do acúmulo de gordura, além de efeitos antidiabético, antioxidante, cardioprotetor e neuroprotetor (Zhang et al., 2021a), sendo um composto produzido por diferentes espécies de plantas, incluindo videiras, amendoins e romãs (Pallàs Et Al., 2013; Smoliga; Baur; Hausenblas, 2011). Sobre os seus efeitos no cérebro em específico, sabe-se que ele possui propriedades antioxidantes, além de ser capaz de modular o sistema colinérgico e atenuar danos em regiões como o hipocampo e córtex pré-frontal, repercutindo melhoras na aprendizagem e no humor (Rebas et al., 2020). O tratamento com o resveratrol demonstrou reduzir a liberação de citocinas pró-inflamatórias e função mitocondrial através de processos de modulação da SIRT1 (Zhao et al., 2021). Entretanto, muitos aspectos da sua atuação no organismo ainda permanecem obscuros, mas sabe-se que o alvo de atuação do resveratrol é no metabolismo (Baur et al., 2006).

Em uma revisão recente a respeito dos efeitos dos polifenóis em lesões cerebrais precoces, similares aos presentes na PC, observou-se que o resveratrol foi o composto mais comum utilizado nos estudos (Pontes et al., 2023). Em contextos de eventos hipóxicos-isquêmicos, observou que o resveratrol atenuou os danos nas substâncias brancas e cinzas do cérebro (Pan et al., 2016). De forma similar, em modelos de desafios imunológicos através da exposição à LPS, o resveratrol reduziu a expressão de citocinas pró inflamatórias, especificamente a IL-1 β e TNF- α , e preveniu a ativação de micróglia (Broderick et al., 2020; Zhang et al., 2013).

Por essa razão, o resveratrol tem sido investigado amplamente na isquemia (ZHU et al., 2022), no qual acredita-se que os seus efeitos terapêuticos se encontram na da inibição da cascata necroinflamatória e, consequentemente, reduz a apoptose e os danos subjacentes à lesão cerebral (Chao et al., 2020; Yang et al., 2018). Esse efeito ocorre devido à redução da produção de biomarcadores, como Bax, bcl-2 e caspase-3 (Le et al., 2019). Adicionalmente, o resveratrol atua também reduzindo o estresse oxidativo, traduzindo-se em uma redução da atividade da

lactato desidrogenase (LDH) e aumento da atividade da superóxido dismutase (SOD) (Chao et al., 2020; Yang et al., 2018). Essa redução do estresse oxidativo associada a redução da neuroinflamação faz com que o resveratrol melhore a função cerebral e promova um efeito protetor e recuperador no tecido nervoso (Lei; Chen, 2018).

Por outro lado, o resveratrol não somente protege contra danos isquêmicos, mas também atua promovendo respostas adaptativas e plásticas, na qual observa-se, após a sua utilização, um aumento na sobrevivência e proliferação celular no cérebro (Shen et al., 2016). Por isso, além de inibir a ativação das micróglias e dos astrócitos, o resveratrol atua intensificando processos como a proliferação, diferenciação, migração e sobrevivência dos neurônios (Yu et al., 2021). Em termos de doses, observa-se que a utilização de 10mg/kg até 40 mg/kg apresentou efeitos promissores reduzindo danos em regiões motoras, como o estriado e córtex, e em regiões implicadas em processos de aprendizagem, como o hipocampo (Li et al., 2020).

Apesar desses efeitos pós lesão, o resveratrol também apresenta efeitos promissores quando utilizados de forma preventiva. Assim, a sua utilização antes da hipóxia-isquemia resultou em uma redução do estresse oxidativo, função mitocondrial, astrogliose, do tamanho da lesão, e protegeu os processos de mielinização dos neurônios (Arteaga et al., 2015). Em termos comportamentais, o pré-tratamento também protegeu contra os prejuízos na aprendizagem, no qual os animais que receberam o pré-tratamento apresentaram um índice de discriminação similar aos animais controles (Arteaga et al., 2015).

De um modo geral, os efeitos do resveratrol no cérebro e no comportamento ocorre através de múltiplas, distintas e complexas vias (Pallàs et al., 2013; Smoliga; Baur; Hausenblas, 2011), mas a função mitocondrial é uma das suas vias de atuação (Da Silva Souza et al., 2023b). Dados indicam que, no pré e pós-evento isquêmico, o tratamento com o resveratrol manteve constante a quantidade de células com membrana interna mitocondrial intacta, além de apresentar uma elevada quantidade de cardiolipina (Arteaga et al., 2015). De forma similar, a preservação da função mitocondrial e redução do estresse oxidativo demonstrou estar relacionado com a melhora das sequelas motoras presentes na PC (Da Silva Souza et al., 2023b). Além disso, o tratamento pós lesão demonstrou alterar a distribuição de fibras musculares e reduziu a quantidade de micróglias ativadas no cerebelo, promovendo melhorias no neurodesenvolvimento (Pereira et al., 2024).

Assim, entende-se que o resveratrol atua na função mitocondrial, no balanço oxidativo e na cascata inflamatória no cérebro em regiões sensoriais, como o córtex somatossensorial, e em regiões motoras, como o cerebelo (Da Silva Souza et al., 2023a; Miguel et al., 2021; Pereira et al., 2024). Curiosamente, o resveratrol apresentam também efeitos benéficos em regiões

envolvidas em processos mais robustos, como a aprendizagem, tomada de decisão, flexibilidade cognitiva, como o córtex pré frontal e o hipocampo, no qual reduziu a inflamação nessas regiões (Ma et al., 2020b). A morfologia dos neurônios também é afetada pelo resveratrol, no qual observa-se uma redução da atrofia dos dendritos e nas ramificações dos neurônios piramidais de CA1 do hipocampo e um aumento dos níveis da SIRT1 (Khanna et al., 2020).

Por essa razão, entende-se que os polifenóis atuam em aspectos morfológicos, funcionais, metabólicos e comportamentais (Calado et al., 2023b; Da Silva Souza et al., 2023b; Pereira et al., 2024). Essas modificações ocorrem através de mecanismos epigenéticos (Pontes et al., 2023). Em consonância com isso, observou-se que a expressão do miRNAs, um gene envolvido em processos de apoptose, teve a sua expressão suprimida após o tratamento com o resveratrol (Bian; Shan; Chen, 2017). Além de reduzir a da cascata inflamatória, o tratamento com o resveratrol promove um aumento da neuroplasticidade através do aumento da proliferação celular e da expressão do CREB-1 no hipocampo (Calado et al., 2023b).

Esses achados são cruciais na medida em que se entende a necessidade de modificações morfológicas nos neurônios hipocampus para formação de memórias de longo prazo (Faria, 2020). Essa potente capacidade modificar a função neurológica através do aumento da plasticidade e de modificações funcionais e morfológicas nos neurônios, além de reduzir a cascata deletério da neuroinflamação, faz com que o resveratrol seja considerado um composto promissor no tratamento de doenças neurológicas. Por essa razão, acredita-se que o tratamento neonatal com resveratrol possa atenuar os prejuízos na formação de novas memórias através da redução da neuroinflamação na paralisia cerebral.

3. HIPÓTESE

A memória e aprendizagem são prejudicadas na paralisia cerebral através do aumento da neuroinflamação no hipocampo de ratos, e o tratamento neonatal com o resveratrol é capaz de atenuar os prejuízos na memória episódica e a neuroinflamação do hipocampo na paralisia cerebral experimental.

4. OBJETIVOS

4.1 OBJETIVO GERAL

Investigar as repercussões da paralisia cerebral e do tratamento neonatal com resveratrol sobre a memória episódica e a neuroinflamação no hipocampo de ratos.

4.2 OBJETIVOS ESPECÍFICOS

Avaliar em ratos submetidos ou não ao modelo de paralisia cerebral associado ou não ao tratamento neonatal com resveratrol

1. A memória episódica de reconhecimento de objetos no P22 e P27;
2. A memória espacial no P23, P24 e P25;
3. O comportamento semelhante à ansiedade no P22, P28 e P29;
4. O peso corporal no P0 e P29;
5. O peso absoluto e relativo do encéfalo no P29;
6. A expressão dos genes IL-1 β , IL-6, TNF- α , CREB-1 e ChAT no hipocampo;
7. A ativação das micróglias no DG, CA1 e CA3 do hipocampo;
8. A proliferação celular no DG, CA1 e CA3 do hipocampo.

5 MATERIAIS E MÉTODOS

5.1 QUESTÕES ÉTICAS

O projeto é de cunho experimental com animais, onde todos os procedimentos foram realizados em conformidade com as diretrizes do Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais do *National Institute of Health Guide for Care and Use of Laboratory Animals* (8ª ed). O projeto foi submetido e aprovado pela Comissão de Ética em Uso Animal (CEUA- 0082/2022) da UFPE.

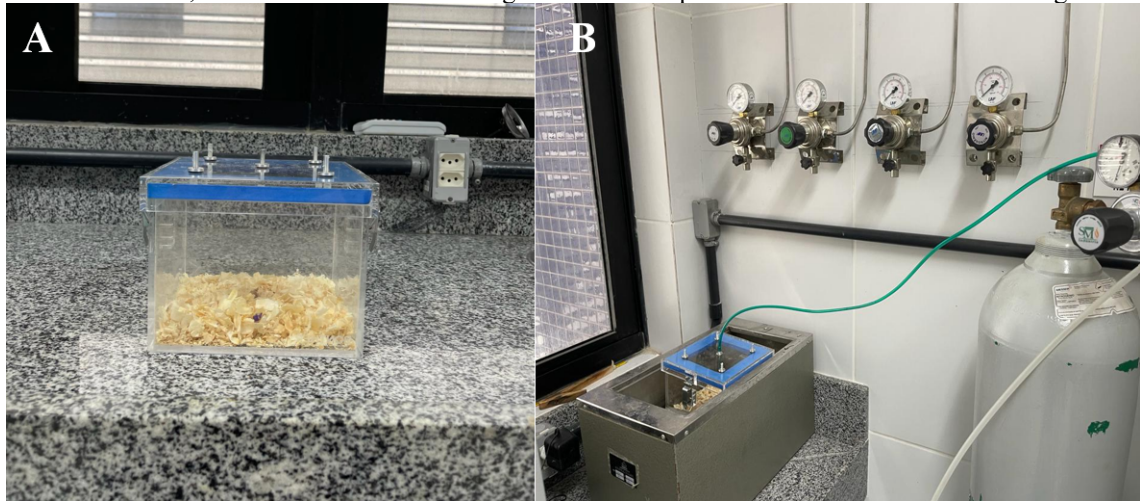
5.2 ANIMAIS E CONDIÇÕES DO BIOTÉRIO

O presente estudo foi realizado no Laboratório de Estudos em Nutrição e Instrumentação Biomédica (LENIB), no Departamento de Nutrição da UFPE, e na Unidade de Estudos em Nutrição e Plasticidade Fenotípica, no Centro de Ciências da Saúde da UFPE. Nesse estudo, foram utilizados filhotes ratos machos *Rattus Norvegicus Albinus* da linhagem Wistar provenientes do biotério de criação do Departamento de Nutrição da UFPE, no qual os animais foram mantidos no biotério de manutenção do Departamento de Nutrição da UFPE com temperatura de 22 ± 2 °C, ciclo claro-escuro invertido de 12/12 horas (Ciclo claro - 20:00 as 8:00h; Ciclo escuro - 08:00 às 20:00 h), alojados em gaiolas de polipropileno (46cmx34cmx20cm) forrada com maravalha estéril e com acesso livre a água e dieta.

5.3 MODELO EXPERIMENTAL DA PARALISIA CEREBRAL

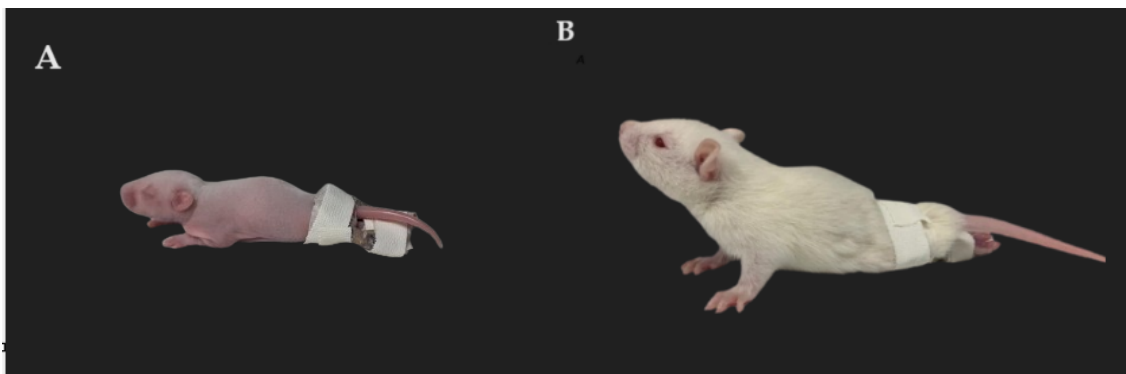
O modelo experimental de paralisia cerebral foi baseado nos experimentos de Strata et al. (2004), Coq et al. (2008) e Marcuzzo et al. (2010) que associaram a anóxia perinatal e restrição sensório-motora das patas posteriores. Visto isso, os animais foram submetidos a dois episódios de anóxia pós-natal, no dia do nascimento (P0) e primeiro dia pós-natal (P1). Para isso, foram colocados dentro de uma câmara de vidro parcialmente imersa em água a 37°C e expostos a nitrogênio (100%) a 9L/min por 12 minutos cada (figura 8). Feito isso, os filhotes foram colocados em temperatura ambiente para recuperação e depois devolvidos as suas respectivas mães. No segundo dia (P2) ao vigésimo oitavo dia (P28) de vida pós natal, foi realizada a restrição sensório-motora durante 16 horas por dia, nas 8 horas restantes a livre movimentação do animal foi permitida. Para a imobilização sensório-motora, utilizou-se um molde de epóxi em um formato que permite apenas movimentos limitados da articulação do quadril, deixando os membros posteriores estendidos com o auxílio de fita adesiva complementar ao molde reduzido (figura 9), sem que a eliminação de urina, de fezes e os cuidados maternos fossem prejudicados (Strata et al., 2004). Esse modelo mimetiza as características fenotípicas da PC diplégica espástica (Coq et al., 2008, 2020; Pereira et al., 2021).

Figura 8: Materiais utilizados para realização da anóxia perinatal no P0 e P1 por 12min. A **figura A** mostra a câmara de vidro em que os animais foram colocados. A **figura B** mostra os materiais utilizados para a anóxia; banho maria à 37°C, câmara de vidro imersa na água e conectada por uma cânula ao cilindro de nitrogênio.



Fonte: Autor (2024).

Figura 9: Filhotes do grupo PC com as patas posteriores em extensão com o auxílio de uma órtese de epóxi fixada nas patas com auxílio de fita microporosa e de fita esparadrapo para reproduzir as alterações fenotípicas da PC. **Figura A** mostra um animal com a órtese no P7. A **figura B** demonstra um animal com a órtese no P17.



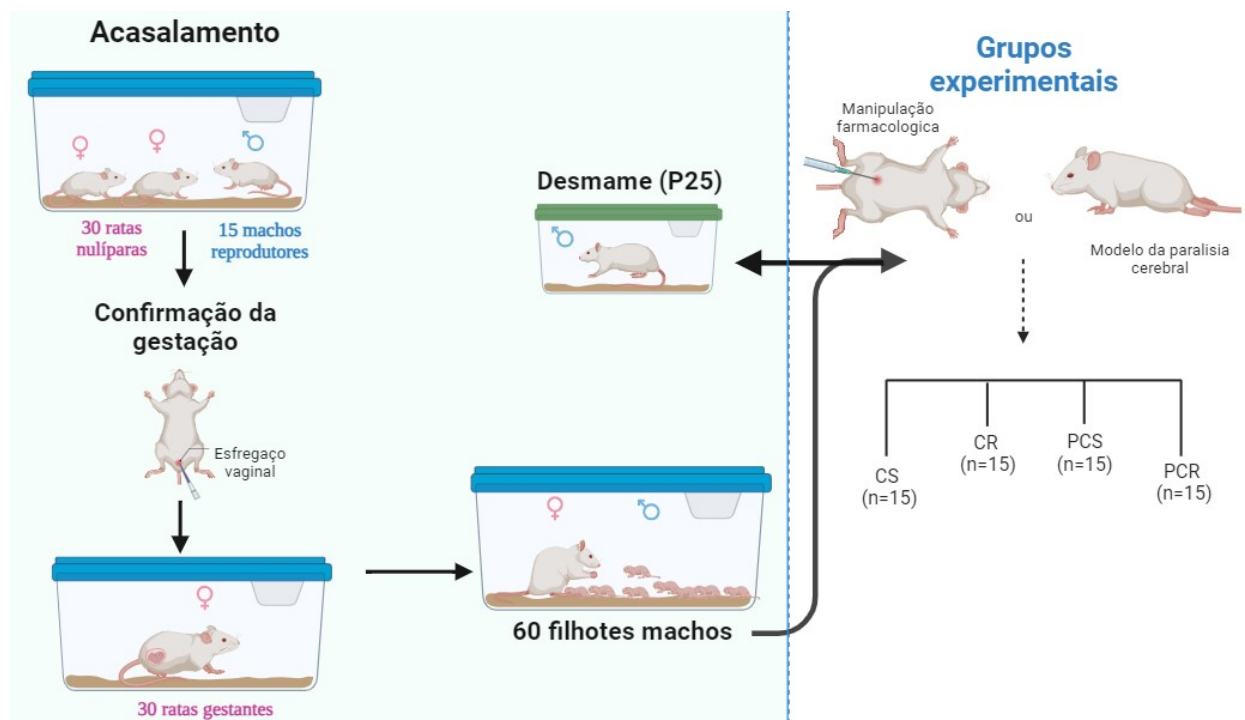
Fonte: Autor (2024).

5.4 GRUPOS EXPERIMENTAIS

Os filhotes foram obtidos através de 30 ratas nulíparas da espécie *Rattus Norvegicus Albinus* da linhagem Wistar, provenientes do biotério de criação do Departamento de Nutrição da UFPE. Essas ratas foram colocadas para acasalar com machos reprodutores (na proporção de duas fêmeas para um macho), não consanguíneos, com idade entre 90 e 120 dias, e peso corporal entre 220g e 260g. A confirmação da gestação foi feita através do esfregaço vaginal, quando foi detectada a presença de espermatozóide na secreção vaginal, das ratas gestantes foram então separadas do macho e distribuídas em gaiolas individuais durante o todo o período de gestação. No dia do nascimento dos filhotes, foi realizada a distribuição dos filhotes machos em quatro grupos experimentais de forma aleatória, onde foram incluídos somente filhotes machos saudáveis e com o peso corporal adequado (6 a 8 gramas), as fêmeas que nasceram na ninhada foram utilizadas apenas para completar a ninhada com 8 filhotes até o dia do desmame.

Os grupos experimentais foram formados de acordo com a indução da PC e da manipulação farmacológica administrada nos filhotes: i) Controle Salina (CS, n=15) formado por filhotes que receberam substância salina; ii) Controle Resveratrol (CR, n=15) formado por filhotes que receberam resveratrol; iii) PC Salina (PCS, n=15), formado por filhotes submetidos ao modelo experimental da paralisia cerebral e que receberam substância salina; PC Resveratrol (PCR, n=15), formado por filhotes submetidos ao modelo experimental da paralisia cerebral e que receberam resveratrol. Cada ninhada foi composta por 8 filhotes que permaneceram com as mães até o 25º dia pós-natal quando eles foram desmamados. Após o desmame, os machos foram colocados em gaiolas individuais, no qual permaneceram até a eutanásia por decapitação ou perfusão no 29º dia pós-natal.

Figura 10: Distribuição dos grupos experimentais. 60 filhotes machos divididos nos seguintes grupos: Controle salina (n=15), Controle Resveratrol (n=15), PC Salina (n=15) e PC Resveratrol (n=15).



Fonte: Autor (2024).

5.5 MANIPULAÇÃO FARMACOLÓGICA COM O RESVERATROL

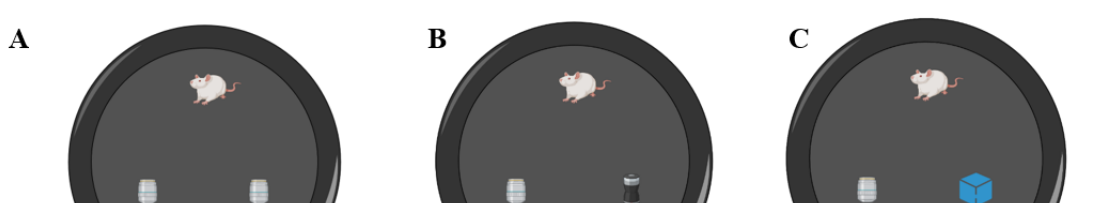
Após o nascimento dos animais, os filhotes machos foram alocados de forma aleatória conforme os grupos experimentais. A administração da droga foi feita diariamente do P3 ao P21 por via intraperitoneal (Girbovan e Plamondon, 2015). Os animais foram distribuídos em: 1- tratados com resveratrol (dose diária, 10mg/kg) e 2-salina (0,9% NaCl), onde volume de injeção foi de 0,1 ml / 100g peso do rato). Os ratos foram pesados diariamente e o volume de injeção foi ajustado para corresponder ao peso corporal do animal.

5.6 TESTE DE RECONHECIMENTO DO NOVO OBJETO

A memória episódica foi avaliada através do teste de reconhecimento do novo objeto (do inglês- *novel object recognition test*), que é configurado como um teste rápido e eficiente para testar diferentes fases e tipos de memória e aprendizagem em ratos. Além de ser um teste adaptável para verificação de diferentes tipos de memória (Lueptow, 2017). O teste foi realizado em duas etapas, inicialmente ocorreu a sessão de treinamento no P22, após o término da manipulação farmacológica. Para o treinamento, o animal foi colocado no campo aberto com dois objetos idênticos posicionados no centro do campo, em uma distância de 5cm de um objeto para o outro, e foi permitido que o filhote explorasse por 5min tais objetos (Lueptow, 2017). Finalizando essa etapa, o animal foi retirado e colocado de volta a sua gaiola para que ocorresse o armazenamento das informações de curto prazo. Em paralelo a isso, o campo e os objetos foram limpos com álcool 70% para que não houvesse pistas olfativas. Entretanto, um dos objetivos foi substituído por um objeto não familiar. Em seguida, o animal foi colocado novamente no campo para a sessão do teste, no qual essa etapa foi filmada um período de 5min para posterior análise.

Após um período de 5 dias, o animal foi submetido a uma nova sessão de teste para avaliar a memória de longo prazo. Assim, no P27 o animal foi devolvido ao campo aberto, na presença de dois objetos no centro: o familiar (presente em todas as etapas) e um novo objeto (Lueptow, 2017) durante 5 minutos de duração (figura 11). Todos os vídeos foram analisados por um avaliador cego enquanto aos grupos experimentais. Serão contabilizados o tempo de exploração de cada objeto, o tempo de exploração total e a quantidade de exploração realizada em cada objeto. Após isso, foi calculado o índice de discriminação (ID), utilizando a fórmula matemática $[DI = (\text{tempo gasto explorando o objeto novo}) - (\text{tempo gasto explorando o objeto familiar}) / (\text{tempo total de exploração})]$ e o índice de reconhecimento (IR) que foi calculado utilizando a fórmula: $[IR = (\text{tempo explorando o objeto novo} / (\text{tempo total de exploração}) \times 100 (\%)]$.

Figura 11: Ilustração das três etapas do teste de reconhecimento do novo objeto realizado. A **figura A** ilustra a sessão de treinamento com os dois objetos idênticos. A **figura B** demonstra o primeiro teste para verificar a memória episódica de curto prazo. Por fim, **figura C** demonstrando o reteste para avaliar a retenção de longo prazo. Figura criada com o Biorender.



Fonte: Autor (2024).

5.7 TESTE DE LABIRINTO EM T

A memória espacial dos animais foi avaliada através do teste de Labirinto em T, sendo tida como uma tarefa de rota-aprendizagem, na qual não depende de estímulos aversivos ou estímulos visuais (Shoji et al., 2012). Esse é um teste comportamental utilizado para avaliar a aprendizagem do animal (Wu et al., 2018), no qual o labirinto é formado por uma estrutura de plexiglass preto em formato de T. Ele possui portas deslizantes que dividem o espaço e é composto por uma haste de 87×20 cm, cercada por paredes de 20 cm de altura. Na extremidade, há dois braços medindo 40×10 cm cada. A 1 cm da ponta de cada braço, existe um espaço circular de 1 cm de diâmetro e 1,5 cm de profundidade, destinado à colocação de recompensas (figura 12). O teste foi realizado quatro vezes por dia em um período de 3 dias consecutivos, no P25-P27 durante 5min no máximo e sempre no período escuro do biotério.

Visando a adaptação ao ambiente, 30min antes do experimento os animais foram transferidos para a sala de análise de comportamento anexa ao biotério. Finalizando a adaptação ao ambiente, o animal foi colocado no braço inicial, a parte inferior do “T”, que é a parte inicial da corrida, com a porta deslizante fechada. Após isso, abriu-se a porta deslizante e foi permitido que o animal explorasse todo o labirinto. Uma vez que o rato passe com as quatro patas para o braço esquerdo ou direito, a porta deslizante era fechada, impedindo assim a saída do animal, deixando-o agora explorar durante 30s todo o braço comprometido que ele escolheu (figura 12). Por fim, o filhote foi devolvido à sua gaiola por 1min e repetiu-se a corrida. No término das duas primeiras corridas, o animal foi devolvido à gaiola por um período de 10min, e repetiu-se as duas etapas anteriores novamente, completando um total de 4 corridas diárias. Após cada corrida a maravalha foi trocada e o labirinto limpo com álcool 70% para eliminar pistas olfativas (Wu et al., 2018). Esse procedimento foi feito por três dias consecutivos. Ao todo foram 12 execuções no final do experimento, onde foi calculada a quantidade de vezes de alternância do animal por escolha correta do animal. O cálculo foi feito por conjunto (a cada duas tentativas) e verificou se o animal escolheu o lado oposto (escolha correta) do lado escolhido anteriormente.

Figura 12: Teste do Labirinto em T no P25 para avaliar a memória espacial e a capacidade de alternância do animal. O teste foi realizado por três dias consecutivos no P25, P26 e P27 em 4 tentativas diárias, onde foi calculada a porcentagem de escolhas corretas (quando o animal alterna de braço) no labirinto.

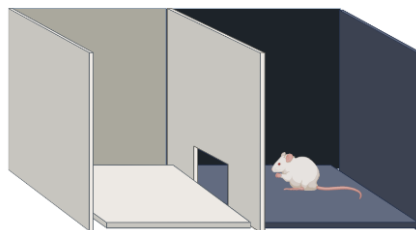


Fonte: Autor (2024).

5.8 CAIXA CLARO ESCURO

No P22 e P29, os animais foram colocados em uma caixa 40cmx40cm e com uma altura de 80cm, possuindo uma parede que divide a caixa em duas extremidades, uma totalmente branca (o lado claro) e outra totalmente preta (o lado escuro), havendo apenas uma entrada central que permite a transição do animal entre os dois lados (figura 13 e 14). O animal foi colocado no lado escuro da caixa, durante o período claro do biotério, e foi permitida a exploração do aparato por um período de 5min. O teste foi filmado por uma câmera acoplada ao teto. Os vídeos foram analisados com o auxílio do software Anymaze para os seguintes parâmetros: tempo de permanência e número de entradas no lado claro e escuro.

Figura 13: Ilustração da Caixa Claro-Escuro. Um aparato constituído por um lado totalmente claro e por outro totalmente escuro, conectados por uma entrada central no aparato. Aumento no número de entradas e tempo de permanência no lado escuro é um indicador de traços comportamentais semelhantes à ansiedade. Figura criada no Biorender.



Fonte: Autor (2024).

Figura 14: Animal realizando o teste da caixa claro escuro no P22. Um teste comportamental baseado na capacidade do conflito do animal de preferir lugares escuros, mas também de explorar o novo (o claro), utilizado para avaliar traços comportamentais semelhantes à ansiedade.

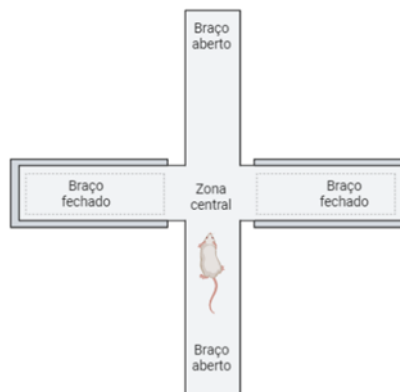


Fonte: Autor (2024).

5.9 LABIRINTO ELEVADO EM CRUZ

Para avaliar os parâmetros comportamentais relacionados à ansiedade, foi utilizado também o Labirinto Elevado em Cruz (LEC) (Pellow et al., 1985) no P28. O aparato é constituído por uma estrutura de madeira com quatro braços a 50cm de distância do chão, onde cada braço possui 50cm de comprimento x 10cm de largura. Dois desses braços possuem paredes de 40cm de altura e, por isso, são denominados de “braços fechados” do LEC, enquanto os outros dois não possuem paredes e, por isso, são chamados de “braços abertos” do LEC (figura 15). Visando a diminuição da probabilidade de ocorrência de acidentes durante a exploração dos roedores, os braços abertos do LEC possuem bordas com elevação de 1 cm. O ponto de encontro entre os quatros braços é conhecido como “zona central” do Labirinto e possui as dimensões de 10cm x 10cm.

Figura 15: Ilustração do Labirinto Elevado em Cruz. Possuindo 2 braços fechados, uma zona central e dois braços abertos. Criada no Biorender.



Fonte: Autor (2024).

Os filhotes foram transferidos do biotério para a sala de sala de comportamentos 30min antes do início do teste para que ocorresse a adaptação ao ambiente. Após o fim da adaptação, o animal foi colocado na área central do aparato com a cabeça em direção a um dos braços abertos durante um período de 10min (figura 16). O labirinto foi limpo com álcool 70% após o uso de cada animal para eliminar a influência de odores na performance do animal seguinte. As sessões foram filmadas no escuro com uma câmera acoplada ao teto posicionada acima da área central durante todo o experimento. Os vídeos foram analisados no software Anymaze para os seguintes parâmetros: número de entradas, tempo de permanência nos braços abertos e nos fechados, como também o tempo de permanência na área central (Cavalcanti et al., 2021; Takada et al., 2016).

Figura 16: Teste do Labirinto Elevado em Cruz com animal do grupo PC Salina no P28. Foram analisados os parâmetros de tempo de permanência, quantidade entradas e número de bolos fecais nos braços abertos, braços fechados e na zona central do aparato.



Fonte: Autor (2024).

5.10 EUTANÁSIA E PESO DOS ANIMAIS E DO ENCÉFALO

Todos os animais tiveram o seu peso registrado diariamente do P0-P29 utilizando uma balança de precisão (Marte, capacidade de 1 kg e sensibilidade de 0,01 g) do Laboratório de Estudos em Nutrição e Instrumentação Biomédica da UFPE. Metade dos animais (n=8, por grupo experimental), foram eutanasiados por decapitação no P29 após um período de 4 horas de jejum. Em seguida, o encéfalo foi retirado para obtenção do peso absoluto. Após isso, o hipocampo foi rapidamente removido e armazenado em freezer -70 °C para análises de expressão gênica.

5.11 EXTRAÇÃO DO RNAm E EXPRESSÃO GÊNICA NO HIPOCAMPO

Após a decapitação, os hipocampos passaram por análises de expressão gênica pelo método de RT-PCR (em português, reação da transcriptase reversa seguida pela reação em cadeia da polimerase) para avaliar biomarcadores da neuroinflamação e da plasticidade sináptica. O mRNA total foi extraído do hipocampo com o reagente TRIzol, utilizando o

método do isotiocianato de guanidina (Chomczynski & Sacchi, 1987), de acordo com as instruções do fabricante (Invitrogen, Carlsbad, CA, EUA). Os pellets de RNA foram lavados com etanol a 75% e centrifugados a 7500× durante 5 minutos a 4°C, secos ao ar e fervidos em água ultrapura tratada com DEPC. A quantificação do mRNA foi efetuada em um espectrofotômetro NanoDrop 2000 (Thermo Scientific, EUA) e a pureza foi avaliada utilizando uma gama de 260/280 nm. Como exemplo, apenas com um rácio superior a 1,90. O gene Housekeeping Gapdh foi usado como controle interno para a análise de PCR. A expressão relativa do mRNA dos genes foi calculada usando o método comparativo 2- $\Delta\Delta C_t$ (Livak; Schmittgen, 2001).

5.12 RT-PCR E EXPRESSÃO GÊNICA DA IL-1B, IL-6, TNF-A, CREB-1 e CHAT NO HIPOCAMPO

A reação em cadeia da polimerase em tempo real (RT-PCR) foi posteriormente realizada para β 2-microglobulina (β 2M) como normalizador e para os genes de interesse: IL-1 β , IL-6, TNF- α , CREB-1 e ChAT (tabela 1). utilizando o SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, EUA). As sequências foram obtidas a partir da base de dados NCBI e foram elaboradas utilizando o Primer Input Software (v.0.4.0). O gene Housekeeping foi utilizado como controle interno para a análise por PCR. A expressão relativa de mRNA dos genes foi calculada usando o método comparativo 2- $\Delta\Delta C_t$ (Livak; Schmittgen, 2001).

Tabela 1: Primers utilizado para a análise de expressão gênica por PCR.

Genes	Forward primer	Reverse primer	Amplico n Size
B2M	TGACCGTGATCTTTCTGGTG	ACTTGAATTTGGGGAGTTTTCTG	73 bp
IL-6	AAGGAGTGGCTAAGGACCAA	GTTTGCCGAGTAGACCTCAT	89 bp
TNF-α	AAGCATGATCCGAGATGTGG	AGTAGACAGAAGAGCGTGGT	141 bp
CHAT	CCTGGAAAAGGCTCCCCAAA	CCCAAACCGCTTCACAATGG	178 bp
CREB	GACGGAGGAGCTTGTACCAC	TGGCATGGATACCTGGGCTA	156 bp

Fonte: Autor (2024)

5.13 PERFUSÃO TRANSCARDÍACA

A outra metade dos animais (n=7, por grupo experimental) foram eutanasiados por perfusão transcardíaca. Primeiramente, os filhotes foram anestesiados profundamente com quetamina (100mg/kg) e xilazina (12mg/kg,) para abertura da cavidade torácica. Logo após, foi introduzida uma cânula para perfusão no ventrículo esquerdo do coração, acoplada a uma bomba peristáltica que é acionada em velocidade compatível com a manutenção da integridade dos vasos sanguíneos. Inicialmente, foi infundido 150 ml de solução salina tamponada em tampão fosfato. A perfusão com salina foi seguida com a infusão de 200 ml de solução fixadora (4% de paraformaldeído). Ao final da passagem do fixador, os encéfalos foram retirados do

crânio. Por fim, os encéfalos foram pós-fixados na mesma solução fixadora overnight e em seguida acrescida de sacarose (30%) por 24-48h. Por fim, os encéfalos foram congelados em n-hexano e armazenados em freezer -70° até o momento da criosecção e análise imuno-histoquímica.

5.14 DETERMINAÇÃO DA DENSIDADE E ATIVAÇÃO DAS MICRÓGLIAS NO HIPOCAMPO (IBA+)

Os encéfalos removidos por perfusão passaram por cortes coronais de 30µm de espessura no criostato, sendo coletados cortes do hipocampo posterior (entre -1,82 e -2,18 mm posterior ao bregma). Para coloração das micróglia, os cortes do hipocampo foram incubados em 10% de H₂O₂ em metanol e posteriormente em 10% de H₂O₂ em tampão fosfato (0,1 M, pH 7,4), contendo 3% de Triton X-100 (PBT). Feito isto, as secções foram incubadas a 4°C por 48h em anticorpo primário (rabbit anti-Iba1 / IAF1, 1: 10.000, Wako) diluído com 5% de soro de cavalo em PBT (Saavedra et al., 2021). Após isso, os cortes foram incubados por um período de 2h no anticorpo secundário em temperatura ambiente (biotinylated anti-rabbit antibody, 1:750, Sigma-Aldrich). Após isso, os cortes dos hipocampos foram incubados com soluções do complexo avidina-biotina peroxidase (ABC Elite Kit; Vector Laboratories, Burlingame, CA, EUA) e uma solução do kit de coloração de diaminobenzidina (DAB Kit; Vector Laboratories) para realizar a marcação das micróglia (Saavedra et al., 2021).

Os cortes dos encéfalos foram montados em lâminas gelatinizadas e lamínulas com Cytooseal (Thermo Scientific, EUA). Para avaliação das células microgliais Iba1+ do hipocampo, 3 campos por secção foram selecionados, de um total de 4-6 secções por cortes (n=7 animais por grupo) no DG, CA1 e CA3 do hipocampo para realizar a contagem de micróglia. Os campos selecionados foram analisados com microscópio óptico com aumento de 20x. O software ImageJ foi utilizado para a análise do número de células/área (Saavedra et al., 2021). As células microgliais com um pequeno *soma* e poucos a numerosos processos foram consideradas micróglia ramificadas (tipos I-III), enquanto aquelas com uma grande soma ou corpo amebóide, e processos mais espessos e curtos, foram consideradas micróglia ativadas (tipos IV-V) (Saavedra et al., 2021); Foram apresentados os dados de densidade de micróglia (n/mm³ – Total de células dividido pela área avaliada x 1.000.000 x 0,001 x 40) e porcentagem de ativação das micróglia relativo ao total.

5.15 PROLIFERAÇÃO CELULAR NO HIPOCAMPO APÓS ADMINISTRAÇÃO DE BROMODEOXIURIDINA (BRDU)

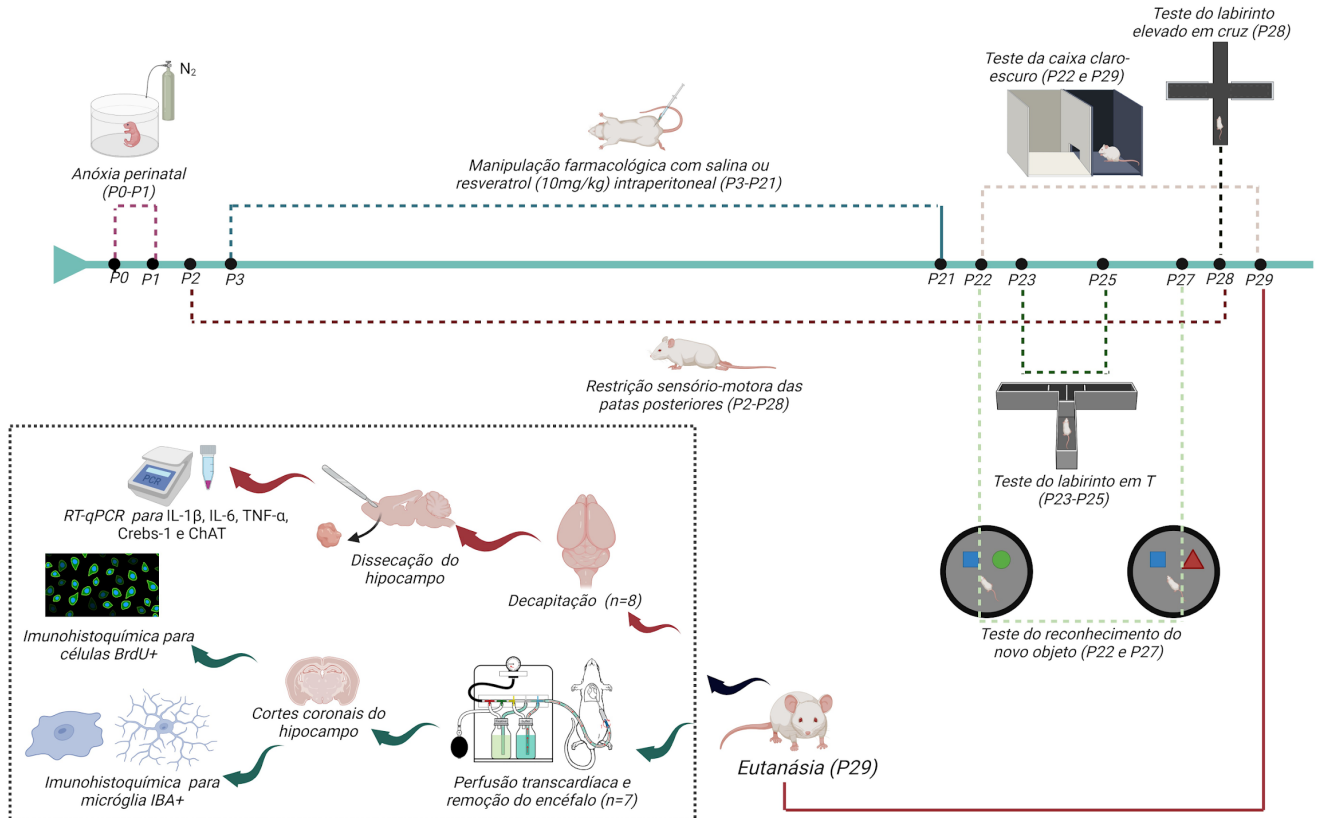
Para verificar a proliferação celular no hipocampo, os animais que foram eutanasiados por perfusão (n=7, por grupo experimental) receberam previamente, no P5 e P6, uma injeção intraperitoneal por dia de BrdU (5-bromo-2'-deoxiuridina- BrdU, Sigma-Aldrich Co. LLC) uma

dose de 50mg/kg. O BrdU é um análogo da timidina que marca o DNA durante a fase S do ciclo celular (Eriksson et al., 1998), pois é um marcador mitótico utilizado para identificar neurônios recém-nascidos (Cameron; McKay, 2001; Taupin, 2007). Assim, o BrdU é utilizado na investigação da proliferação celular (Hancock et al., 2009). Por isso, as secções foram submetidas a um processo de imuno-histoquímica, no qual os cortes do hipocampo foram lavados em substância tamponada com fosfato (PB) e incubadas em Triton (X-100 0,3%, St. Louis, MO, USA) contendo 10% de peróxido de hidrogênio. Subsequentemente, as secções foram incubadas em metanol absoluto e lavadas em PB e incubadas em formamida (50% em SSC, solução de sal de citrato de sódio, Sigma-Aldrich) a 65°C durante 2 horas. Após o processo de lavagem, ocorreu a desnaturação do ADN em HCl (1N) a 37°C. Posteriormente, as secções foram incubadas em solução tampão de borato (pH 8,4) (Diaz-Chávez et al., 2020).

Após esta fase, as secções foram incubadas durante a noite (4°C) numa solução com anticorpo primário anti-BrdU (mouse anti-BrdU, 1: 30.000 Roche Molecular). No dia seguinte, as secções foram lavadas 4min a 5 min em PBS e incubadas no escuro durante 2h com anticorpo secundário biotinilado (1: 750, Vector Laboratories e desenvolvido em complexo avidina-biotina (kit Elite ABC, Vector Laboratories) e diaminobenzidina (kit de coloração DAB, Vector Laboratories). Posteriormente, as secções foram lavadas e montadas em lâminas com Cytoseal após secagem e cobertas com lamínulas. Por fim, as imagens foram captadas utilizando um microscópio óptico com uma lente objetiva de 20x em 4 secções por animal. O número de células BrdU+ no hipocampo foi contado por um investigador cego para cada grupo experimental. Os limites das áreas DG, CA1 e CA3 do hipocampo foram delineados digitalmente (figura 17).

Figura 17: Linha do tempo de todos os experimentos realizados: anóxia perinatal, restrição sensório-motora, manipulação farmacológica, teste da caixa claro-escuro, teste do reconhecimento do novo objeto, teste do labirinto elevado em cruz e sacrifício. Figura criado no *Biorender*.

Linha do tempo dos experimentos



Fonte: Autor (2024).

5.16 ANÁLISE ESTATÍSTICA

Foi utilizado o software GraphPadPrism® versão 8.0.2 para análises dos dados e construção dos gráficos. Os dados obtidos foram quanto à normalidade da distribuição dos dados através do teste de Shapiro-Wilk. Se constatada distribuição normal, foram realizados os testes paramétricos adequados como o Anova 2-Way para comparação dos grupos nas análises realizadas em apenas uma idade do animal, ou o Anova 2-Way Medidas repetidas para comparação dos grupos nas análises realizadas em mais de uma idade. Nos dados sem distribuição normal, foram adotados testes estatísticos não paramétricos como o teste de Kruskal-Wallis e Friedman. Seguido pelo teste post-hoc de Tukey. Os resultados foram expressos como média $\pm$ e erro padrão da média. O nível de significância utilizado foi de 5%.

6. RESULTADOS

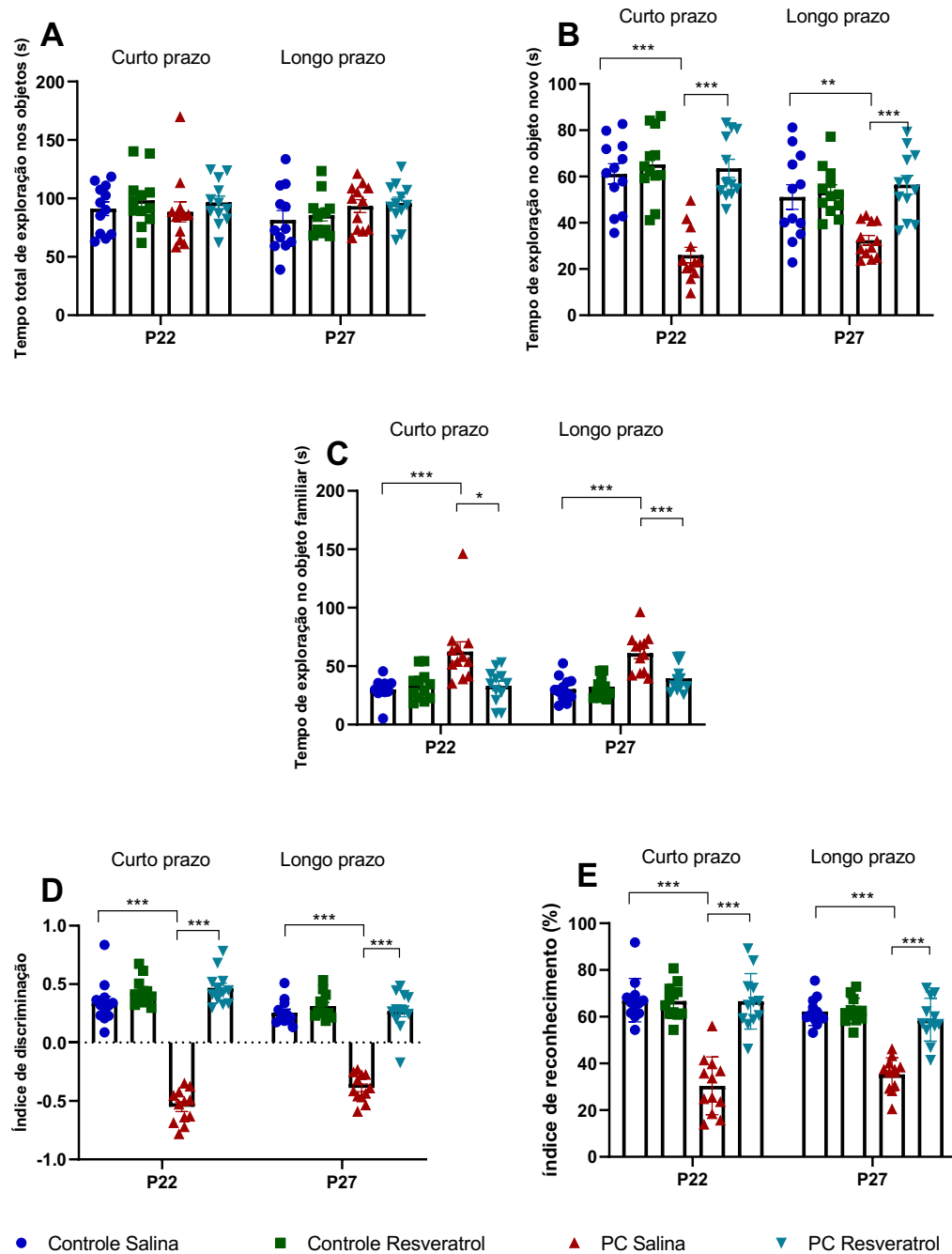
5.17 6.1 MEMÓRIA DE RECONHECIMENTO

A memória episódica reconhecimento foi avaliada pelo teste de reconhecimento do novo objeto no P22 e P27. No P22, notou-se diferenças no tempo de exploração do objeto novo (Kruskal-Wallis statistic 24.62; $p < 0,001$), tempo de exploração no objeto familiar (Kruskal-Wallis statistic 18.48; $p = 0,003$), no índice de discriminação [$F(3, 88) = 7,491$; $p = 0,0002$] e no índice de reconhecimento [$F(3, 88) = 7,491$; $p = 0,0002$] entre os animais CS vs. PCS e PCS vs. PCR. Entretanto, não foram encontradas diferenças no tempo total de exploração entre nenhum grupo experimental ($p > 0,05$). Os animais do grupo PCS apresentaram um tempo de exploração no objeto novo reduzido (PCS = $26,08 \pm 3,342$ vs. CS = $61,13 \pm 4,380$; $p = 0,001$) (figura 18B) e apresentaram um aumento no tempo de exploração no objeto familiar em comparação com os animais do grupo CS (PCS = $62,41 \pm 8,346$ vs. CS = $30,06 \pm 2,708$; $p = 0,007$) (figura 18C). Estes animais consequentemente apresentaram um índice de discriminação (PCS = $-0,549 \pm 0,041$ vs. CS = $0,342 \pm 0,054$; $p < 0,0001$) e índice de reconhecimento (PCS = $22,53 \pm 2,03$ vs. CS = $67,11 \pm 2,67$; $p < 0,0001$) (figuras 18D e 18E) menor. Os filhotes do grupo PCR apresentaram aumento no tempo de exploração no objeto novo (PCR = $63,56 \pm 3,915$ vs. PCS = $26,08 \pm 3,342$; $p = 0,0006$) (figura 18B), uma redução no tempo de exploração do objeto familiar (PCR = $33,09 \pm 4,906$ vs. PCS = $62,41 \pm 8,346$; $p = 0,0106$) (figura 18C), um aumento do índice de discriminação (PCR = $0,467 \pm 0,042$ vs. PCS = $-0,549 \pm 0,041$; $p < 0,0001$) e no índice de reconhecimento (PCR = $73,36 \pm 2,092$ vs. PCS = $22,53 \pm 2,032$; $p < 0,0001$) (figuras 18D e 18E) em relação ao grupo PCS.

No P27, as diferenças persistiram para o tempo de exploração no objeto novo [$F(3,33) = 9,905$; $p < 0,0001$], no objeto familiar [$F(3,33) = 14,24$; $p < 0,0001$] e para o índice de discriminação [$F(3, 88) = 7,491$; $p = 0,0002$] e índice de reconhecimento [$F(3, 88) = 7,491$; $p = 0,0002$]. Não foram encontradas diferenças no tempo total de exploração dos objetos [$F(3, 33) = 1,439$; $P = 0,2490$] (figura 18A). Os animais do grupo PCS apresentaram uma redução no tempo de exploração de objetos novos (PCS = $32,42 \pm 2,147$ vs. CS = $51,13 \pm 5,371$; $p = 0,0028$) (figura 18B) e apresentaram um aumento no tempo de exploração no objeto familiar (PCS = $61,04 \pm 4,818$ vs. CS = $30,44 \pm 3,00$; $p < 0,0001$) (figura 18C). Consequentemente, apresentaram uma redução no índice de discriminação (PCS = $-0,387 \pm 0,034$ vs. CS = $0,254 \pm 0,030$; $p < 0,0001$) e no índice de reconhecimento (PCS = $30,61 \pm 1,68$ vs. CS = $62,73 \pm 1,52$; $p < 0,0001$) (figuras 18D e 18E) quando comparados com os animais do grupo controle salina. Os animais do grupo PCR, no entanto, apresentaram um aumento no tempo de exploração do objeto novo

(PCR= $56,43 \pm 4,059$ vs. PCS= $32,42 \pm 2,147$; $p=0,0001$) e uma redução no tempo de exploração no objeto familiar (PCR= $39,58 \pm 3,378$ vs. PCS= $61,04 \pm 4,818$; $p=0,0014$) (figuras 18B e 18C). Por isso, apresentaram um aumento no índice de discriminação (PCR= $0,270 \pm 0,050$ vs. PCS= $-0,387 \pm 0,034$; $p<0,0001$) e no índice de reconhecimento (PCR= $63,50 \pm 2,489$ vs. PCS= $30,61 \pm 1,683$; $p<0,0001$) (figuras 18D e 18E) comparado com os filhotes do grupo PC Salina.

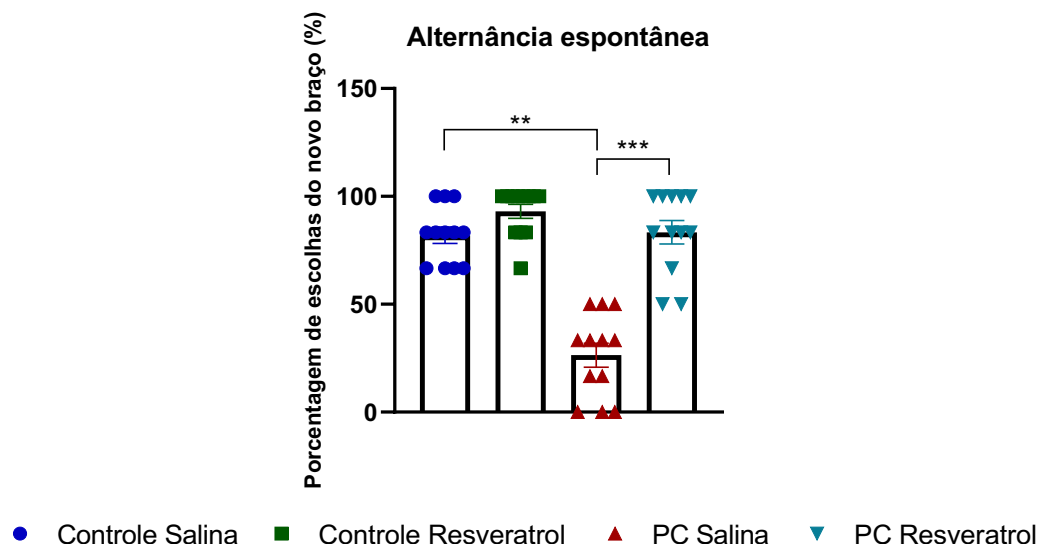
Figura 18: Teste de reconhecimento de novos objetos no P22 e P27 nos quatro grupos experimentais CS (n=12), CR (n=12), PCS (n=12) e PCR (n=12). **Figura A** apresenta o tempo total de exploração dos animais. **Figura B** apresenta o tempo de exploração do objeto novo. **Figura C** apresenta o tempo de exploração do objeto familiar. **Figura D** demonstra o índice de discriminação de novos objetos. **Figura E** demonstra o índice de reconhecimento. Os valores foram expressos como média $\pm$ erro padrão da média. ANOVA two-way multiple comparisons Tukey's post-hoc test ou Kruskal-Wallis statistic. * $p<0,05$; ** $p<0,01$; *** $p<0,001$.



5.18 6.2 MEMÓRIA ESPACIAL

A memória espacial dos animais foi avaliada através do teste de labirinto em T nas idades 25, 26 e 27 de vida pós natal através da porcentagem de alternância total. Foi constatada diferenças na alternância espontânea dos animais (Kruskal-Wallis statistic 29,52; $p < 0,0001$). Os animais do grupo PC Salina apresentaram uma quantidade menor de alternância no labirinto, escolhendo o mesmo braço que já havia sido explorado ($PCS = 25,00 \pm 5,618$ vs. $CS = 81,94 \pm 3,815$; $p = 0,0016$). Contudo, os animais do grupo PC Resveratrol apresentaram um aumento na alternância ($PCR = 83,33 \pm 5,428$ vs. $PCS = 25,00 \pm 5,618$; $p = 0,0003$), (figura 13).

Figura 19: Porcentagem da alternância (escolhas seguidas de lados opostos do labirinto) no teste do Labirinto em T nas idades 25, 26 e 27 de vida pós natal dos quatros grupos experimentais CS (n=12), CR (n=12), PCS (n=12) e PCR (n=12), foram realizadas 4 corridas por dia. Os filhotes do grupo PCS apresentam uma maior porcentagem de escolhas erradas no teste ($p=0,0016$) comparado ao grupo CS. Enquanto o tratamento com resveratrol aumentou a porcentagem de escolhas corretas ($p=0,0003$). Não houve diferenças significativas entre o grupo CS e CR. Valores expressos em média $\pm$ erro padrão da média. Kruskal-Wallis statistic e Tukey's post-hoc test. * $p<0,05$; ** $p<0,01$; *** $p<0,001$.



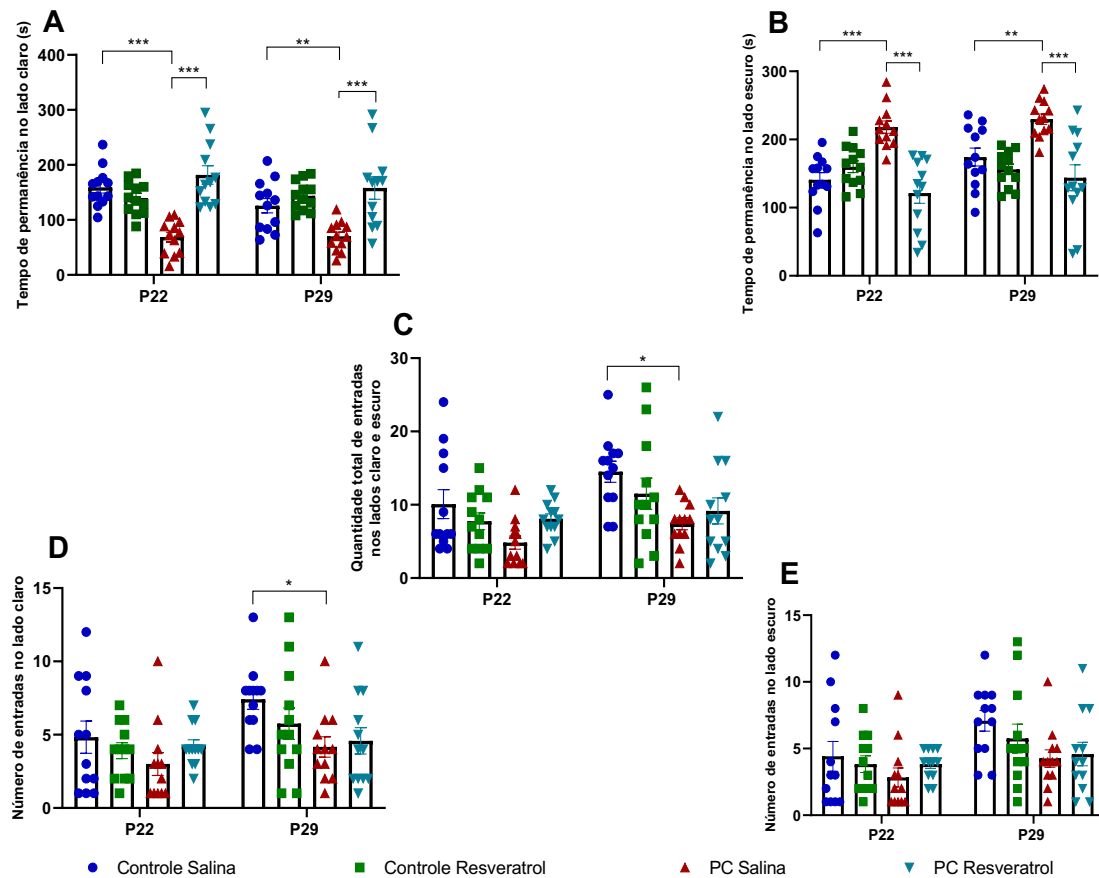
6.3 COMPORTAMENTO SEMELHANTE À ANSIEDADE: CAIXA CLARO-ESCURO

A ansiedade traço foi avaliada através do teste da caixa claro escuro no P22 e P29 dos animais. No P22, foram observadas diferenças no tempo de permanência no claro [$F(3, 33) = 15,89$; $p<0,0001$] e no tempo de permanência no lado escuro [$F(3, 33) = 15,94$; $p<0,0001$]. Não foi possível observar diferenças na quantidade de entradas no lado claro, no lado escuro ou na quantidade total de transições entre os lados da caixa entre os grupos experimentais. Os animais do grupo PC Salina passaram menos tempo explorando o lado claro da caixa ($PCS = 68,72 \pm 8,841$ vs. $CS = 159,1 \pm 10,34$; $p<0,0001$) e ficaram mais tempo no lado escuro ($PCS = 231,3 \pm 8,841$ vs. $CS = 140,9 \pm 10,36$; $p<0,0001$), sinalizando traços comportamentais semelhantes à ansiedade. No entanto, os animais do grupo PC Resveratrol apresentaram um aumento no tempo de permanência do lado claro ($PCR = 181,7 \pm 16,75$ vs. $PCS = 68,71 \pm 8,841$; $p<0,0001$) e uma redução no tempo de permanência no lado escuro ($PCR = 118,1 \pm 16,75$ vs. $PCS = 231,3 \pm 8,841$; $p<0,0001$) (figura 20).

No P29 os animais foram testados novamente. Notando diferenças no tempo de permanência no lado claro [$F(3, 33) = 8,868$; $p=0,0002$], no tempo de permanência no lado escuro [$F(3, 33) = 9,005$; $p=0,0002$], na quantidade total de entradas no lado claro e escuro [$F(3, 33) = 3,714$; $p=0,0209$] e na quantidade de entradas no lado claro [$F(3, 33) = 3,269$;

$p=0,0334$]. Os animais do grupo PC Salina apresentaram um menor tempo de permanência no lado claro (PCS= $70,23 \pm 7,717$ vs. CS= $125,8 \pm 13,16$; $p=0,0227$), um aumento no tempo de permanência no lado escuro (PCS= $229,8 \pm 7,717$ vs. CS= $174,2 \pm 13,16$; $p=0,0220$), uma redução na quantidade de entradas no lado claro (PCS= $4,167 \pm 0,6945$ vs. CS= $7,417 \pm 0,6904$; $p=0,0354$) e uma redução na quantidade total de entradas no lado claro e escuro (PCS= $7,417 \pm 0,8207$ vs. CS= $14,50 \pm 1,449$; $p=0,0174$). Os animais do grupo PC Resveratrol apresentaram um aumento no tempo de permanência no lado claro (PCR= $158,1 \pm 20,50$ vs. PCS= $70,23 \pm 7,17$; $p=0,0002$), e uma redução no tempo de permanência no lado escuro (PCR= $141,4 \pm 20,39$ vs. PCS= $229,8 \pm 7,717$; $p=0,0002$). Não foram encontradas diferenças na quantidade de entradas no claro, na quantidade de entradas no escuro e na quantidade total de entradas no claro e escuro entre os grupos PC Resveratrol e PC Salina ($p>0,05$). Também não foram observadas diferenças entre os controles em nenhum dos parâmetros avaliados (figura 20).

Figura 20: Tempo de permanência e número de transições entre os lados claro e escuro no teste da caixa claro-escuro no P22 e P29 dos quatro grupos experimentais CS (n=12), CR (n=12), PCS (n=12) e PCR (n=12). **Figura A** mostra o tempo gasto no lado claro, **figura B** o tempo gasto no lado escuro, **figura C** o número total de entradas no lado claro e escuro, **figura D** o número de entradas no lado claro e **figura E** o número de entradas no lado escuro. Os valores foram expressos como média $\pm$ erro padrão da média. ANOVA two-way multiple comparisons e Tukey's post-hoc test or Kruskal-Wallis statistic. * $p<0,05$; ** $p<0,01$; *** $p<0,001$.

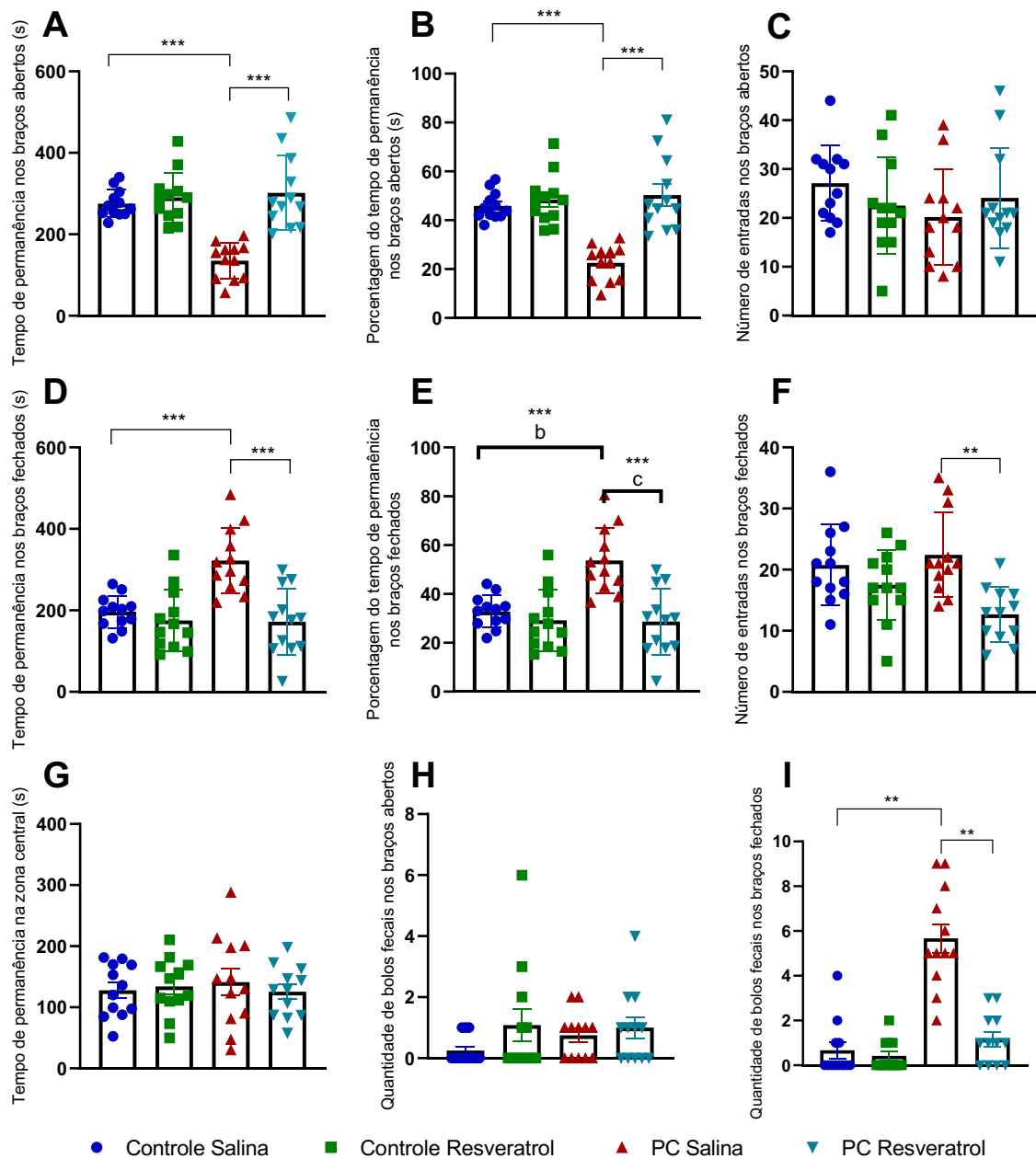


6 6.4 COMPORTAMENTO SEMELHANTE À ANSIEDADE: LABIRINTO ELEVADO EM CRUZ

A ansiedade traço também foi avaliada no P28 em outro aparato, no Labirinto Elevado em Cruz, no qual observou-se diferenças no tempo de permanência [$F(3, 33) = 21,29$; $p < 0,0001$] e na porcentagem do tempo de permanência nos braços abertos [$F(3, 33) = 21,29$], no tempo de permanência [$F(3, 33) = 13,12$; $p < 0,0001$], na porcentagem do tempo de permanência [$F(3, 33) = 13,12$; $p < 0,0001$], na quantidade de entradas [$F(3, 33) = 7,051$; $p = 0,0009$] e no número de bolos fecais nos braços fechados (Kruskal-Wallis statistic = 28,83; $p < 0,0001$). De forma similar a caixa claro escuro, os animais do grupo PC Salina apresentaram um menor tempo de permanência (figura 21A) (PCS= $135,4 \pm 12,69$ vs. CS= $275,4 \pm 9,751$; $p < 0,0001$), uma menor porcentagem de tempo (figura 21B) (PCS= $22,56 \pm 2,114$ vs. CS= $45,90 \pm 1,625$; $p < 0,0001$) nos braços abertos do labirinto. Por consequência, apresentaram um maior tempo de permanência (figura 21D) (PCS= $322,4 \pm 23,01$ vs. CS= $196,7 \pm 11,35$; $p = 0,0004$), uma maior porcentagem de tempo (figura 21E) (PCS= $53,74 \pm 3,835$ vs. CS= $32,79 \pm 1,892$; $p = 0,0004$) e uma quantidade maior de bolos fecais (figura 21I) (PCS= $5,667 \pm 0,6435$ vs. CS= $0,6667 \pm 0,3553$; $p < 0,0001$) nos braços fechados.

Os animais do grupo PC Resveratrol apresentaram um aumento no tempo de permanência (figura 21A) (PCR= $301,9 \pm 26,21$ vs. PCS= $135,4 \pm 12,69$; $p < 0,001$) e, por consequência, um aumento na porcentagem no tempo de permanência (figura 21B) (PCR= $50,32 \pm 4,369$ vs. PCS= $22,56 \pm 2,114$; $p < 0,0001$) nos braços abertos. Além disso, os animais do grupo PC Resveratrol passaram menos tempo nos braços fechados (figura 21D) (PCR= $172,4 \pm 23,55$ vs. PCS= $322,4 \pm 23,01$; $p < 0,001$) e, por consequência, apresentaram uma menor porcentagem no tempo de permanência (figura 21E) (PCR= $28,73 \pm 3,924$ vs. PCS= $53,74 \pm 3,835$; $p < 0,0001$) e uma redução no número de entradas (figura 21F) (PCR= $12,67 \pm 1,281$ vs. PCS= $22,42 \pm 1,990$; $p = 0,0009$) e de bolos fecais (figura 21I) (PCR= $1,167 \pm 0,3218$ vs. PCS= $5,667 \pm 0,6434$; $p = 0,0038$) nos braços fechados. Não foram constatadas diferenças entre os animais do grupo Controle Salina e Controle Resveratrol, e nem na quantidade de entradas e de bolos fecais nos braços abertos, e no tempo de permanência na zona central entre os grupos experimentais (figura 21 F, G e H).

Figura 21: Performance dos animais no Labirinto Elevado em Cruz no P28 dos quatros grupos experimentais CS (n=12), CR (n=12), PCS (n=12) e PCR (n=12). As **figuras A, B e C** mostram o tempo de permanência, porcentagem do tempo de permanência e quantidade de entradas nos braços abertos, respectivamente. **Figuras D, E e F** mostram o tempo de permanência, porcentagem do tempo de permanência e quantidade de entradas nos braços fechados, respectivamente. **Figura G** mostra o tempo de permanência na zona central. As **figuras H e I** mostram a quantidade de bolos fecais nos braços abertos e fechados. Os valores foram expressos como média $\pm$ erro padrão da média. ANOVA two-way multiple comparisons Tukey's post-hoc test ou Kruskal-Wallis statistic. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

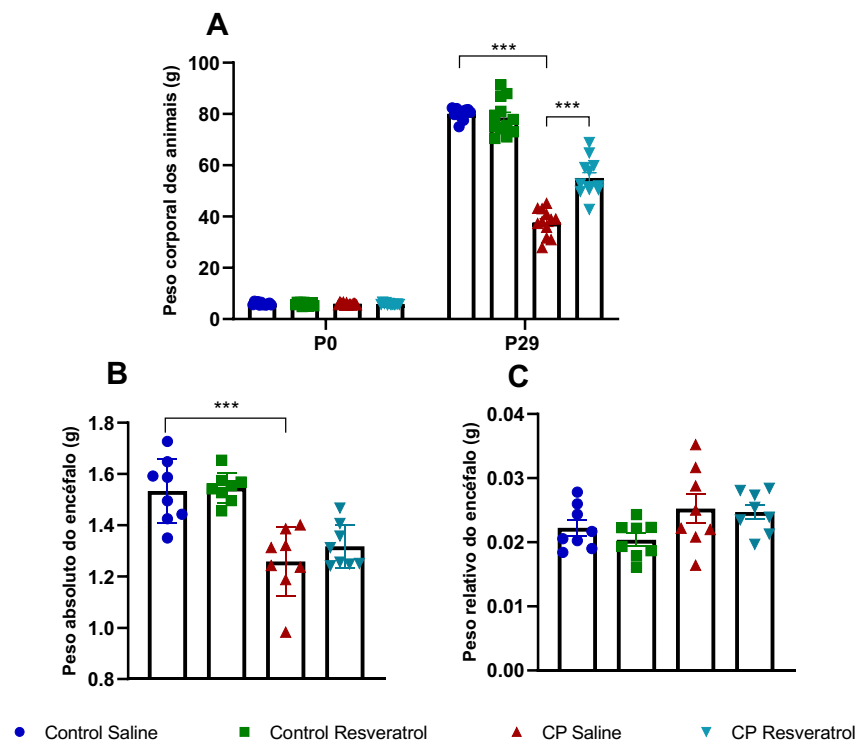


6.5 PESO DOS ANIMAIS E DO ENCÉFALO

Os animais no P0, antes dos experimentos, os animais não apresentaram diferenças no peso corporal. Entretanto, após o término dos experimentos, no P29, foram constatadas diferenças entre os grupos experimentais [$F(3, 33) = 137,3$; $p < 0,0001$]. Os animais do grupo PC Salina apresentaram o menor peso corporal ($PCS = 37,65 \pm 1,528$ vs. $CS = 80,04 \pm 0,6119$; $p < 0,0001$). No entanto, os animais do grupo PC Resveratrol apresentaram uma proteção contra a perda do peso ($PCR = 55,05 \pm 2,093$ vs. $PCS = 37,65 \pm 1,528$; $p < 0,0001$), apresentando um peso maior quando comparado com os animais do grupo PC Salina. Não foram constatadas diferenças entre o grupo Controle Salina e Controle Resveratrol ($CS = 80,04 \pm 0,6119$ vs. $CR =$

78,66 $\pm$ 1,994; $p > 0,055$) (figura 22A). Em relação ao peso do encéfalo *ex vivo*, foram encontradas diferenças [F (3, 21) = 14,66; $p < 0,0001$], na qual os animais do grupo PC Salina apresentaram uma redução no peso do encéfalo (PCS= 1,259 $\pm$ 0,04726 vs. CS= 1,533 $\pm$ 0,04471; $P < 0,001$). Curiosamente, os animais do grupo PC Resveratrol não demonstraram alterações no peso do encéfalo (PCR= 1,318 $\pm$ 0,03006 vs. PCS= 1,259 $\pm$ 0,04726; $p > 0,05$), quando comparado com os animais do grupo PC Salina. Não foram constatadas diferenças entre os animais do grupo Controle Salina e Controle Resveratrol (figura 22B). Por outro lado, quando o peso do encéfalo foi relativizado pelo peso corporal, não se observou diferenças no peso relativo do relativo (figura 22C).

Figura 22: Peso corporal (P0 e P29) e do encéfalo (P29) dos animais sacrificados por decapitação dos quatros grupos experimentais CS (n=8), CR (n=8), PCS (n=8) e PCR (n=8). **Figura A** mostra o peso corporal dos animais, a **figura B** mostra o peso absoluto do encéfalo e a **figura C** mostra o peso do encéfalo relativizado pelo peso corporal dos animais. Os animais do grupo PCS apresentaram uma redução no peso do encéfalo comparado ao grupo CS ($p < 0,0001$). Valores expressos em média $\pm$ erro padrão da média. ANOVA two-way múltiplas comparações do teste post-hoc de Tukey. * $p < 0,05$; ** $P < 0,01$; *** $p < 0,001$.

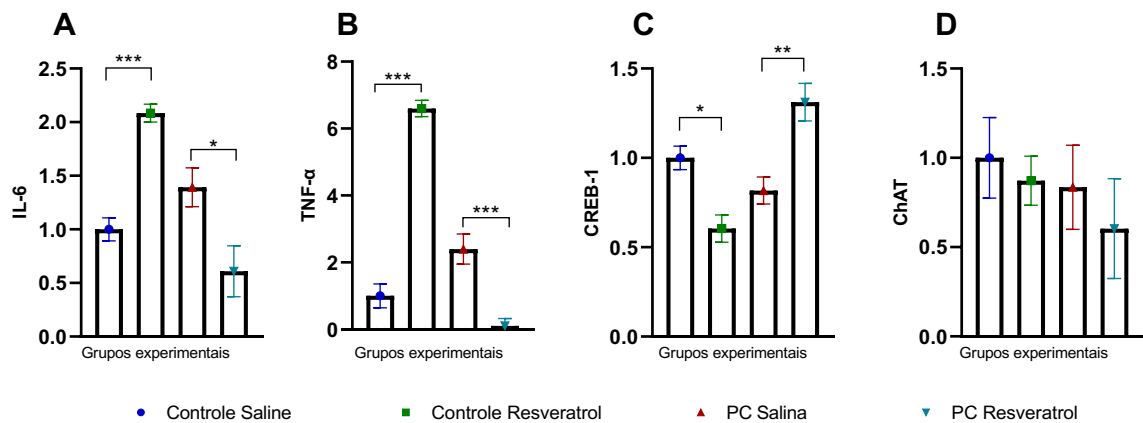


6.6 EXPRESSÃO GÊNICA

A análise de expressão dos genes pró inflamatórios revelou diferenças entre os grupos experimentais na expressão dos genes IL-6 (F (1, 19) = 10,99; $P = 0,0036$) e TNF- α [F (1, 17) = 61,62; $P < 0,0001$]. O tratamento neonatal com resveratrol reduziu a expressão da IL-6 no grupo PC Resveratrol quando comparado com os animais do grupo PC Salina (PCR= 0,609205 $\pm$ 0,581588 vs. PCS= 1,392811 $\pm$ 0,404912; $P = 0,0175$). A expressão gênica do TNF- α também

foi reduzida nos animais do grupo PC Resveratrol quando comparado aos animais do grupo PC Salina (PCR= $0,1 \pm 0,5$ vs. PCS= $2,4 \pm 1$; $P= 0,0007$). No entanto, houve um aumento na expressão da IL-6 (CS= $1 \pm 0,260384$ vs. CR= $2,084932 \pm 0,204809$; $P= 0,0006$) e TNF- α (CS= $1 \pm 0,8$ vs. CR= $6,6 \pm 0,6$; $P<0,0001$) nos animais do grupo Controle Resveratrol quando comparado aos animais do grupo Controle Salina (Figura 23A e B). Enquanto isso, na análise da expressão de genes relacionados com a plasticidade cerebral, os testes revelaram diferenças na expressão do CREB-1 [$F(1, 20) = 10,20$; $p=0,0046$], mas não se observaram constar diferenças na expressão do ChAT [$F(1, 19) = 0,9161$; $p=0,3505$]. Os animais do grupo PC Resveratrol apresentaram um aumento na expressão do CREB-1 em comparação os animais do grupo PC Salina (PCR= $1,311908 \pm 0,257985$ vs. PCS= $0,817147 \pm 0,185024$; $p= 0,0020$). Entretanto, os animais do grupo Controle Resveratrol apresentaram uma redução nos níveis do CREB-1 (CR= $0,604299 \pm 0,188108$ versus CS= $1 \pm 0,160555$; $p=0,0137$) (figura 23C). Não foram observadas diferenças nos níveis do ChAT (figura 23d).

Figura 23: RT-PCR dos genes IL-6 (CS n=6; CR n=6; PCS n= 5; PCR n= 6), TNF- α (CS n=5; CR n=6; PCS n=5; PCR n=5) CREB-1 (CS n=6; CR n=6; PCS n= 6; PCR n= 6) e genes ChAT (CS n=6; CR n=6; PCS n= 5; PCR n= 6) do hipocampo de animais sacrificados por decapitação no P29. **Figura A** mostra a expressão da IL-6, **figura B** mostra a expressão do TNF-a, **figura C** mostra a expressão do CREB-1 e a **figura D** mostra a expressão do ChAT. Valores expressos como média $\pm$ erro padrão da média. ANOVA two-way multiple comparisons e pos teste de Tukey's. * $p<0,05$; ** $P<0,01$; *** $p<0,001$.



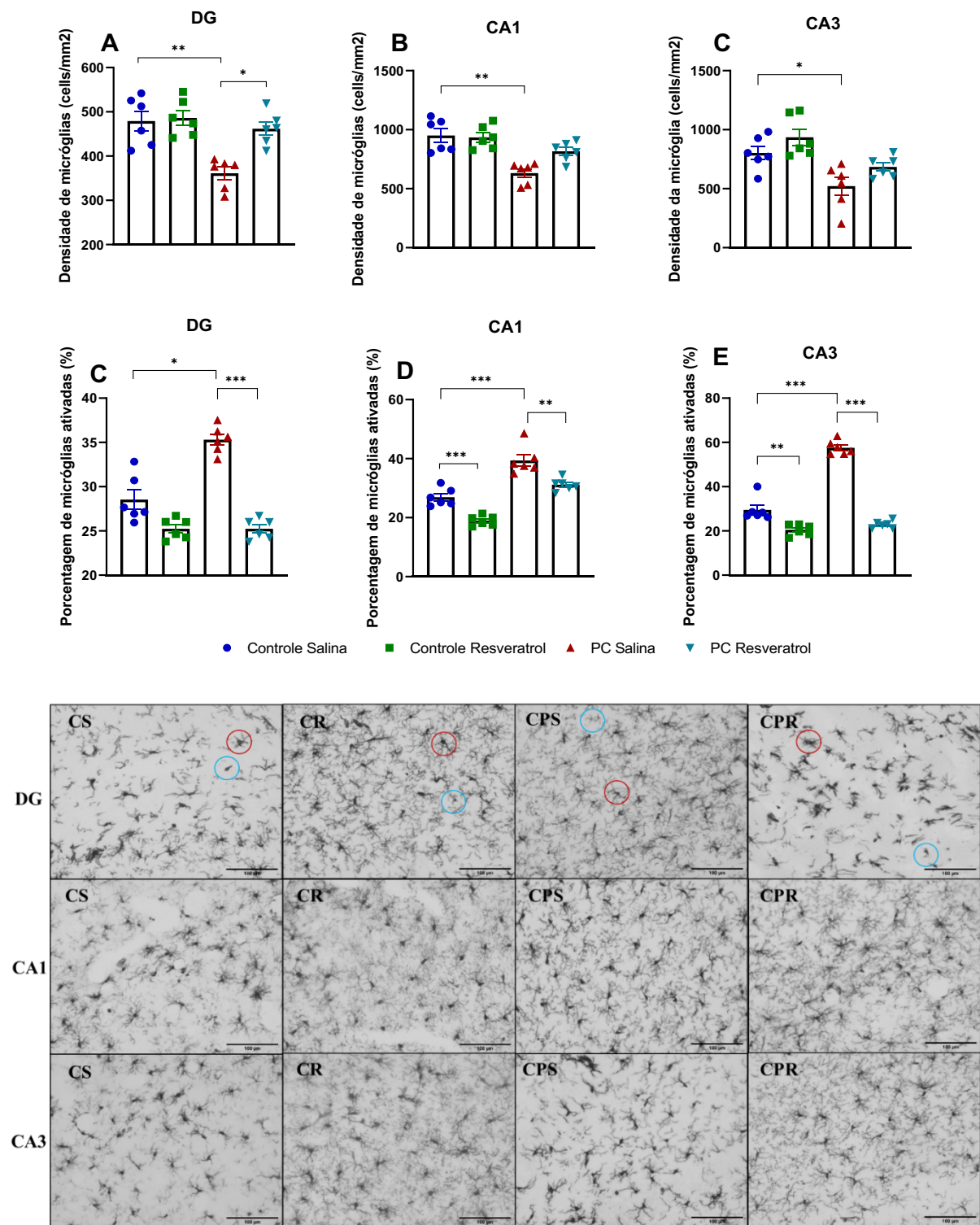
6.7 PERFIL DAS MICRÓGLIAS NO HIPOCAMPO

Através da análise histoquímica para verificação de células Iba+ no hipocampo, foram constadas diferenças na densidade de micróglia no DG [$F(3, 15) = 9,998$; $p=0,0007$], CA1 [$F(3, 15) = 9,683$; $p=0,0008$] e no CA3 [$F(3, 15) = 7,388$; $p=0,0029$] do hipocampo. Além disso, foi observada diferenças na porcentagem de micróglia ativadas no DG [$F(3, 15) = 46,17$; $p<0,0001$], CA1 [$F(3, 15) = 48,10$; $p<0,0001$] e no CA3 [$F(3, 15) = 147,9$; $p<0,0001$]. Os animais do grupo PC Salina apresentaram uma redução na densidade das micróglia no DG (PCS= $361,2 \pm 14,09$ vs. CS= $479,1 \pm 22,04$; $p=0,0020$), CA1 (PCS= $631,4 \pm 36,30$ vs. CS=

951,1 $\pm$ 56,80; $p=0,0013$) e no CA3 (PCS= 522,3 $\pm$ 76,30 vs. CS= 803,0 $\pm$ 57,55; $p= 0,0350$) (figura 24A, B e C). Apesar dessa redução na densidade de células, os animais do grupo PC Resveratrol apresentaram um aumento na quantidade de micróglia no DG (PCR= 461,8 $\pm$ 15,10 vs. PCS= 361,2 $\pm$ 14,09; $p= 0,0074$) quando comparado com os animais do grupo PC Salina (figura 24A). Os animais do grupo PC Salina apresentaram uma redução na densidade das micróglia no DG (PCS= 361,2 $\pm$ 14,09 vs. CS= 479,1 $\pm$ 22,04; $p=0,0020$), CA1 (PCS= 631,4 $\pm$ 36,30 vs. CS= 951,1 $\pm$ 56,80; $p=0,0013$) e no CA3 (PCS= 522,3 $\pm$ 76,30 vs. CS= 803,0 $\pm$ 57,55; $p= 0,0350$) (figura 24A, B e C). Apesar dessa redução na densidade de células, o grupo PC Resveratrol apresentou um aumento na quantidade de micróglia no DG (PCR= 461,8 $\pm$ 15,10 vs. PCS= 361,2 $\pm$ 14,09; $p= 0,0074$) (figura 24A).

Em relação ao perfil das micróglia no hipocampo, os animais do grupo PC Salina apresentaram um aumento na porcentagem de micróglia ativadas no DG (PCS= 35,30 $\pm$ 0,6264 vs. CS= 28,54 $\pm$ 1,080; $p<0,0001$), no CA1 (PCS= 39,38 $\pm$ 1,964 vs. CS=26,93 $\pm$ 1,227; $p<0,0001$) e CA3 (PCS= 57,58 $\pm$ 1,286 vs. CS= 29,50 $\pm$ 2,130; $p<0,0001$) (figura 24D, E e F). Todavia, os animais do grupo PC Resveratrol apresentaram uma redução na porcentagem de micróglia ativadas no DG (PCR= 25,24 $\pm$ 0,4723 vs. PCS= 35,30 $\pm$ 0,6264; $p <0,0001$), CA1 (PCR= 31,18 $\pm$ 0,8415 vs. PCS= 39,38 $\pm$ 1,964; $p= 0,0014$) e CA3 (PCR= 22,93 $\pm$ 0,6704 vs. PCS= 57,58 $\pm$ 1,286; $p <0,0001$) (figura 24D, E e F). Os filhotes do grupo Controle Resveratrol também apresentaram uma redução na porcentagem de micróglia ativadas no CA1 (CR= 18,98 $\pm$ 0,6863 vs. CS=26,93 $\pm$ 1,227; $p= 0,0018$) e no CA3 (CR= 20,48 $\pm$ 1,024 vs. CS= 29,50 $\pm$ 2,130; $p=0,0020$) (figura 24E e F).

Figura 24: imunohistoquímica para células Iba1+ no hipocampo. As **figuras A-C** mostram densidade celular no DG, CA1 e CA3. As **figuras D-E** mostram a porcentagem de micróglia ativadas nas regiões do DG, CA1 e CA3. Os grupos experimentais foram divididos aleatoriamente de acordo com o modelo de paralisia cerebral ou controle com tratamento com resveratrol ou solução salina. Os valores foram expressos como média $\pm$ erro padrão da média. ANOVA two-way multiple comparisons e Tukey's post-hoc test. * $p<0.05$; ** $P<0.01$; *** $p<0.001$.



Fonte: Autor (2024).

6.8 PROLIFERAÇÃO CELULAR NO HIPOCAMPO

A partir da análise por imunohistoquímica para verificação de células positivas para BrdU+, foram observadas diferenças na quantidade de células no DG [F (3, 18) = 17,87; $p < 0,000$], CA1 [F (3, 18) = 69,96; $p < 0,0001$] e CA3 [F (3, 18) = 56,75; $p < 0,0001$]. Os filhotes

do grupo PC Salina demonstraram uma redução na quantidade de células marcadas para BrdU+ no DG (figura 25A) (PCS= $1139 \pm 40,50$ vs. CS= $2489 \pm 140,2$; $p < 0,0001$), CA1 (figura 25B) (PCS= $314,6 \pm 20,96$ vs. CS= $1491 \pm 72,70$; $p < 0,0001$) e CA3 (figura 25C) (PCS= $287,9 \pm 19,93$ vs. CS= $1361 \pm 82,06$; $p < 0,0001$). Enquanto isso, os animais do grupo PC Resveratrol apresentaram um aumento na quantidade de células no CA1 (PCR= $766,4 \pm 66,71$ vs. PCS= $314,6 \pm 20,96$; $p = 0,0003$) (figura 25B) e no CA3 (PCR= $698,9 \pm 70,14$ vs. PCS= $287,9 \pm 19,93$; $p = 0,0008$) (figura 25C). Entretanto, os animais do grupo Controle Resveratrol apresentaram efeitos opostos, observando uma redução na quantidade de células no DG (CR= $1668 \pm 206,2$ vs. CS= $2489 \pm 140,2$; $p = 0,0020$), (figura 25A) CA1 (CR= $563,0 \pm 41,97$ vs. CS= $1491 \pm 72,70$; $p < 0,0001$) (figura 25B) e CA3 (CR= $520,7 \pm 19,81$ vs. CS= $1361 \pm 82,06$; $p < 0,0001$) (figura 25C).

Figura 25: imuno histoquímica para células BrdU+ no hipocampo. **Figura A** mostra o número de células no DG. **Figura B** mostra o número de células em CA1. **Figura C** mostra o número de células em CA3. Os grupos experimentais foram divididos aleatoriamente de acordo com o modelo de paralisia cerebral ou com tratamento com resveratrol ou solução salina: Controle salina (n=7), Controle Resveratrol (n=7), PC Salina (n=7), PC Resveratrol (n=7). Os valores são expressos como média $\pm$ erro padrão da média. ANOVA two-way multiple comparisons e Tukey's post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

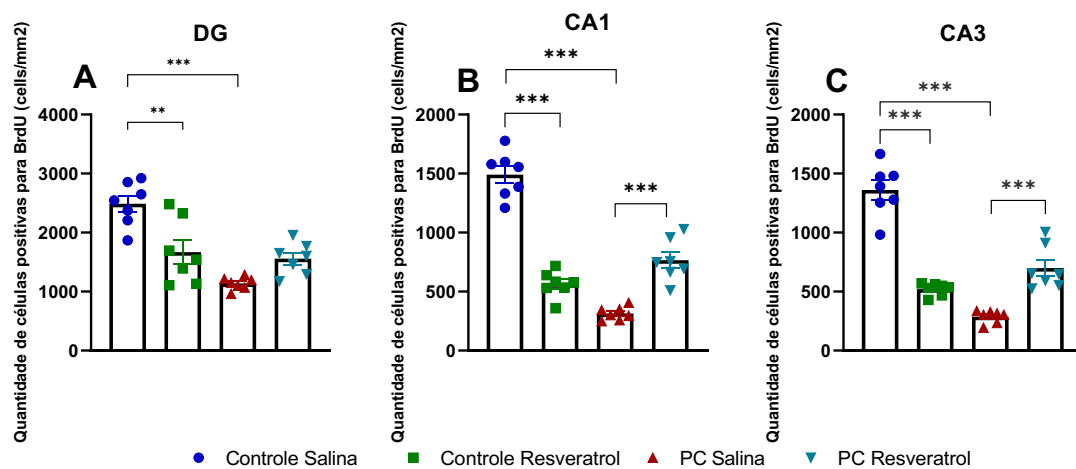
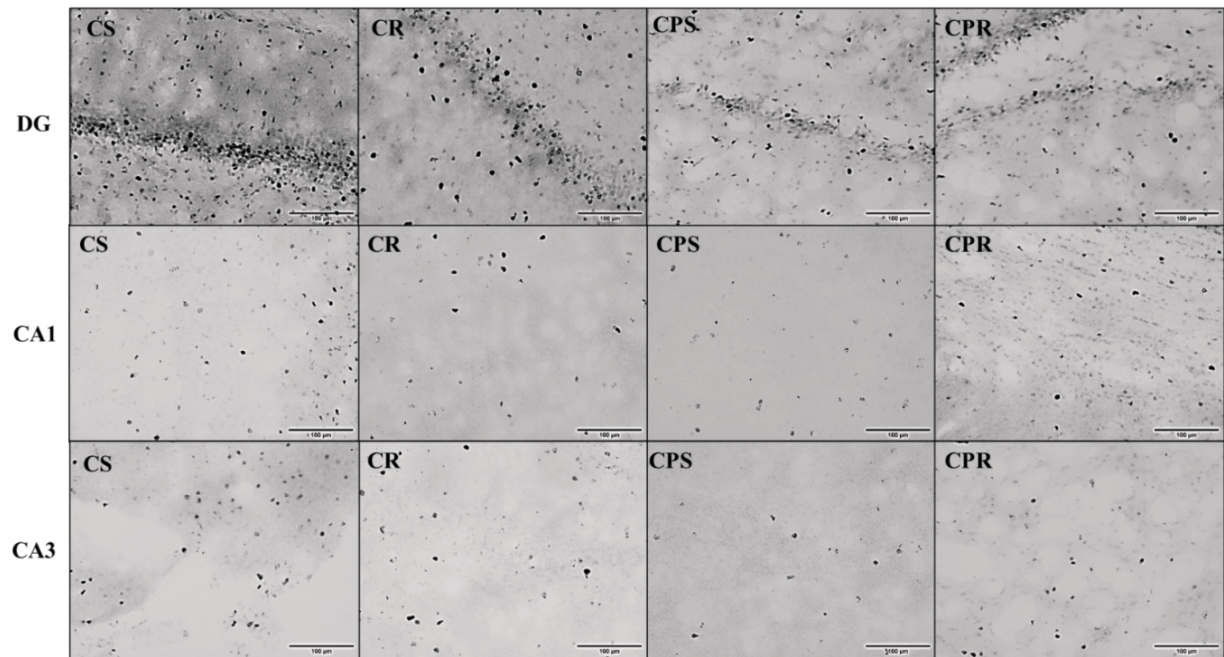


Figura 26: imunohistoquímica para células BrdU+ no hipocampo. A linha 1 mostra o DG, a linha 2 mostra o CA1 e a linha 3 o CA3 do hipocampo, respectivamente. Cada coluna representa um grupo experimental: CS (Controle Salina), CR (Controle Resveratrol), PCS (PC Salina) e PCR (PC Resveratrol), respectivamente.



Fonte: Autor (2024).

7 DISCUSSÃO

Os resultados deste estudo demonstram o impacto negativo da paralisia cerebral através da associação da anoxia perinatal e da restrição sensório motora na memória episódica e no comportamento emocional semelhante à ansiedade, impactando a aprendizagem dos animais, atrelados a prejuízos no hipocampo de ratos. Nas análises comportamentais, os animais apresentaram prejuízos em tarefas de aprendizagem visual espacial de curto e longo prazo, como o reconhecimento de objetos na localização espacial. Ademais, os animais também apresentaram alterações no comportamento semelhante à ansiedade em paradigmas que avaliam a ansiedade traço. Curiosamente, essas perturbações comportamentais estão correlacionadas com o aumento da expressão de genes pró inflamatórios e ativação das micróglias, reduzindo mecanismos neuroplásticos como a proliferação celular e os níveis de Creb-1, no hipocampo de ratos jovens. Apesar disso, o tratamento neonatal com o resveratrol demonstrou resultados promissores na atenuação desses danos, facilitando a formação de novas memórias episódicas e reduzindo os prejuízos hipocampais.

Anteriormente, estudos que utilizaram o mesmo modelo de PC observaram resultados promissores na utilização dos polifenóis durante o período crítico, como o resveratrol (Da Silva Souza et al., 2023a; Pereira et al., 2024) e kaempferol (Visco et al., 2023) nas sequelas neuromusculares, no qual observaram uma redução dos prejuízos motores na locomoção, marcha, coordenação motora e controle postural. Esses estudos observaram que a utilização do resveratrol promoveu uma redução do estresse oxidativo e melhora da função mitocondrial no córtex somatossensorial, promovendo um aumento na força muscular e no controle postural dos animais com PC (Da Silva Souza et al., 2023a). De forma similar, o tratamento com o resveratrol foi capaz de reduzir a ativação das micróglias no cerebelo e modificou a distribuição das fibras musculares, repercutindo em melhoras no neurodesenvolvimento dos animais (Pereira et al., 2024). Por outro lado, a utilização do Kaempferol promoveu um efeito similar ao resveratrol, reduzindo os biomarcadores do estresse oxidativo e a ativação das micróglias no giro denteado do hipocampo (Visco et al., 2022b), no qual promoveu uma melhora na atividade locomotora através do aumento da distância percorrida no campo aberto e na redução na quantidade de episódios imóveis.

Recentemente, os polifenóis vem sendo alvo de estudo a respeito dos seus efeitos em lesões cerebrais similares a PC devido os seus efeitos terapêuticos pré e pós lesão (Gouveia et al., 2022; Pontes et al., 2023; Visco et al., 2022a), como o resveratrol, no qual acredita-se que os efeitos terapêuticos da sua utilização no período crítico ocorre via plasticidade fenotípica,

melhorando aspectos do metabolismo cerebral e promovendo aumento de respostas neuroplásticas (Pontes et al., 2023). Em uma meta análise, o resveratrol destacou-se como um dos compostos com efeitos promissores nas sequelas comportamentais, no qual reduziu os prejuízos na formação de novas memórias (Calado et al., 2023a), de forma similar aos resultados observados neste estudo.

Estudos indicam que a lesão cerebral experienciada de forma precoce impacta o desenvolvimento cerebral, no qual observa-se um aumento nos marcadores do estresse oxidativo e da neuroinflamação que, por sua vez, impactam o desenvolvimento motor e a aprendizagem (Calado et al., 2023a; Da Conceição Pereira et al., 2021; Delcour et al., 2012). Acredita-se a formação de novas memórias é prejudicada por esse processo envolve uma série de mudanças em níveis sinápticos e moleculares (Dudai; Eisenberg, 2004; Josselyn; Köhler; Frankland, 2015; Kandel, 2001a, 2001b) e, por isso, necessita do funcionalmente adequado cerebral, sobretudo, dos mecanismos da neuroplasticidade (Faria, 2020). Como observado neste estudo, o modelo experimental da PC foi capaz de reduzir os níveis do CREB-1 um elemento essencial na consolidação de memórias de longo prazo, sugerindo o impacto da PC em mecanismos de neuroplasticidade.

Nos testes comportamentais de aprendizagem, os animais submetidos à PC apresentaram dificuldades na aprendizagem de curto e longo prazo, no qual observou-se uma redução do índice de discriminação no NORT e uma redução na quantidade de escolhas diferentes no Labirinto em T. Isso pode ser entendido na medida em que a lesão cerebral do tipo hipóxica e isquêmica, por definição, resulta em uma deficiência nos níveis de oxigênio e glicose no cérebro, impactando negativamente o desenvolvimento cerebral (Orso et al., 2021; Trollmann; Gassmann, 2009). À vista disso, o cérebro é uma estrutura que necessita de um grande suprimento de oxigênio e glicose, em especial o hipocampo para a formação de novas memórias e, por isso, é considerada uma das estruturas mais afetadas em condições de hipóxia e isquemia (Almli et al., 2000; Orso et al., 2021; Visco et al., 2021). Por essa razão, nessas condições clínicas nota-se a presença de prejuízos na aprendizagem associados a desequilíbrios sinápticos no hipocampo (Spahic et al., 2022; Takada et al., 2016), tais como os prejuízos observados neste estudo. E, apesar do dano ser experienciado no início da vida, as alterações cerebrais perduram em outras fases da vida, ecoando em prejuízos na formação de novas memórias (Azevedo et al., 2020).

Apesar da PC promover prejuízos em animais jovens, o tratamento neonatal com o resveratrol atenuou os danos no reconhecimento de objetos e na localização espacial. Isso pode

ter ocorrido porque o resveratrol é um potente agente antioxidante e anti inflamatório, no qual os seus efeitos atuam em diversos sistemas fisiológicos, inclusive no sistema nervoso, promovendo um aumento da função mitocondrial e da plasticidade sináptica (Bollinger et al., 2019; Da Silva Souza et al., 2023a) e, como postulado anteriormente, a plasticidade sináptica é a base molecular da memória e aprendizagem (Kandel, 2001b, 2001a; Peng et al., 2022), evidenciando possíveis mecanismos de atuação do resveratrol na facilitação da formação de novas memórias.

Corroborando com essa hipótese, um estudo observou que a utilização do resveratrol melhorou a aprendizagem após a lesão cerebral através da melhora da plasticidade sináptica, no qual promoveu um aumento na quantidade de espinhas dendríticas no DG do hipocampo e aumentou a expressão de proteínas como as sinapsinas que estão implicadas na regulação da liberação de neurotransmissores durante as sinapses (Peng et al., 2022) e os neurotransmissores, como a serotonina e o glutamato, atuam no processo de formação de memórias de curto prazo, como também a serotonina ativa a via PKA que inicia o processo de transcrição gênica para a formação de memórias de longo prazo (Faria, 2020; Kandel, 2001a).

Adicionalmente, os animais do grupo PC Salina não apresentaram somente prejuízos na aprendizagem, mas também apresentaram alterações no comportamento emocional, no qual observou-se um aumento dos traços comportamentais similares à ansiedade nos testes da caixa claro escuro e do labirinto elevado em cruz. Estudos anteriores demonstram que a privação de oxigênio impacta o hipocampo de diferentes formas, no qual pode repercutir em prejuízos na aprendizagem e/ou no comportamento emocional (Soares et al., 2013b). Assim, a hipóxia-isquemia precoce não prejudica somente o comportamento motor, sensorial e de aprendizagem, mas impacta também o comportamento emocional (Muntsant et al., 2019). Isso ocorre porque o hipocampo é uma região cerebral diferente, sendo dividida na porção ventral e dorsal, e cada uma vai possuir conexões com estruturas cerebrais distintas e, por isso, pode apresentar prejuízos comportamentais de forma diferente a depender da lesão (Fanselow; Dong, 2010).

A região ventral do hipocampo parece estar associada ao comportamento emocional porque tem ligações com áreas como a amígdala e o hipotálamo (Fanselow; Dong, 2010; Kjelstrup et al., 2002). Descobertas interessantes indicam que na PC há um aumento da ativação das micróglia no hipotálamo (Costa-de-Santana et al., 2023), danificando estruturas envolvidas no comportamento emocional. O modelo adotado no presente estudo utiliza dois diferentes insultos para mimetizar o fenótipo da PC, no qual expõe os animais a diferentes agressões de

forma precoce (Coq et al., 2008; Strata et al., 2004) e o estresse precoce produz comportamentos similares à ansiedade como observado no presente estudo.

O tratamento neonatal com resveratrol demonstrou efeitos ansiolíticos nos animais com, no qual observou-se, no Labirinto Elevado em Cruz, que os animais do grupo PC Resveratrol passaram menos tempo nos braços fechados do labirinto no P28, e na caixa claro escuro permaneceram menos tempo no lado escuro da caixa no P22 e no P29. Esses resultados sugerem que os efeitos ansiolíticos do resveratrol perduraram após o final da manipulação. O resveratrol é um composto natural capaz de atravessar a barreira hematoencefálica e modular as funções cerebrais, modificando, não somente estruturas relacionadas com a aprendizagem, mas modulando o humor produzindo efeitos antidepressivos e ansiolíticos (Shayganfard, 2020).

A hiperativação e a desregulação do eixo límbico hipotálamo-pituitária-adrenal parecem estar associadas a comportamentos antigênicos em ratos submetidos ao stress pós-traumático (Li et al., 2018), e esse eixo é alvo de atuação do resveratrol no qual a modulação parece reduzir comportamentos ansioso, aumentando o tempo de permanência nos braços abertos do Labirinto Elevado em Cruz (Li et al., 2018), de forma similar a observada neste estudo em animais com PC. Além disso, o resveratrol promoveu um aumento na expressão de CREB e BDNF associadas a redução dos comportamentos ansiosos (Li et al., 2018), mas acredita-se que o efeito ansiolítico do resveratrol encontra-se na sua habilidade de ativar a SIRT1 (Fan et al., 2018). Esses achados estão em consonância com o presente estudo, no qual nota-se uma redução dos comportamentos ansiosos em animais jovens tratados com o resveratrol em uma dose baixa (10 mg/kg).

O presente estudo constatou que os animais com PC apresentavam uma redução no peso do corpo e do cérebro. Estudos anteriores relataram que os animais com paralisia cerebral apresentavam um desenvolvimento somático prejudicado (Gouveia et al., 2023; Lacerda et al., 2017; Pereira et al., 2021; Visco et al., 2022b). Isso acontece porque os animais estão sujeitos a fatores estressantes no início da vida, bem como a utilização da restrição sensório-motora pode dificultar a capacidade do animal para se movimentar (Coq et al., 2008; Strata et al., 2004), prejudicando assim o comportamento alimentar. A lesão cerebral modifica o comportamento motor dos animais afetando também o comportamento alimentar através da redução dos ciclos mastigatórios e modificações no comportamento de saciedade (Costa-de-Santana et al., 2023; Lacerda et al., 2017). Estudos anteriores também encontraram um déficit nos músculos masseter e digástrico envolvidos na mastigação e deglutição, sinalizando possíveis vias para esse baixo peso corporal.

O presente estudo realizou uma intervenção durante o período crítico e observou que o tratamento neonatal com resveratrol foi capaz de atenuar os danos somáticos. Embora não tenhamos avaliado aspectos específicos das medidas antropométricas dos animais, uma revisão sistemática com meta-análise observou que a suplementação com resveratrol pode reduzir o peso corporal, diminuindo a quantidade de gordura e a circunferência abdominal e promovendo o aumento do tecido muscular (Tabrizi et al., 2020). Os resultados indicam que o resveratrol é capaz de regular o metabolismo energético celular através do aumento da expressão de Sirt1 e da regulação das funções mitocondriais, protegendo assim contra a acumulação de lipídios (Imamura et al., 2017; Zhao et al., 2017). Embora o presente estudo não tenha observado os efeitos do resveratrol em modelos de desnutrição, o resveratrol pode assim afetar os distúrbios metabólicos, reduzindo a acumulação de lipídios e aumentando o armazenamento de glicogênio nas células musculares e hepáticas (Gong; Guo; Zou, 2020), no qual observou-se que o resveratrol modifica a distribuição de fibras musculares na PC (Pereira et al., 2024). Esses dados fornecem algumas indicações sobre os mecanismos que podem estar subjacentes ao efeito do resveratrol nas alterações metabólicas que afetam o peso corporal na PC.

Por outro lado, prejuízos no hipocampo podem alterar comportamentos como a aprendizagem e o comportamento emocional como visto anteriormente, no qual observa-se que um fenômeno que ocorre no hipocampo após a lesão cerebral é a hiper expressão de citocinas inflamatórias que estão atreladas aos prejuízos motores na PC (Gouveia et al., 2023). As citocinas, como o fator de necrose tumoral e as interleucinas, são pequenas glicoproteínas que atuam como vetores de comunicação entre as células e estão envolvidas no controle de funções celulares cruciais, como o desenvolvimento, a diferenciação e a morte celular (Girard et al., 2009), mas quando liberadas no sistema nervoso podem ter efeitos deletérios (Girard et al., 2009). Dessa maneira, são células envolvidas em condições basais e inflamatórias do cérebro (Erta et al., 2015; Erta; Quintana; Hidalgo, 2012). As alterações na expressão das interleucinas também podem afetar a função cerebral e o comportamento, na qual descobertas indicam que as desregulações da expressão da IL-6 produzem um comprometimento comportamental em casos de lesão cerebral (Sanchis et al., 2020). Isto acontece porque a neuroinflamação é um dos principais fatores que contribuem para as perturbações neurológicas e para o comprometimento comportamental (Brambilla, 2019) e vem sendo associada com o desenvolvimento da PC (Fragopoulou et al., 2019; Jiang et al., 2018).

Apesar dos mecanismos da hipóxia não serem totalmente compreendidos, achados indicam que os baixos níveis de oxigênio e de glicose durante a hipóxia-isquemia têm um

impacto negativo na reserva de energia necessária para a sobrevivência das células e provocam um aumento da excitotoxicidade (Chen et al., 2020; Deb; Sharma; Hassan, 2010; Northington; Chavez-Valdez; Martin, 2011). Posteriormente, ocorre uma desregulação dos níveis de radicais livres e a produção de espécies reativas de oxigênio no cérebro conduzindo ao stress oxidativo e a respostas neuro inflamatórias, causando assim danos nos tecidos (Deb; Sharma; Hassan, 2010; Wu et al., 2021). Essa cascata de reações deletérias do estresse oxidativo e da neuroinflamação vem sendo no modelo de PC, no qual observa-se alterações no peso corporal, locomoção, força, coordenação motora e aprendizagem (Calado et al., 2023b; Da Silva Souza et al., 2023a; Gouveia et al., 2023; Pereira et al., 2024; Visco et al., 2022b).

No entanto, foi demonstrado que o tratamento neonatal com resveratrol (10mg/kg) reduz a expressão de IL-6 e TNF- α no hipocampo de animais com PC. Os estudos sugerem que o resveratrol é um composto com poderosas propriedades anti-inflamatórias (Meng et al., 2021; Rebas et al., 2020; Yu et al., 2021), decorrente da ativação da via de sinalização Sirt-1/NF- κ B (Cong et al., 2021; Zhao et al., 2021). Descobertas anteriores sugerem que o resveratrol é capaz de inibir a neuroinflamação em modelos de isquemia através da inibição da expressão do gene miR-155 e, consequentemente, da inibição da micróglia do tipo M2 (Ma et al., 2020a).

Estudos anteriores demonstram que as modificações negativas nos mecanismos de neuroplasticidade no giro denteado após uma lesão cerebral, tais como a redução da migração celular, podem estar associadas a uma perturbação da memória a longo prazo (Zhang et al., 2021c). Em conformidade com esses achados, os resultados do presente estudo fornecem novas evidências de que, na paralisia cerebral, há um aumento na ativação das micróglia, associado a uma redução na proliferação celular nas regiões do DG, CA1 e CA3 do hipocampo, o que impacta na aprendizagem de animais jovens. Neste contexto, o tratamento neonatal com resveratrol demonstrou ser capaz de reduzir a ativação da micróglia no DG, CA1 e CA3.

Estudos anteriores descobriram que a super expressão de IL-6 e a ativação da micróglia no hipotálamo, cerebelo e hipocampo estão associadas às sequelas motoras e comportamentais da PC (Costa-de-Santana et al., 2023; Gouveia et al., 2023), e, associado a ativação da micróglia, ocorre uma redução da proliferação celular (Visco et al., 2022b). Isso porque a micróglia é um elemento essencial no desenvolvimento do cérebro, no qual, em condições típicas, opera em processos de eliminação sináptica (Paolicelli et al., 2011; Schafer et al., 2012; Wang et al., 2020) em especial no hipocampo, visto que é uma região marcada pela neurogênese e necessita de uma reorganização sináptica em condições de aprendizagem (Toni et al., 2007).

Assim a micróglia desempenha um papel essencial na poda sináptica, promovendo a reorganização do hipocampo (Wang et al., 2020), sendo essencial, inclusive, em processos de esquecimento (Wang et al., 2020).

Mesmo sendo uma célula que opera em processos basais do organismo, a micróglia é uma das primeiras células a responder a insultos, no qual a sua ativação ocorre em condição de inflamação (Fan et al., 2023; Guruswamy; Elali, 2017; Zhang et al., 2021b). A ativação da micróglia no hipocampo pode ocorrer em excesso em resposta a uma lesão cerebral, prejudicando a função adequada do hipocampo (Gouveia et al., 2023), bem como a alteração do metabolismo e do perfil microglial contribui para a neurotoxicidade (Haukedal et al., 2023). De forma semelhante a estudos anteriores (Gouveia et al., 2023; Visco et al., 2022b), a ativação das micróglia e o aumento dos biomarcadores da neuroinflamação no hipocampo estão presentes em condições de prejuízos de aprendizagem na PC como constatado neste estudo.

Adicionalmente, o tratamento neonatal com resveratrol provocou um aumento da proliferação celular nas regiões CA1 e CA3 do hipocampo, evidenciando efeitos promissores em relação à atenuação das sequelas da PC. Evidências anteriores observaram que o insulto precoce (pré-natal ou pós-natal) tem um impacto direto na geração de novos neurônios no hipocampo, causando um declínio na produção de células troncos neurais (Visco et al., 2021). Para além disso, a redução dos mecanismos neuroplásticos, tais como a expressão do gene CREB-1, o BDNF e o processo de neurogênese, estão associados a perturbações da memória em lesões cerebrais precoces (Calado et al., 2023a). Assim, sugerimos que a redução da ativação das micróglia associada a um aumento da proliferação celular no hipocampo dorsal está associada a uma melhora na formação de memórias episódicas de longo prazo em animais jovens.

Apesar dos efeitos benéficos em relação à paralisia cerebral, surpreendentemente, os animais controles tratados com resveratrol apresentaram um aumento na expressão dos genes IL-6 e TNF- α e uma redução no número de células BrdU⁺ no DG, CA1 e CA3. Estudos anteriores com polifenóis durante o período crítico observaram o mesmo fenômeno, uma melhora em condições de PC nos marcadores do estresse oxidativo e da proliferação celular, mas um aumento em condições típicas (Da Silva Souza et al., 2023a; Visco et al., 2022b, 2023), sinalizando a necessidade de um maior compreensão dos efeitos desses compostos no período crítico antes de recomendações clínicas. Mas, por outro lado, acredita-se que isso tenha ocorrido porque os animais controles não precisam dessa suplementação, visto que estão em

condições típicas de metabolismo e funcionalmente, diferentes dos animais com PC que apresentam uma série de lacunas e excessos que comprometem o seu desenvolvimento.

Em contrapartida, os animais de controles tratados com resveratrol não apresentaram prejuízos de memória, isso ocorre porque a entende-se a memória enquanto um processo comportamental complexo que envolve uma série de contextos ambientais e, de forma interna e privada ao organismo, uma cascata de mecanismos bioquímicos e estruturas neurais envolvidas nesse processo. Por exemplo, em particular o córtex pré-frontal dorsolateral, que está relacionado com a capacidade de processar e manipular a informação sensorial antes de esta ser armazenada como memória de longo prazo (Carmichael; Lockhart, 2012; Funahashi, 2006). Assim, existem mecanismos não explorados para o entendimento da formação de memória de longo prazo em condições de PC e de tratamento com resveratrol. Assim, o tratamento neonatal com resveratrol parece ter efeitos benéficos na resposta neuro-inflamatória presente em ratos jovens com paralisia cerebral, mas são necessários estudos adicionais para compreender melhor os efeitos a longo prazo dos polifenóis.

8 CONSIDERAÇÕES FINAIS

O presente estudo observou prejuízos na formação e evocação da memória de reconhecimento e de localização espacial e um aumento dos comportamentos similares à ansiedade em ratos jovens com PC, repercutindo em dificuldades na aprendizagem dos animais. Associado a esses prejuízos, notou-se uma série de modificações epigenéticas através do aumento da expressão das citocinas IL-6, TNF- α e redução do CREB-1. Além disso, a PC promoveu um aumento da ativação das micróglias e redução da proliferação celular no DG, CA1 e CA3 do hipocampo. O tratamento neonatal com resveratrol foi capaz de reduzir a neuroinflamação, a ativação das micróglias e promover o aumento da proliferação celular. Esse conjunto de modificações epigenéticas contribuíram para uma melhora na aprendizagem visuoespacial dos animais.

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APÊNDICE A - RESVERATROL REDUCES NEUROINFLAMMATION AND HIPPOCAMPAL MICROGLIA ACTIVATION AND PROTECTS AGAINST IMPAIRMENT OF MEMORY AND ANXIETY-LIKE BEHAVIOR IN EXPERIMENTAL CEREBRAL PALSY

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Resveratrol Reduces Neuroinflammation and Hippocampal Microglia Activation and Protects Against Impairment of Memory and Anxiety-Like Behavior in Experimental Cerebral Palsy

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Abstract

Cerebral palsy (CP) is a neurodevelopmental disorder characterized by motor and postural impairments. However, early brain injury can promote deleterious effects on the hippocampus, impairing memory. This study aims to investigate the effects of resveratrol treatment on memory, anxiety-like behavior, and neuroinflammation markers in rats with CP. Male Wistar rats were subjected to perinatal anoxia (P0-P1) and sensory-motor restriction (P2-P28). They were treated with resveratrol (10 mg/kg, 0.1 ml/100 g) or saline from P3-P21, being divided into four experimental groups: CS ($n = 15$), CR ($n = 15$), CPS ($n = 15$), and CPR ($n = 15$). They were evaluated in the tests of novel object recognition (NORT), T-Maze, Light–Dark Box (LDB), and Elevated Plus Maze (EPM). Compared to the CS group, the CPS group has demonstrated a reduced discrimination index on the NORT ($p < 0.0001$) and alternation on the T-Maze ($p < 0.01$). In addition, the CPS group showed an increase in permanence time on the dark side in LDB ($p < 0.0001$) and on the close arms of the EPM ($p < 0.001$). The CPR group demonstrated an increase in the object discrimination index ($p < 0.001$), on the alternation ($p < 0.001$), on the permanence time on the light side ($p < 0.0001$), and on the open arms ($p < 0.001$). The CPR group showed a reduction in gene expression of IL-6 ($p = 0.0175$) and TNF- α ($p = 0.0007$) and an increase in Creb-1 levels ($p = 0.0020$). The CPS group showed an increase in the activated microglia and a reduction in cell proliferation in the hippocampus, while CPR animals showed a reduction of activated microglia and an increase in cell proliferation. These results demonstrate promising effects of resveratrol in cerebral palsy behavior impairment through reduced neuroinflammation in the hippocampus.

Keywords Brain injury · Cognition, Emotional behavior · Episodic memory · Polyphenols

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Introduction

The perinatal period is considered critical for the development of mammals, especially that of the brain [1]. Environmental exposures during gestation and/or the first years of life can thus lead to the development of adaptive or maladaptive phenotypes, favoring the emergence of disorders, such as cerebral palsy (CP), in later stages of life [2–4]. Hypoxia–ischemia and infections in this period are therefore one of the main risk factors for the development of CP [5–7]. CP is a neurodevelopmental disorder with severe repercussions for the organism, characterized by severe impairment of movement and posture [6], and is one of the main causes of physical and cognitive disability in childhood [6, 8, 9].

Although locomotion and motor coordination are impaired by a brain injury [10], studies indicate that the hippocampus is a region sensitive to oxygen deprivation [11] or exposure to inflammation [12, 13]. Different regions of the brain may be affected in CP, depending on the type of injury, time of exposure, and the extent of the injury [14]. Impairment of maturation of the hippocampus of animals with cerebral palsy has thus been observed, and a reduction in the expression of antioxidant enzymatic genes with reduced cell proliferation has also been noted [15]. Studies thus indicate that perinatal brain injuries impair the maturation of the hippocampus, with consequences for behavior and have noted an increase in anxiety-like behaviors [16, 17] and impairment of the formation of new memories in adulthood [18].

Memory is a complex ability associated with learning. Learning can be described as the organism's ability to acquire information, or skills, while memory is defined as the organism's ability to consolidate and recover this information [19]. Memory is associated with synapses and the synthesis of new proteins (or changes in existing proteins) in regions of the brain such as the hippocampus [19–21]. The formation of new memories involves changes in the brain, which are known as engrams [22]. The long-term episodic memories formation involves a cascade of molecular mechanisms such as the expression of CREB-1 (CAMP responsive element binding protein 1) and suppression of CREB-2 (CAMP responsive element binding protein 2) [19, 20, 23–26]. Studies also indicate that BDNF (brain-derived neurotrophic factor) synthesis is involved in the formation of long-term memories [19, 20, 23–26]. Additionally, the hippocampal choline acetyltransferase (ChAT) has been identified as one of the genes that act on memory [27, 28]. The ChAT reduction is also associated with the cognitive deficit present in Alzheimer's disease [27, 28]. Thus, the appropriate development of the hippocampus and synaptic connections plays a crucial role in the formation of new memories [19, 26, 29, 30].

Memory can also, however, be altered, both at the time of information recovery, when this information can be consolidated [31], and also by epigenetic mechanisms. Brain injuries and inadequate nutrition at a critical period can lead to memory formation impairment in adulthood [32–34]. Studies show that brain injuries early in life increase the expression of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α in the hippocampus [35, 36]. Neuroinflammation is an important aspect of early brain injuries, such as those caused by CP [37], which can impair maturation of the hippocampus and cause abnormalities relating to memory and behavior [35, 38–40].

By contrast, findings indicate that neonatal polyphenol supplementation is capable of attenuating hippocampal damage by reducing oxidative stress and microglial activation in the hippocampus [15]. This occurs because polyphenols are able to modulate metabolic and brain activity [41, 42]. Polyphenols are natural metabolite compounds commonly found in plants and foods, and their chemical structure contains at least one aromatic ring linked to a hydroxyl [43]. They have antioxidant and anti-inflammatory effects [44–46]. Resveratrol is a polyphenol with anti-inflammatory effects that is found in grapes and red wine and in nuts [47]. Studies indicate promising effects on the brain, improving mitochondrial function and preventing microglia activation and thereby reducing TNF- α , IL-1 β , IL-6, and nitric oxide expression in the hippocampus and improving behavior and the capacity to form new memories [47–53].

The present study thus aimed to assess the effects of neonatal resveratrol treatment on memory formation, anxiety-like behavior, pro-inflammatory markers, and the expression of genes involved in memory, microglial activation, and cell proliferation in the hippocampus of rats submitted to a cerebral palsy model. We hypothesized that neonatal exposure to resveratrol reduces the severity of problems present in cerebral palsy related to the formation of new memories and abnormal emotional behavior by reducing neuroinflammation and microglial activation and increasing cell proliferation in the hippocampus.

Materials and Methods

Animals and Animal Housing Conditions

A randomized, controlled, pre-clinical study was conducted using Wistar rats from the colony of the Federal University of Pernambuco's Department of Nutrition. All animals were kept under standard conditions at a temperature of 22 ± 2 °C, with an inverted light–dark cycle of 12/12 h (light cycle—20:00 to 8:00 h; dark cycle—08:00 to 20:00 h), housed in polypropylene cages (46 cm $\times$ 34 cm $\times$ 20 cm) lined with sterile wood shavings, with free access to food and water.

Experimental Groups

The male pups ($n=60$) were obtained from nulliparous female rats ($n=30$) placed to mate with reproductive males ($n=15$) at the proportion of two females to one male. The rats were consanguineous, aged between 90 and 120 days, with a body weight of between 220 and 260 g. Once pregnancy had been confirmed by detecting spermatozoa in vaginal smear, the pregnant rats were placed in separate individual cages until the birth of the pups. After birth, the male rat pups were randomly distributed into four experimental groups, consisting only of healthy male pups (weighing 6–8 g). The female offspring were used only to complete the litter of eight pups until weaning. The experimental groups were divided accordingly by exposure to cerebral palsy and pharmacological manipulation using saline or resveratrol, as follows: (i) control saline (CS, $n=15$); (ii) control resveratrol (CR, $n=15$); (iii) cerebral palsy saline (CPS, $n=15$); (iv) cerebral palsy resveratrol (CPR, $n=15$). Each litter consisted of eight pups that remained with their mothers until P25 (25th postnatal day of life), when they were weaned and the male rats placed in individual cages until subjected to euthanasia (Fig. 1).

Experimental Model of Cerebral Palsy

The CP phenotype was reproduced using a model that combines perinatal anoxia and sensory-motor restriction of the hindlimbs [54–56]. The model is applied to male pups only, because males are more prone to developing CP [57]. This model reproduces phenotypic characteristics of diplegic-spastic CP [10, 54]. The pups underwent two episodes of anoxia, at P0 and P1 (0th and 1st postnatal day of life), during which they were placed in a glass chamber, partially immersed in water at 37 °C, and exposed to nitrogen 100% (White Martins, Brazil) at 9 l/min for 12 min on each occasion. No more than four males were exposed to anoxia in each experiment. The pups were then placed at room temperature for recovery (breathing, skin color, and postural reflexes) and were then returned to their respective mothers [54, 55]. Sensory motor restriction of the hindlimbs was performed from P2 to P28 (2nd to 28th postnatal day of life) for 16 h a day. The procedure was performed from 4 pm until 8 am the next day. This involved fixing an orthosis in such a way as to enable only limited movement of the hip joint, leaving the hindlimbs extended with the support of surgical and adhesive tape, without hindering maternal care or the elimination of urine and feces.

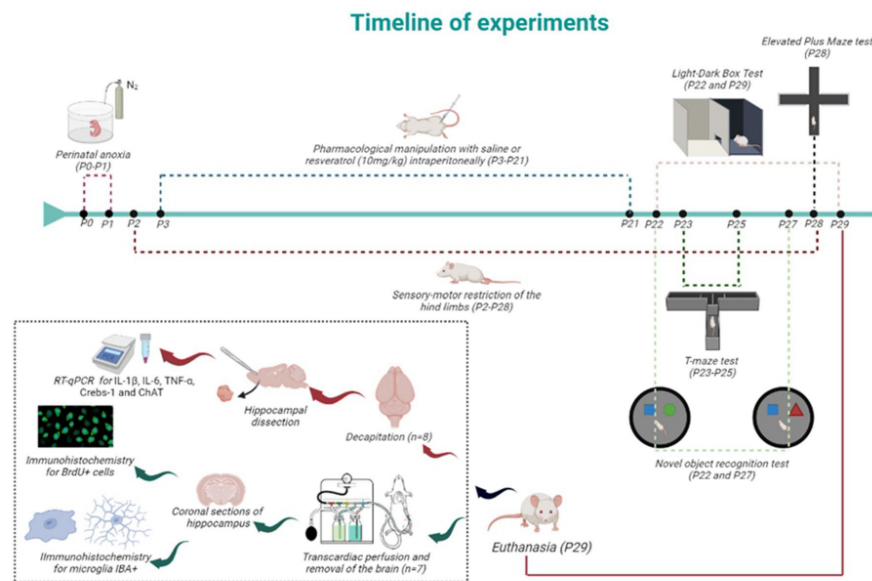


Fig. 1 Summary of all experiments performed on male offspring. The picture created with BioRender

Neonatal Treatment with Resveratrol or Saline

Male pups were randomly divided into groups to assess the effects of the intervention on brain injury, receiving treatment with saline or resveratrol by way of a daily peritoneal injection at 9 am from P3 to P21 (3rd to 21st postnatal day of life). The animals were distributed into the following groups: treatment with resveratrol (daily dose of 10 mg/kg) and treatment with saline (0.9% NaCl), injected at a volume of 0.1 ml/100 g of the rat's weight. Resveratrol (Sigma, St. Louis, MO, USA) was diluted according to Girbovan and Plamondon, 2015. Resveratrol was diluted in 50% ethanol and 50% in a vehicle consisting of 0.9% saline solution (NaCl) containing 20% hydroxypropyl β -cyclodextrin (Sigma, St. Louis, MO, USA) [58]. To 10 mg of resveratrol, 0.25 ml of ethanol was used to dissolve it and 0.25 ml of saline solution was added. The final substance was stored in aliquots in a freezer at -20°C . After that, the resveratrol was only unfrozen at the time of application. The rats were weighed daily, and the injection volume was adjusted to match the animal's body weight.

Evaluation of Novel Object Recognition Memory Test

The animals were randomized for the behavioral tests, with at least twelve animals per group for each test. The novel object recognition test (NORT) [53] was used to assess the formation of new episodic memories. The experiment was conducted during the dark period of the cycle. The training session was conducted on the 22nd day of life (P22). The animals were placed one at a time in an entirely black circular open field with two identical objects and allowed to freely explore the objects. After completion of training, each animal was returned to the cage, and the open field and objects were cleaned with ethanol (70%). The familiar object (big rectangular, acrylonitrile butadiene styrene) on the right side was subsequently placed once again in the field, after the open field and objects were cleared, while the object on the left side was replaced with a novel object (small cube, acrylonitrile butadiene styrene). The animal was placed again in the open field and was filmed for 5 min to assess the formation of short-term memories. At P27, the test was repeated with another novel object different (conical object, polyethylene) from the familiar object in order to assess the animals' ability to acquire and retrieve long-term memories [53]. All videos were analyzed by an evaluator blinded to the experimental groups and treatments, and the novel object discrimination index (DI) and recognition index (RI) was calculated. The mathematical formula used to calculate DI is as follows: $[\text{DI} = (\text{time spent exploring the novel object}) - (\text{time spent exploring the familiar object}) / (\text{total exploration time})]$. This result can vary between $+1$

and -1 . The formula used to calculate RI is as follows: $[\text{RI} = (\text{time spent exploring the novel object}) / (\text{total exploration time}) \times 100 (\%)]$ [59].

Evaluation of Memory Using the T-Maze Test

The animals' spatial memory was assessed using the T-Maze test [60, 61]. The maze consists of a black plexiglass structure shaped like an upper-case letter "T", with sliding doors dividing the paradigm. The experiment was conducted in sets of two trials each to assess the animal's alternation behavior [60, 61]. The test was applied four times daily (two sets of two) for three consecutive days (P24–P27). Thirty minutes prior to the experiment, the animals were placed in the behavior room for adaptation. Each animal was placed at the bottom of the "T" and was allowed to explore the paradigm. Once the animal had chosen one arm of the maze, the sliding door of the chosen arm was closed. The animal was then returned to its cage, and the maze was cleaned. The same procedure was then repeated to complete the first set of two. On conclusion of the test, each animal was returned to its cage for 10 min, the maze was cleaned, and the procedure repeated, giving a total of four runs per day. By the end of the experiment, six sets of tests had been conducted, and the number of new arm choices was calculated [60, 61].

Evaluation of Anxiety-Like Behavior Using the Light–Dark Box Test

The Light–Dark Box (LDB) test was used to evaluate the effects of brain injury and treatment on anxiety-like emotional behavior of animals at P22 and P29 (22nd and 29th postnatal day of life). The apparatus consisted of a 40 cm $\times$ 40 cm box with a height of 80 cm, with a central wall that divides the box into two sides, one completely white (the light side) and one completely black (the dark side), with only one central entrance allowing the animal to move from one side to the other. The animal was placed in the dark side of the box during the light period of the daily cycle and was allowed to explore the apparatus for a period of 5 min. The test was filmed by a camera attached to the ceiling. The videos were analyzed by a blinded evaluator using Anymaze software to establish the following parameters: permanence time, and number of entries into the light and dark side of the box.

Evaluation of Anxiety-Like Behavior Using the Elevated Plus Maze Test

The Elevated Plus Maze (EPM) test consists of a task that assesses anxiety-like behavior in rodents [56]. On P28 (28th postnatal day of life), the pups were transferred to the behavior room for 30 min of adaptation to the environment prior

commencing the test. Each animal was placed in the central area of the apparatus and allowed to explore it for 10 min. The test was performed in the dark, and the animals were filmed using a camera attached to the ceiling [11, 57]. All videos were analyzed by an evaluator blinded to the experimental groups and treatments using Anymaze software to establish the following parameters: the number of entries and permanence time in the open and closed arms, and permanence time in the central area.

Euthanasia and Body and Brain Weight of Animals

The weight of each of the animals was measured daily from P0-P29 using a precision balance (Marte, capacity of 1 kg and sensitivity of 0.01 g). Half of the animals ($n = 8$ per experimental group) were euthanized by decapitation at P29 after a 4-h fasting period. The brain was then removed and weighed in an electronic digital balance (Marte AUW220, capacity of 220 g and sensitivity of 0.1 mg). The hippocampus was removed shortly thereafter and stored in a freezer at -70°C for gene expression analysis.

RNA Extraction

Total RNA was extracted from dissected hippocampal tissue with TRIzol reagent using the guanidine isothiocyanate [62] method in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA pellets were washed in 75% ethanol, centrifuged at $7500 \times g$ for 5 min at 4°C , air-dried, and boiled in DEPC-treated ultrapure water. RNA quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and purity was assessed using the 260/280 nm range.

RT-PCR Evaluation of Hippocampal IL-1 β , IL-6, TNF- α , Krebs-1, and ChAT Gene Expression

Real-time polymerase chain reaction (RT-PCR) was subsequently performed for $\beta 2$ -microglobulin ($\beta 2\text{M}$) as a normalizer and for the genes of interest, IL-1 β , IL-6, TNF- α , Krebs-1, and ChAT (Table 1) using the SuperScript[®] III Platinum[®] SYBR[®] Green One-Step qRT-PCR Kit (Invitrogen, USA). Sequences were obtained from the NCBI database and were designed using Primer3 Input Software

(v.0.4.0) [63]. The housekeeping gene GAPDH was used as the internal control for PCR analysis. The relative mRNA expression of the genes was calculated using the $2^{-\Delta\Delta\text{Ct}}$ comparative method [64].

Euthanasia and Brain Histology

The remaining animals ($n = 7$) were euthanized by transcardiac perfusion for evaluation of cell proliferation and microglial activation in the hippocampus. The pups were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and xylazine (12 mg/kg), and the thoracic cavity was opened and transcardiac perfusion was performed using 150 ml of saline (0.9% NaCl) and 200 ml of fixative solution (4% paraformaldehyde). After perfusion, the brain was removed and stored, and post-fixed in the same fixative solution overnight. Sucrose (30%) was added for the 24 h–48 h period. The brains were cryosectioned at a thickness of 30 μm into coronal sections to collect the posterior portion of the hippocampus (between -1.82 and -2.18 mm, posterior to the bregma) in a -30°C Leica cryostat. The sections were kept in an antifreeze solution for immunohistochemical processing.

Profile of Hippocampal Microglia

Analysis of the microglia profile was conducted using brain sections incubated first in 10% H_2O_2 in methanol and subsequently in 10% H_2O_2 in phosphate buffer (0.1 M, pH 7.4) containing 3% Triton X-100 (PBT). The sections were then incubated at 4°C for 48 h in primary ionized calcium-binding adapter molecule 1 antibody (Iba1) (rabbit anti-Iba1/IAF1, 1: 30,000, Wako), diluted with 5% horse serum in PBT [65]. The sections were subsequently incubated in a secondary antibody (biotinylated anti-rabbit; 1:750, Sigma-Aldrich) for a period of 2 h at 4°C . The brain sections were then incubated in solutions of avidin–biotin–peroxidase complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) and a solution of the diaminobenzidine staining kit (DAB Kit; Vector Laboratories) to stain the microglia [65]. Sections from each group were processed in parallel to avoid non-specific staining effects [15]. The brain sections were then mounted on gelatinized slides and covered with Cytoseal coverslips (Thermo Scientific, USA).

Table 1 Primers used in PCR analysis

Gene	Forward primer	Reverse primer	Amplicon size
B2M	TGACCGTGATCTTCTGGTG	ACTTGAATTGGGGAGTTTCTG	73 bp
IL-6	AAGGAGTGGCTAAGGACCAA	GTTTGCCGAGTAGACCTCAT	89 bp
TNF- α	AAGCATGATCCGAGATGTGG	AGTAGACAGAAGAGCGTGGT	141 bp
CHAT	CCTGGAAAAGGCTCCCAAA	CCCAAACCGCTTCACAATGG	178 bp
CREB	GACGGAGGAGCTTGACCAC	TGGCATGGATACCTGGGCTA	156 bp

Evaluation of Iba1 + microglial cells in the hippocampus ($n = 7$ per experimental group) was conducted by selecting two fields per section from a total of four sections randomly selected by brain section from the CA1, CA3, and dentate gyrus regions of the posterior hippocampus, giving a total of eight photographs per area of the hippocampus for each animal. The selected fields were examined using a $20\times$ objective microscope. A blinded researcher used ImageJ software (<https://imagej.nih.gov>) to count the number of cells/area and classify the profile of the microglia according to the previous descriptions. Microglial cells with a small soma and few to numerous processes were considered to be branched microglia (types I–III), while those with a large soma or amoeboid body and thicker, shorter processes were considered to be activated microglia (types IV–V) [65, 66]. The data presented relate to microglia density (n/mm^3 —total cells divided by the area assessed $\times 1,000,000 \times 0.001 \times 40$) and the percentage of microglia activation relative to the total [15].

Cell Proliferation in the Hippocampus

To investigate cell proliferation in the hippocampus, animals to be euthanized by perfusion ($n = 7$ per experimental group) received, at P5 and P6, an intraperitoneal injection of 50 mg/kg of BrdU (5-bromo-2'-deoxyuridine- BrdU, Sigma-Aldrich Co. LLC). Cryosections subsequently underwent immunohistochemical analysis. BrdU is a thymidine analog that marks DNA during the S phase of the cell cycle [59], as it is a mitotic marker used to identify newborn neurons [60, 61]. BrdU is therefore used to assess cell proliferation [62]. The brains were cut into 30- μm sections and these were washed in a phosphate-buffered substance (PB) and incubated in Triton (X-100 0.3%, St. Louis, MO, USA) containing 10% hydrogen peroxide. The sections were then incubated in absolute methanol and washed in PB and incubated in formamide (50% in sodium citrate salt solution, Sigma-Aldrich) at 65°C for 2 h. After washing, denaturation of DNA was performed in HCl (1N) at 37°C . The sections were then incubated in borate buffer solution (pH 8.4) [63].

The sections were subsequently incubated overnight (4°C) in a solution of primary anti-BrdU antibody (mouse anti-BrdU, 1: 30,000, Roche Molecular). The following day, the sections were washed for 4 to 5 min in PBS and incubated in the dark for 2 h with biotinylated secondary antibody (1: 750, Vector Laboratories) and developed in an avidin–biotin complex (Elite ABC kit, Vector Laboratories) and diaminobenzidine (DAB staining kit, Vector Laboratories). The brain sections were subsequently washed, dried, and mounted on slides with Cytoseal, and covered with coverslips. Finally, images were captured using a $20\times$ objective optical microscope of 4–5 sections per animal. The number of BrdU + cells in the hippocampus was counted by a

blinded researcher for each experimental group [15]. The boundaries of the dentate gyrus (DG) granular cell layer (GCL) and the subgranular zone (SGZ), and CA1 and CA3 regions of the hippocampus were digitally delineated.

Statistics

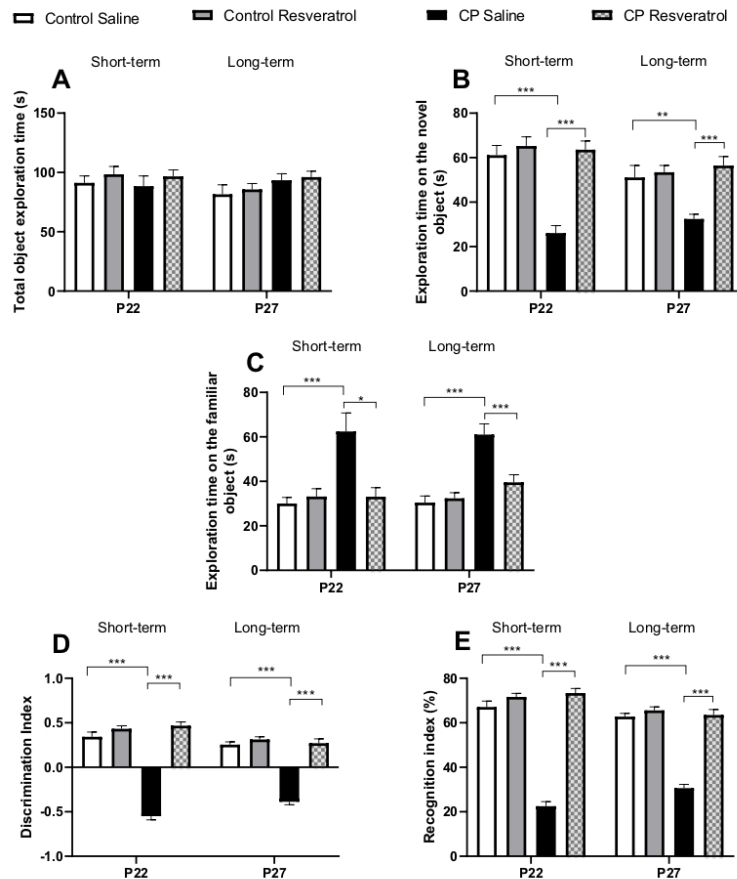
The data obtained were tested for normality using the Shapiro–Wilk test. If a normal distribution was identified, appropriate parametric tests, such as the two-way ANOVA test (for comparison of the groups at only one age of the animal) or repeated measures two-way ANOVA (for comparisons of the performance of animals at more than one age) were applied. For data found not to be distributed normally, non-parametric statistical tests, such as the Kruskal–Wallis and Friedman tests, were employed. The results were expressed as mean $\pm$ standard error of the mean. The significance level was set at 5%. GraphPadPrism® version 8.0.2 software was used for data analysis and creation of the graph.

Results

Resveratrol Provides Protection Against Object Recognition Memory Impairment

Recognition memory was assessed using the novel object recognition test at P22 and P27. On P22, significant differences were found between the CS and the CPS groups and between the CPS and the CPR animals for exploration time in relation to a novel object [$F(3.33) = 29.42$; $p < 0.0001$], exploration time in relation to a familiar object [$F(3.33) = 8.723$; $p = 0.0002$], for the discrimination index [$F(3.88) = 7.491$; $p = 0.0002$], and for the recognition index [$F(3.88) = 7.491$; $p = 0.0002$]. However, no significant differences in total exploration time were found between any of the experimental groups ($p > 0.05$). Animals in the CPS group showed reduced exploration time in relation to a novel object (CPS = 26.08 ± 3.342 vs. CS = 61.13 ± 4.380 ; $p = 0.001$) (Fig. 2B) and spent more time exploring a familiar object compared to those in the CS group (CPS = 62.41 ± 8.346 vs. CS = 30.06 ± 2.708 ; $p = 0.007$) (Fig. 2C). These animals consequently presented a lower discrimination index (CPS = -0.549 ± 0.041 vs. CS = 0.342 ± 0.054 ; $p < 0.0001$) and recognition index (CPS = 22.53 ± 2.03 vs. CS = 67.11 ± 2.67 ; $p < 0.0001$) (Fig. 2D and E). This suggests impairment in the object recognition memory of animals submitted to the cerebral palsy model. The pups in the CPR group exhibited increased novel object exploration time (CPR = 63.56 ± 3.915 vs. CPS = 26.08 ± 3.342 ; $p = 0.0006$) (Fig. 2B), reduced exploration time for the familiar object (CPR = 33.09 ± 4.906 vs. CPS = 62.41 ± 8.346 ; $p = 0.0106$) (Fig. 2C), and an

Fig. 2 Novel object recognition test on P22 and P27 in the four experimental groups: CS ($n=12$), CR ($n=12$), CPS ($n=12$), and CPR ($n=12$). **A** Total exploration time of the animals, where no significant differences were found. **B** Exploration time on the novel object, where the CPS group showed a shorter time ($p<0.001$), while the CPR group demonstrated exploration similar to the CS group. **C** Exploration time on the familiar object, where the CPS group showed a longer time compared to the CS group ($p<0.05$ and $p<0.001$) and CPR group ($p<0.0001$ and $p<0.0001$). **D** Novel object discrimination index where the pups from the CP group showed a worse index ($p<0.001$) relative to the CS and CPR group. **E** Recognition index where the animals from the CP group showed a worse index relative to the CS and CPR groups. Values are expressed as mean $\pm$ standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$



increase in the discrimination index (CPR = 0.467 ± 0.041 vs. CPS = -0.549 ± 0.041 ; $p<0.0001$) and recognition index (CPR = 73.36 ± 2.092 vs. CPS = 22.53 ± 2.032 ; $p<0.0001$) (Fig. 2D and E) compared to the CPS group.

After 5 days, the novel object recognition test was repeated to check long-term recognition memory. On P27, differences persisted for exploration time in relation to a novel object [$F(3,33)=9.905$; $p<0.0001$], in relation to a familiar object [$F(3,33)=14.24$; $p<0.0001$] and for the novel object discrimination index [$F(3,88)=7.491$; $p=0.0002$] and recognition index [$F(3,88)=7.491$; $p=0.0002$]. No significant differences, however, were found in the total exploration time for the objects [$F(3,33)=1.439$; $p=0.2490$] (Fig. 2A). Animals in the CPS group performed less well in relation to novel object exploration (CPS = 32.42 ± 2.147 vs. CS = 51.13 ± 5.371 ; $p=0.0028$)

(Fig. 2B) and spent more time exploring a familiar object (CPS = 61.04 ± 4.818 vs. CS = 30.44 ± 3.00 ; $p<0.0001$) (Fig. 2C), and consequently presented the worst discrimination index (CPS = -0.387 ± 0.034 vs. CS = 0.254 ± 0.030 ; $p<0.0001$) and recognition index (CPS = 30.61 ± 1.68 vs. CS = 62.73 ± 1.52 ; $p<0.0001$) (Fig. 2D and E), providing evidence of the presence of episodic memory impairment in animals with cerebral palsy compared to the CS group. The animals in the CPR group, however, presented the longest exploration time for a novel object (CPR = 56.43 ± 4.059 vs. CPS = 32.42 ± 2.147 ; $p=0.0001$) and a reduction in exploration time for a familiar object (CPR = 39.58 ± 3.378 vs. CPS = 61.04 ± 4.818 ; $p=0.0014$) (Fig. 2B and C), thereby increasing the discrimination index (CPR = 0.270 ± 0.050 vs. CPS = -0.387 ± 0.034 ; $p<0.0001$) and recognition index (CPR = 63.50 ± 2.489 vs. CPS = 30.61 ± 1.683 ; $p<0.0001$)

(Fig. 2D and E) indicating that resveratrol improved the long-term recognition memory of animals with cerebral palsy (Fig. 2).

Resveratrol Facilitates Recovery of Novel Spatial Memories

The spatial memory of the animals was assessed using the T-Maze test at ages P25, P26, and P27, using the overall percentage of new arm choices. Significant differences were found in the animals' spontaneous alternation [$F(3.33) = 44.30$; $p < 0.0001$]. The animals in the CPS group made fewer new arm choices in the maze, opting for the same arm of the maze that had already been explored, compared to the CS group (CPS = 25.00 ± 5.618 vs. CS = 81.94 ± 3.815 ; $p = 0.0016$). However, animals in the CPR group showed a higher number of new arm choices, alternating more often in the maze (CPR = 83.33 ± 5.428 vs. CPS = 25.00 ± 5.618 ; $p = 0.0003$) (Fig. 3).

The CP Model Increases and Resveratrol Reduces Anxiety-Like Behavior

Anxiety was assessed using the LDB test at P22 and P29. At P22, significant differences were observed between the experimental groups in the time spent on the light side [$F(3.88) = 23.68$; $p < 0.0001$], the time spent on the dark side [$F(3.88) = 23.90$; $p < 0.0001$], and the total number of transitions from one side of the box to the other [$F(3.88) = 6.129$; $p = 0.0008$]. At P22, the animals in the CPS group spent less time exploring the light side of the box (CPS = 68.72 ± 8.841 vs. CS = 159.1 ± 10.34 ; $p < 0.0001$)

(Fig. 4A) and more time on the dark side compared to the CS group (CPS = 231.3 ± 8.841 vs. CS = 140.9 ± 10.36 ; $p < 0.0001$) (Fig. 4B). These outcomes suggest that anxiety-like behavior traits had been induced by the CP model, since the animals spent more time in the dark and explored the novelty of the light side less. Animals in the CPR group, however, exhibited an increase in time spent on the light side (CPR = 181.7 ± 16.75 vs. CPS = 68.71 ± 8.841 ; $p < 0.0001$) (Fig. 4A) and a decrease in time spent on the dark side (CPR = 118.1 ± 16.75 vs. CPS = 231.3 ± 8.841 ; $p < 0.0001$) (Fig. 4B), suggesting possible anxiolytic effects of resveratrol in CP. No differences, however, were found between the control groups. Neither were any differences observed between any of the experimental groups in relation to the number of entries into the light and dark sides of the apparatus (Fig. 4C–E).

On P29, the animals in the CPS group were found to spend less time on the light side (CPS = 70.23 ± 7.717 vs. CS = 125.8 ± 13.16 ; $p = 0.0119$) (Fig. 4A), and more time on the dark side (CPS = 229.8 ± 7.717 vs. CS = 174.2 ± 13.16 ; $p = 0.0117$) (Fig. 4B), along with a decrease in the number of entries into the light side (CPS = 4.167 ± 0.6945 vs. CS = 7.417 ± 0.6904 ; $p = 0.0274$) and a reduced total number of entries into the light and dark sides (CPS = 7.417 ± 0.8207 vs. CS = 14.50 ± 1.449 ; $p = 0.0049$) (Fig. 4C), suggesting anxiety-like behavior in the CPS group. The animals in the CPS group showed a reduction in the number of entries into the light side compared to the CS group (CPS = 3.583 ± 0.5833 vs. CS = 6.125 ± 1.292 ; $p = 0.0274$) (Fig. 4D). Animals in the CPR group were found to spend more time on the light side (CPR = 158.1 ± 20.50 vs. CPS = 70.23 ± 7.17 ; $p < 0.0001$) and less time on the

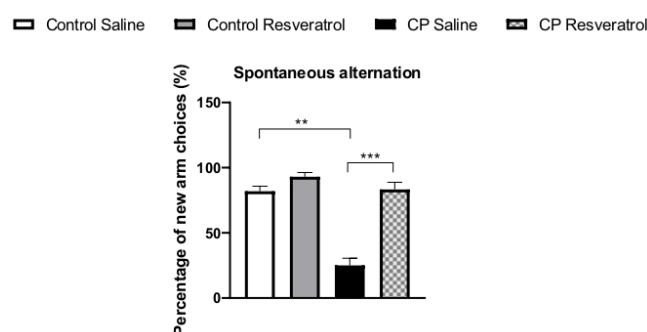


Fig. 3 Percentage of total new arm choices (choices followed by opposite sides of the maze) in the T-Maze test at ages 25, 26, and 27 post-natal day of the four experimental groups: CS ($n = 12$), CR ($n = 12$), CPS ($n = 12$), and CPR ($n = 12$); 4 runs per day were performed. The pups in the CPS group had a higher percentage of the same arm choices in the test ($p = 0.0016$) compared to the CS group,

while resveratrol treatment increased the percentage of new arm choices ($p = 0.0003$). There were no significant differences between the CS and CR groups. Values are expressed as mean $\pm$ standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

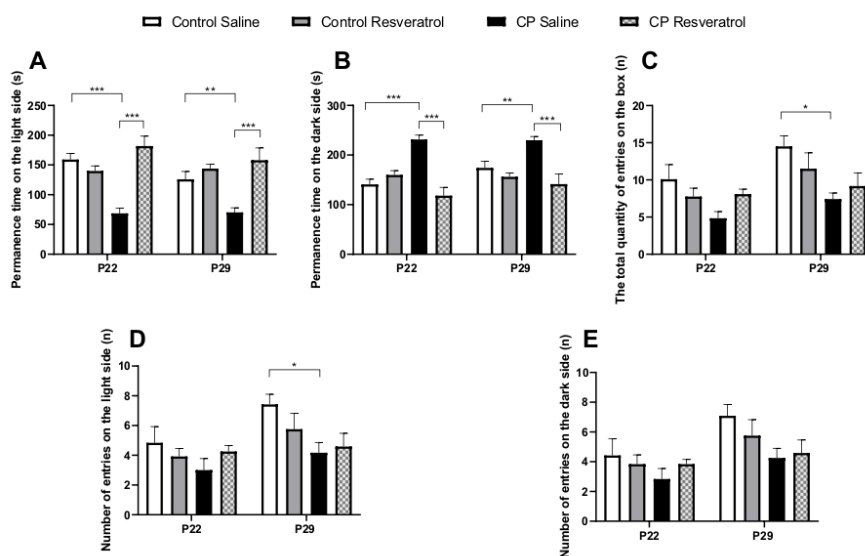


Fig. 4 Spent time and number of transitions between light and dark side in the light–dark box test at P22 and P29 of the experimental groups: CS ($n=12$), CR ($n=12$), CPS ($n=12$), and CPR ($n=12$). **A** Spent time on the light side. **B** Spent time on the dark side. **C** Total number of entries on the light and dark side. **D** Number of entries on

the light side. **E** Number of entries on the dark side. Values expressed as mean $\pm$ standard error of the mean. Tukey's two-way ANOVA repeated measures and multiple comparisons post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

dark side (CPR = 141.4 ± 20.39 vs. CPS = 229.8 ± 7.717 ; $p = 0.0001$) (Fig. 4A and B), suggesting that the anxiolytic effects of resveratrol persisted beyond P29, restoring the CPR animals to the levels of the control animals. We found no significant differences in the number of entries for the light and the dark sides, or in the total number of entries into the light and dark sides between the CPR and CPS groups ($p > 0.05$) (Fig. 4C–E). There were also no differences between control groups for any of the parameters evaluated (Fig. 4).

On P28, anxiety was also assessed using the Elevated Plus Maze and the results demonstrated differences in permanence time [$F(3,33) = 21.29$; $p < 0.0001$] and percentage of permanence time spent in the open arms [$F(3,33) = 21.29$] (Fig. 5A and B); permanence time [$F(3,33) = 13.12$; $p < 0.0001$] (Fig. 5D), the percentage of the permanence time [$F(3,33) = 13.12$; $p < 0.0001$] (Fig. 5E), the number of entries [$F(3,33) = 7.051$; $p = 0.0009$] (Fig. 5F) and the number of fecal boluses (Fig. 5I) in the closed arms [$F(3,33) = 35.66$; $p < 0.0001$]. Similar to the LDB test, animals in the CPS group presented a shorter total permanence time (CPS = 135.4 ± 12.69 vs. CS = 275.4 ± 9.751 ; $p < 0.0001$) and a shorter percentage of time (CPS = 22.56 ± 2.114 vs. CS = 45.90 ± 1.625 ;

$p < 0.0001$) (Fig. 5A and B) in the open arms of the maze compared to the CS group. They consequently also showed an increased permanence time (CPS = 322.4 ± 23.01 vs. CS = 196.7 ± 11.35 ; $p = 0.0004$), an increased percentage of time (CPS = 53.74 ± 3.835 vs. CS = 32.79 ± 1.892 ; $p = 0.0004$), and a larger number of fecal boluses (CPS = 5.667 ± 0.6435 vs. CS = 0.6667 ± 0.3553 ; $p < 0.0001$) in the closed arms (Fig. 5D, E, and I), indicating the presence of anxiety-like behavior in the cerebral palsy animals. Animals in the CPR group, however, showed an increase in permanence time (CPR = 301.9 ± 26.21 vs. CPS = 135.4 ± 12.69 ; $p < 0.001$) and, consequently, an increase in the percentage of permanence time (CPR = 50.32 ± 4.369 vs. CPS = 22.56 ± 2.114 ; $p < 0.0001$) in the open arms compared to the CPS group (Fig. 5A and B). Animals in the CPR group also spent less time in the closed arms (CPR = 172.4 ± 23.55 vs. CPS = 322.4 ± 23.01 ; $p < 0.001$) (Fig. 5D) and, consequently, presented a lower percentage for permanence time (Fig. 5E) (CPR = 28.73 ± 3.924 vs. CPS = 53.74 ± 3.835 ; $p < 0.0001$) and a reduction in the number of entries (CPR = 12.67 ± 1.281 vs. CPS = 22.42 ± 1.990 ; $p = 0.0009$) (Fig. 5F) and fecal boluses (CPR = 1.167 ± 0.3218 vs.

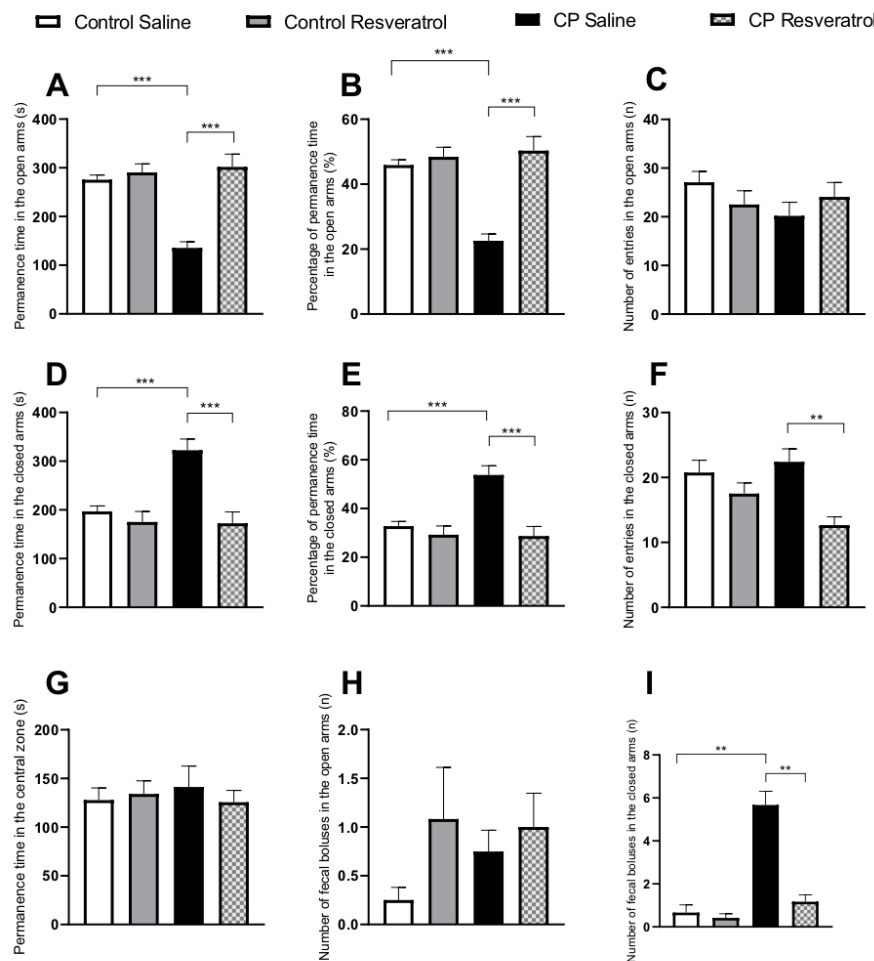


Fig. 5 Performance of animals in the Elevated Plus Maze at P28 of the four experimental groups: CS ($n=12$), CR ($n=12$), CPS ($n=12$), and CPR ($n=12$). **A–C** The permanence time, the percentage of permanence time, and the number of entries in the open arms, respectively. **D–F** The permanence time, the percentage of permanence time, and the number of entries in the closed arms, respectively. **G** The permanence time in the central zone. **H, I** The number of fecal

boluses in the open and closed arms, respectively. Animals in the CPS group show anxiety-like behavioral traits, while the CPR group showed behaviors similar to controls, suggesting therapeutic effects. Values are expressed as mean $\pm$ standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test or Kruskal–Wallis statistic. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

CPS = 5.667 ± 0.6434 ; $p = 0.0038$) (Fig. 5I) in the closed arms. No significant differences were found between animals in the CS and CR groups, nor in the number of entries (Fig. 5C) and fecal boluses (Fig. 5H) in the open arms and the permanence time in the central zone between any of the experimental groups (Fig. 5G).

Treatment with Resveratrol Protects Against Body Weight Loss But Does Not Prevent Brain Weight Loss in Cerebral Palsy

On P0, prior to the initiation of experiments, the animals presented no differences in body weight. By P29, at the end of the experiments, however, significant

differences were found between the experimental groups [$F(3,44) = 144.9$; $p < 0.0001$]. Animals in the CPS group exhibited lower body weight compared to the CS group (CPS = 37.65 ± 1.528 vs. CS = 80.04 ± 0.6119 ; $p < 0.0001$). Treatment with resveratrol attenuated this weight loss in animals in the CPR group compared to animals in the CPS group (CPR = 55.05 ± 2.093 vs. CPS = 37.65 ± 1.528 ; $p < 0.0001$). No significant differences were found between the animals in the CS group and those in the CR group (CS = 80.04 ± 0.6119 vs. CR = 78.66 ± 1.994 ; $p > 0.055$), suggesting that resveratrol did not alter the body weight of the control animals. In relation to *in vivo* brain weight, significant differences were found [$F(3,21) = 14.66$; $p < 0.0001$], with animals in the CP saline group showing a reduction in brain weight in comparison to those in the CS group (CPS = 1.259 ± 0.04726 vs. CS = 1.533 ± 0.04471 ; $p < 0.001$). Animals in the CPR group showed no significant changes in the absolute weight of the brain (CPR = 1.318 ± 0.03006 vs. CPS = 1.259 ± 0.04726 ; $p > 0.05$). No differences were observed between animals in the CS group and those in the CR group nor in relation

to relative brain weight (brain weight/body weight) [$F(3,21) = 2.084$; $p = 0.1330$] (Table 2).

Resveratrol Reduces Gene Expression Associated with Neuroinflammation in Animals with Cerebral Palsy But Not in Healthy Animals

Gene expression analysis of proinflammatory genes revealed significant differences between experimental groups in the expression of the IL-6 [$F(1,19) = 10.99$; $p = 0.0036$] and TNF- α [$F(1,17) = 61.62$; $p < 0.0001$]. Neonatal treatment with resveratrol-reduced IL-6 expression in the CPR group compared to that of animals in the CPS group (CPR = 0.609205 ± 0.581588 vs. CPS = 1.392811 ± 0.404912 ; $p = 0.0175$) (Fig. 6A). TNF- α expression was also reduced in the animals in the CPR group (CPR = 0.1 ± 0.5 vs. CPS = 2.4 ± 1 ; $p = 0.0007$) (Fig. 6B). There was, however, an increase in expression of IL-6 (CS = 1 ± 0.260384 vs. CR = 2.084932 ± 0.204809 ; $p = 0.0006$) and TNF- α (CS = 1 ± 0.8 vs. CR = 6.6 ± 0.6 ; $p < 0.0001$) in the animals in the CR group compared

Table 2 Body and brain weight. The table showed animal weight at P0 and P29 of the four experimental groups: CS ($n = 12$), CR ($n = 12$), CPS ($n = 12$), and CPR ($n = 12$). Before anoxia, sensory motor restric-

tion, and pharmacological manipulation, the animals did not show differences in body weight. On the last day of the experiment, animals in the CPS group showed the lowest body weight

Group	Body weight (g) P0	Body weight (g) P29	Brain absolute weight (g) P29	Brain relative weight (g) P29	<i>p</i> value
Control saline	6.003 ± 0.155	$80.038 \pm 0.612^{b***}$	$1.533 \pm 0.04471^{b***}$	0.02226 ± 0.001208	$p < 0.0001$
Control resveratrol	5.838 ± 0.189	78.655 ± 1.994	1.547 ± 0.02074	0.02039 ± 0.0009867	
CP saline	5.938 ± 0.135	37.647 ± 1.528^c	1.259 ± 0.04726	0.02526 ± 0.002199	$p < 0.0001$
CP resveratrol	5.902 ± 0.103	55.046 ± 2.093	1.318 ± 0.03006	0.02471 ± 0.001131	

Values expressed as mean $\pm$ standard error of the mean. Tukey's two-way ANOVA repeated measures and multiple comparisons post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ^aCS vs. VR; ^bCS vs. CP; ^cCP vs. CR

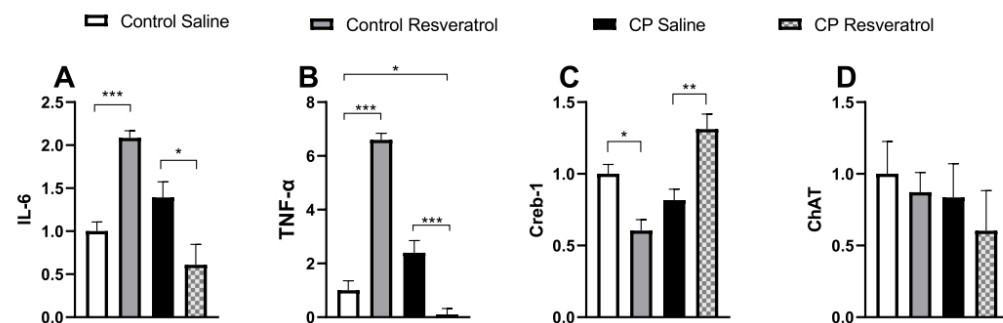


Fig. 6 RT-PCR of IL-6 (CS $n = 6$; CR $n = 6$; CPS $n = 5$; CPR $n = 6$), TNF- α (CS $n = 5$; CR $n = 6$; CPS $n = 5$; CPR $n = 5$), Creb-1 (CS $n = 6$; CR $n = 6$; CPS $n = 6$; CPR $n = 6$), and ChAT (CS $n = 6$; CR $n = 6$; CPS $n = 5$; CPR $n = 6$) genes from the hippocampus of animals sacrificed

by decapitation at P29. Values expressed as mean $\pm$ standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

with those in the CS group (Fig. 6A and B). Analyses of gene expression related to long-term memory consolidation revealed significant differences in Creb-1 (Fig. 6C) gene expression [$F(1, 20) = 10.20$; $p = 0.0046$], but no differences in that of ChAT [$F(1, 19) = 0.9161$; $p = 0.3505$] (Fig. 6D). The animals in the CPR group presented an increase in Creb-1 expression compared to those in the CPS group (CPR = 1.311908 ± 0.257985 vs. CPS = 0.817147 ± 0.185024 ; $p = 0.0020$) (Fig. 6C). Animals in the CR group, however, presented a reduction in Creb-1 levels compared to the CS group (CR = 0.604299 ± 0.188108 vs. CS = 1 ± 0.160555 ; $p = 0.0137$). No significant differences were observed in levels of ChAT (Fig. 6D).

Neonatal Resveratrol Treatment Reduces Microglia Activation in the Hippocampus in the Cerebral Palsy Model

Immunohistochemistry analysis to confirm the presence of Iba1+ cells in the hippocampus revealed significant

differences in the density of microglia in the DG [$F(3, 15) = 9.998$; $p = 0.0007$], CA1 [$F(3, 15) = 9.683$; $p = 0.0008$], and CA3 [$F(3, 15) = 7.388$; $p = 0.0029$]. Significant differences were also observed in the percentage of activated microglia in the DG [$F(3, 15) = 46.17$; $p < 0.0001$] and in CA1 [$F(3, 15) = 48.10$; $p < 0.0001$] and CA3 [$F(3, 15) = 147.9$; $p < 0.0001$]. The animals in the CPS group exhibited a reduction in the density of microglia in the DG (CPS = 361.2 ± 14.09 vs. CS = 479.1 ± 22.04 ; $p = 0.0020$) (Fig. 7A), CA1 (CPS = 631.4 ± 36.30 vs. CS = 951.1 ± 56.80 ; $p = 0.0013$) (Fig. 7B) and in CA3 (CPS = 522.3 ± 76.30 vs. CS = 803.0 ± 57.55 ; $p = 0.0350$) (Fig. 7C) compared to the CS group. Despite this reduction in cell density, the CPR group presented an increase in the number of microglia in the DG compared to CPS group (CPR = 461.8 ± 15.10 vs. CPS = 361.2 ± 14.09 ; $p = 0.0074$) (Fig. 7A). In relation to the profile of microglia in the hippocampus, animals in the CPS group showed an increase in the percentage of activated microglia in the DG (Fig. 7C) (CPS = 35.30 ± 0.6264 vs. CS = 28.54 ± 1.080 ;

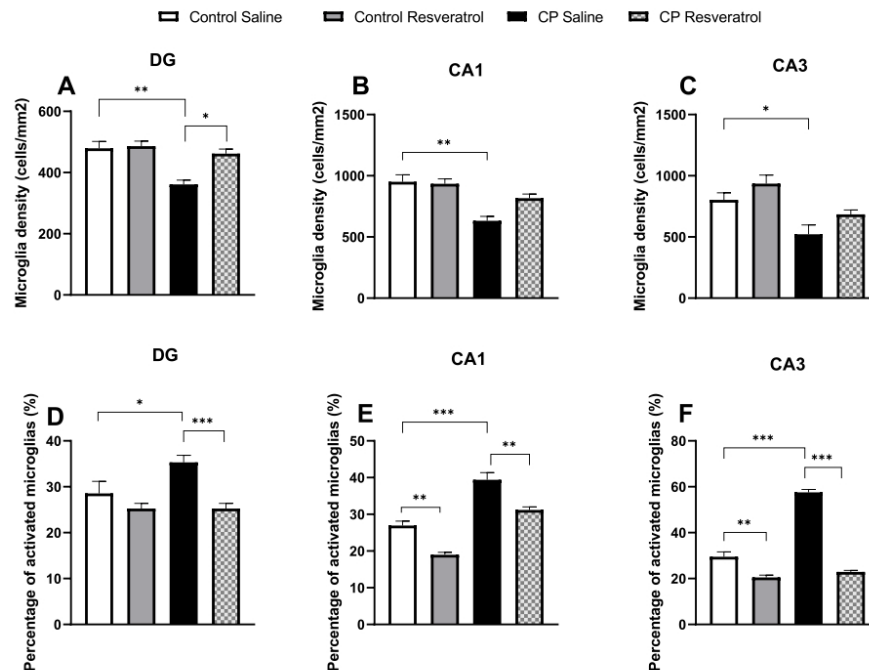


Fig. 7 Immunohistochemistry for Iba1+ cells in the hippocampus. **A, B, C** The cell density in DG, CA1, and CA3, respectively. **D, E, F** The percentage of activated microglia in DG, CA1, and CA3, respectively. The experimental groups were randomly divided according to the cerebral palsy model or control with resveratrol or

saline treatment: control saline ($n=6$), control resveratrol ($n=6$), CP saline ($n=6$), and CP resveratrol ($n=6$). The values expressed as mean $\pm$ standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

$p < 0.0001$), in CA1 (CPS = 39.38 ± 1.964 vs. CS = 26.93 ± 1.227 ; $p < 0.0001$) (Fig. 7D), and in CA3 (CPS = 57.58 ± 1.286 vs. CS = 29.50 ± 2.130 ; $p < 0.0001$) (Fig. 7E) compared to the CS group. Nevertheless, animals in the CPR group showed a reduced percentage of activated microglia in the DG (CPR = 25.24 ± 0.4723 vs. CPS = 35.30 ± 0.6264 ; $p < 0.0001$), CA1 (CPR = 31.18 ± 0.8415 vs. CPS = 39.38 ± 1.964 ; $p = 0.0014$), and CA3 (CPR = 22.93 ± 0.6704 vs. CPS = 57.58 ± 1.286 ; $p < 0.0001$) compared to CPS group (Fig. 7D–F). The pups in the CR group also exhibited a reduction in the percentage of activated microglia in CA1 (CR = 18.98 ± 0.6863 vs. CS = 26.93 ± 1.227 ; $p = 0.0018$) (Fig. 7E) and CA3 (CR = 20.48 ± 1.024 vs. CS = 29.50 ± 2.130 ; $p = 0.0020$) (Fig. 7F) compared to the CS group (Fig. 8).

Resveratrol Treatment Increases Cell Proliferation in the Hippocampus

Immunohistochemistry analysis to identify BrdU+ cells revealed significant differences in the number of cells in the dentate gyrus [$F(3,18) = 17.87$; $p < 0.000$], CA1 [$F(3,18) = 69.96$; $p < 0.0001$], and CA3 [$F(3,18) = 56.75$; $p < 0.0001$]. The pups in the CPS group had fewer cells marked by BrdU+ in the dentate gyrus (CPS = 1139 ± 40.50 vs. CS = 2489 ± 140.2 ; $p < 0.0001$)

(Fig. 8A), CA1 (CPS = 314.6 ± 20.96 vs. CS = 1491 ± 72.70 ; $p < 0.0001$) (Fig. 8B), and CA3 (CPS = 287.9 ± 19.93 vs. CS = 1361 ± 82.06 ; $p < 0.0001$) (Fig. 8C) compared to the CS group. Despite this, animals in the CPR group presented an increased number of BrdU+ cells in the CA1 (CPR = 766.4 ± 66.71 vs. CPS = 314.6 ± 20.96 ; $p = 0.0003$) (Fig. 8B) and CA3 (CPR = 698.9 ± 70.14 vs. CPS = 287.9 ± 19.93 ; $p = 0.0008$) (Fig. 8C) compared to the CPS group. Healthy animals treated with resveratrol, however, exhibited the opposite effect, with a reduction in the numbers of cells in the dentate gyrus (CR = 1668 ± 206.2 vs. CS = 2489 ± 140.2 ; $p = 0.0020$) (Fig. 8A), CA1 (CR = 563.0 ± 41.97 vs. CS = 1491 ± 72.70 ; $p < 0.0001$) (Fig. 8B), and CA3 (CR = 520.7 ± 19.81 vs. CS = 1361 ± 82.06 ; $p < 0.0001$) (Fig. 8C) compared to animals that received saline (Fig. 9).

Discussion

The results of the present study demonstrate the negative impact of the cerebral palsy model through the association of perinatal anoxia at ages 1 and 2 days of postnatal life and sensorimotor restriction at ages 2 to 28 days of postnatal life on episodic memory and anxiety-like behavior associated with developmental impairments in

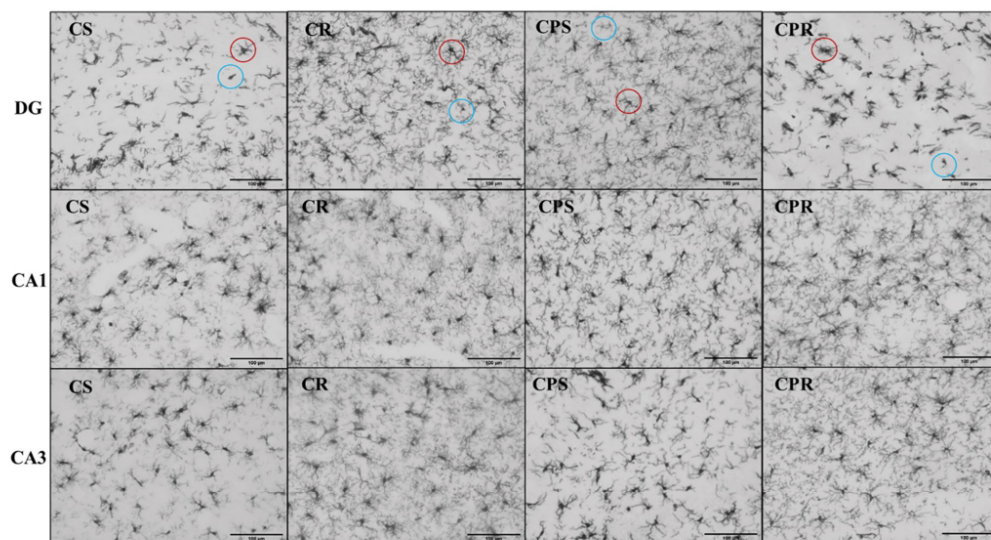


Fig. 8 Immunohistochemistry for Iba1+ cells in the hippocampus. Encircled in red are activated microglia, while encircled in blue are inactivated microglia. Each column represents an experimental group

in the following order: CS, CR, CPS, and CR. Each row of the image represents a region of the hippocampus in the following order: DG, CA1, and CA3

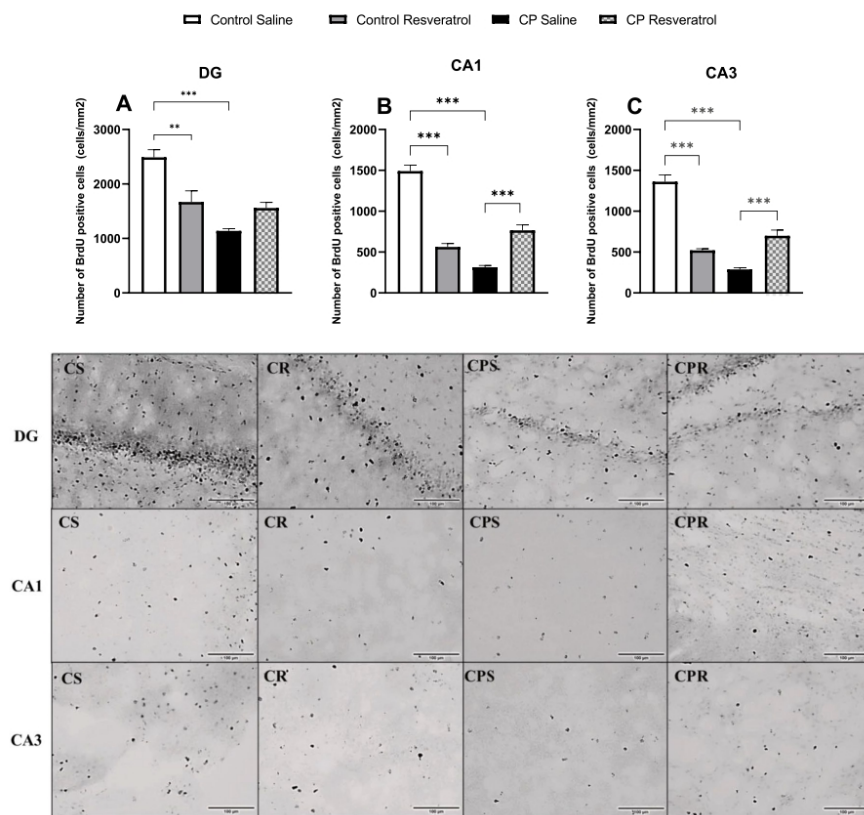


Fig. 9 Immunohistochemistry for BrdU+ cells in the hippocampus. **A** The number of cells in DG. **B** The number of cells in CA1. **C** The number of cells in CA3. The experimental groups were randomly divided according to the cerebral palsy model or control with resveratrol or saline treatment: control saline ($n=7$), control res-

veratrol ($n=7$), CP saline ($n=7$), CP resveratrol ($n=7$). The values expressed as mean $\pm$ standard error of the mean. ANOVA two-way multiple comparisons Tukey's post hoc test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

the hippocampus of young rats. The behavioral analyses in young animals at 21st to 29th postnatal day of life demonstrated impairments on visuospatial memory for object recognition as well as short- and long-term spatial localization. The animals also presented disturbances in relation to anxiety-like emotional behavior in paradigms assessing anxiety. It is interesting to note that these behavioral disturbances are correlated with increased expression of pro-inflammatory genes and microglial activation, leading to a reduction in neuroplastic mechanisms such as cell proliferation and Creb-1 levels, in the hippocampus. Nevertheless, neonatal treatment with resveratrol demonstrated promising results in relation to attenuation of this impairment,

facilitating the formation and recovery of novel episodic memories and reducing hippocampal damage.

Previous studies using models of CP have demonstrated promising results for the use of polyphenols in the critical period of development, such as resveratrol and kaempferol, in the neuromusculoskeletal sequelae present in CP [15, 67, 68]. The neonatal treatment with resveratrol has demonstrated its effectiveness in reducing biomarkers of oxidative stress and mitochondrial biogenesis in the somatosensory cortex, promoting an evolution in the strength and postural development of animals submitted to CP [67]. Similarly, supplementation with kaempferol in the neonatal period has been demonstrated to reduce biomarkers of oxidative stress

and activated microglia in the hippocampus [15], thus promoting an improvement in locomotor activity by increasing the distance traveled in the open field, speed, and a reducing of the number of immobile episodes [15]. These data suggest a possible improvement in the motor deficits present in CP by neonatal treatment with polyphenols.

Despite these studies revealing a negative impact of cerebral palsy on the somatosensory cortex and hippocampus [15, 67, 68], which are regions involved in cognitive and behavioral processes [18, 20, 22, 23, 29], there are still lacunae in the literature about the effects of polyphenols on these sequelae. Furthermore, studies indicate that early brain injury can impact the brain by increasing markers of oxidative stress and neuroinflammation causing impairments in episodic memory [18, 69, 70]. This occurs because the formation of new memories involves a series of changes in synaptic connections and biochemical modifications such as the synthesis of new proteins or alterations in preexisting proteins in the brain [20, 22, 23, 29]. However, the memory should thus be seen as an ability that is related to adequate brain functioning and that can be modulated by environmental factors such as brain injuries early in life [11, 71–73]. The findings of the present study indicate that brain injury can impact the formation and retrieval of episodic memories.

The animals submitted to a cerebral palsy model presented a reduced novel object discrimination index for novel objects in the NORT and a reduction in the number of a novel choices in the T-Maze test. The impaired performance of these animals suggests impairments in spatial memory and in short-term and long-term object recognition memory. The early injury can result in a deficiency of oxygen and glucose in the brain, impairing neurodevelopment [74, 75]. In view of its high level of functioning and consequent need for a greater supply of oxygen and glucose supply, the hippocampus is one of the regions most affected by brain injury, resulting in abnormal development of this region [75–77]. Animals subjected to CP in the present study were found to have short-term memory impairment and this corroborates previous findings indicating that such injuries cause disorders in the dorsal hippocampus and a synaptic disequilibrium that impacts the formation and retrieval of short-term memory [11, 78]. An insult in the critical period can lead to metabolic changes in the brain that persist into adulthood, impairing the maturation of the hippocampus and the formation of new long-term memories [79].

Although CP causes memory formation impairments in young animals, neonatal treatment with resveratrol was found to attenuate impairments in object recognition and spatial recognition memory. Neonatal treatment with polyphenols has demonstrated promising effects in reducing hippocampal impairment resulting from early brain injury [15]. Resveratrol is a polyphenol that has antioxidant and anti-inflammatory properties in the brain, and also improves

synaptic plasticity [80, 81]. Synaptic plasticity is the molecular basis of memory and learning [20, 23, 82]. Treatment with resveratrol at a dose of 100 mg/kg has been shown in previous studies to improve spatial memory after brain injury by increasing synaptic plasticity, leading to increased numbers of dendritic spines in the dentate gyrus of the hippocampus and increased expression of synapsin protein [82]. This indicates the possible mechanisms underlying the memory impairment reduction found in the present study.

In addition to memory impairment, the animals in the cerebral palsy group also exhibited an increase in anxiety-like behaviors in the LDB Test and EPM Test. Previous findings have likewise indicated that brain injury impacts the hippocampus in diverse ways, affecting both spatial memory and anxiety-like behavior [83]. This occurs because the ventral and dorsal regions of the hippocampus are connected to different brain structures and consequently perform different behavioral functions [84]. The ventral region of the hippocampus seems to be associated with emotional behavior because it has connections with areas such as the amygdala and hypothalamus [84, 85]. Interesting findings indicate that in cerebral palsy there is an increased activation of microglia in the hypothalamus, suggesting negative impacts of the injury on regions involved in emotional responses [86]. Moreover, early hypoxia-ischemia increases anxiety-like behavior in association with sensory-motor, tactile, and memory alterations [87]. The model adopted in the present study, however, uses two different insults to mimic cerebral palsy sequelae, exposing the animals to early aggressions [54, 55]. Stress early in life can produce anxiety-like behaviors, as observed in the results of the present study. It should, however, be borne in mind that the animals exposed to this model underwent severe alterations in relation to locomotion [10, 15] and this may have affected the results, despite the fact that the animals performed similarly on two different apparatuses.

Neonatal treatment with resveratrol was found to attenuate anxiety-like behaviors. The animals spent less time on the dark side of the box at P22 and P29. On the EPM, animals in the CP resveratrol group spent less time in the closed arms. These results suggest that the anxiolytic effects of the treatment persisted even after the pharmacological manipulation was completed. Resveratrol is a natural compound capable of crossing the blood–brain barrier and modulating brain function, improving memory and mood, and producing antidepressant and anxiogenic effects [88]. Hyperactivation and dysregulation of the limbic hypothalamic–pituitary–adrenal axis seem to be associated with anxiogenic behaviors in a rat model of post-traumatic stress disorder [89]. Treatment with resveratrol would seem, however, to exert an impact on this axis in so far as it increases permanence time in the open arms of the EPM, as observed in the present study [89], and in addition, reduces the frequency of

freezing behavior by increasing Creb-1 and BDNF levels in the brain [89]. The anxiolytic effect of resveratrol appears to be associated with its ability to activate SIRT1, with animals subjected to a model of chronic normobaric hypoxia showing a reduction in anxiety-like behavior in the LDB, EPM, and open field tests after treatment with resveratrol (20 mg/kg) [90]. This is similar to the results of the present study, which also show that lower doses (10 mg/kg) of resveratrol produce favorable outcomes in juvenile rats.

The present study found that animals with CP exhibited a reduction in the weight of both the body and the brain. Previous studies have reported that animals with cerebral palsy experienced impaired somatic development [10, 15, 37, 91]. This occurs because the animals are subjected to early-life stress factors and the use of sensory-motor restriction may hinder the animal's ability to move about [54, 55], thereby impairing feeding behavior. The literature reports severe deficits in the locomotor activity of such animals [86], which may make it difficult for the animals to feed. Previous studies have also found a deficit in the masseter and digastric muscles involved in mastication [92], which would also hinder feeding behavior and reduce body weight.

The present study conducted an intervention during the critical period and observed that neonatal treatment with resveratrol was capable of attenuating somatic damage. Although we did not evaluate specific aspects of the animals' anthropometric measurements, a systematic review with meta-analysis has observed that resveratrol supplementation can reduce body weight by reducing the quantity of fat and abdominal circumference and promoting an increase in muscle tissue [93]. Findings indicate that resveratrol is capable of regulating cellular energetic metabolism by increasing Sirt1 expression and regulating mitochondrial functions, thereby protecting against lipid accumulation [94, 95]. Although the present study did not observe the effects of resveratrol in malnutrition models, resveratrol may thus affect metabolic disorders by reducing lipid accumulation and increasing glycogen storage in muscle and liver cells [96]. This provides some indication of which mechanisms may underlie the effect of resveratrol on metabolic alterations that affect body weight in cerebral palsy.

The dorsal portion of the hippocampus is connected to brain regions involved in the process of formation, storage, and recovery of episodic memories, while the ventral portion is connected to regions involved in emotional regulation [84]. Alterations in the development of the hippocampus may thus cause significant behavioral impairments. Cytokines, such as tumor necrosis factor and interleukins, are small glycoproteins that act as communication vectors between cells and are involved in the control of crucial cellular functions, such as development, differentiation, and cell death [97]. When released into the nervous system, however, these cytokines can have deleterious effects [76,

97] and are the cells involved in basal and inflammatory conditions in the brain [98, 99]. Alterations in interleukin expression can also affect brain function and behavior. Findings indicate that dysregulations of IL-6 expression produce behavioral impairment in cases of brain injury [100]. This occurs because neuroinflammation is one of the main factors contributing to neurological disorders and impaired behavior [101] and has been associated with the development of cerebral palsy [6, 102].

The mechanisms underlying hypoxia-ischemia are not yet fully understood, but findings indicate that low supplies of oxygen and glucose during hypoxia-ischemia negatively impact the energy reserve needed for cell survival and cause increased excitotoxicity [103–105]. After this, a dysregulation of levels of free radicals and production of reactive oxygen species occur in the brain, leading to oxidative stress and neuroinflammatory responses and causing tissue damage [105, 106]. Nevertheless, neonatal treatment with resveratrol (10 mg/kg) has been shown to reduce the expression of IL-6 and TNF- α in the hippocampus of animals with CP. Studies suggest that resveratrol is a compound with powerful anti-inflammatory properties [48, 52, 107] deriving from activation of the Sirt-1/NF- κ B signaling pathway [108, 109]. Previous findings suggest that resveratrol is capable of inhibiting neuroinflammation in ischemia models by inhibiting miR-155 gene expression and, consequently, inhibiting M2-type microglia [110].

Previous studies demonstrate that abnormalities in neuroplasticity mechanisms in the dentate gyrus after brain injury, such as reduced cell migration, may be associated with long-term memory impairment [111]. The results of the present study provide novel evidence that, in cerebral palsy, there is an increase in microglial activation, associated with a reduction in cell proliferation in the DG, CA1, and CA3 regions of the hippocampus, indicating the neural correlates that may be involved in the cognitive and behavioral impairments observed in CP. In this context, neonatal supplementation with resveratrol was shown to be capable of reducing activation of microglia in the DG, CA1, and CA3. Previous studies have found that overexpression of IL-6 and activation of microglia in the hypothalamus, cerebellum, and hippocampus are associated with the motor and behavior sequelae of CP [37, 86]. However, the reducing microglia activation in the hippocampus promoted a reduction in motor impairment [15]. Microglia play a crucial role in brain development by eliminating excess synapses during postnatal life, operating out the process of synaptic elimination [112–114]. However, the hippocampus is a region marked by neurogenesis, resulting in synaptic reorganization [115]. Thus, microglia act in synaptic pruning and are essential in synaptic reorganization induced by neurogenesis [114], to acting in the natural process of forgetting [114]. Besides, the microglia are the first cells to respond to insults by mediating inflammatory

responses [116–118]. The activation of microglia in the hippocampus can occur in excess in response to brain injury impairing the adequate function of the hippocampus [37], and alteration of microglial metabolism and profile contributes to neurotoxicity stage [119]. For this reason, we suggest that reduced microglial activation in the hippocampus is related to improved memory impairment, as observed in our study. Previously, we verified that reducing microglial activation in the hippocampus reduced the motor sequelae of CP [15, 37].

Additionally, the neonatal treatment with resveratrol caused an increased cell proliferation in the CA1 and CA3 regions of the hippocampus, providing evidence of promising effects in relation to attenuating CP sequelae. Previous evidence has observed that early insult (prenatal or postnatal) directly impacts the generation of new neurons in the hippocampus, causing a decline in the production of neural stem cells [76]. Furthermore, the reduction of neuroplastic mechanisms such as CREB-1 gene expression, BDNF, and the neurogenesis process are associated with memory impairment in early brain injuries [18]. Therefore, we suggest that reduced microglial activation associated with increased cell proliferation in the dorsal hippocampus is associated with a reduction in episodic memory impairment in young animals. Despite the beneficial effects in relation to cerebral palsy, the control animals treated with resveratrol showed an increase in the expression of IL-6 and TNF- α genes and a reduction in numbers of BrdU⁺ cells in the DG, and the CA1 and CA3 regions. This occurs because healthy animals do not necessarily need this supplementation during the neonatal period, different from animals with CP which have been exposed to an early brain injury and present a series of developmental dysfunctions.

Nevertheless, the control animals treated with resveratrol did not present memory impairments. This occurs because memory is a complex cognitive process and various neural structures and biochemical mechanisms participate in this process, for example, the prefrontal cortex, in particular the dorsolateral prefrontal cortex, which is related to the ability to process and manipulate sensory information before it is stored as a long-term memory [120, 121]. The prefrontal cortex is a region involved in executive attention, working memory, decision-making, and emotional regulation [122, 123]. These abilities directly affect the process of formation of long-term memories. Besides, previous studies with polyphenols have demonstrated that supplementation during the critical period has beneficial effects on the brain and behavior of animals with cerebral palsy, but has contrary effects on control animals [15, 81]. For this reason, histological, molecular, and behavioral tests to obtain a more comprehensive response

have been performed. Thus, we did not obtain impairments in memory and anxiety-like behavior in the CR animals. Therefore, neonatal treatment with resveratrol would thus appear to have beneficial effects on the neuroinflammatory response present in young rats with cerebral palsy, but additional studies are needed to better comprehend the long-term effects of polyphenols.

The present study observed impairments in the formation and recovery of visuospatial episodic object recognition and spatial location memory and an increase in anxiety-like behaviors in young animals with cerebral palsy. Increased neuroinflammation and reduced cell proliferation in the dentate gyrus, and the CA1 and CA3 regions of the animals' hippocampus were also observed. Neonatal treatment with resveratrol was able to reduce neuroinflammation in the hippocampus and promote an increase in cell proliferation and reduction in episodic memory impairment and in anxiety-like behavior.

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Author Contribution All authors contributed to the study conception and design. C.M.S.S.C: conceptualization, data collection, analysis of result and decided on the manuscript's structure. R.M.C: intellectual supervision and analysis of results. S.C.P, V.S.S, and L.N.F.B: data collection, results analysis, and writing. O.H.S.J and C.J.L: conducting the gene expression experiments and analysis of results. P.A.R.J, O.G.Q, and L.T: conducting the immunohistochemistry procedure and intellectual supervision. A.E.T: supervision, conceptualization, formal analysis, and decided on the manuscript's structure.

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Data Availability The datasets analyzed during the study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval The present study was conducted in accordance with the recommendations of the National Council for Animal Control and Experimentation (CONCEA-Brazil) and was not initiated until approval had been obtained from the Ethics Committee on Animal Use (process number CEUA- 0082/2022) of the Federal University of Pernambuco (UFPE). All animal experiments were conducted in accordance with the "National Institute of Health Guide for Care and Use of Laboratory Animals" [124].

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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APÊNDICE B – THERAPEUTIC ADVANCES FOR TREATING MEMORY IMPAIRMENTS IN PERINATAL BRAIN INJURIES WITH IMPLICATIONS FOR CEREBRAL PALSY: A SYSTEMATIC REVIEW AND META-ANALYSIS OF PRECLINICAL STUDIES

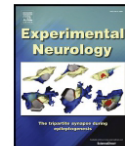
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Review Article

Therapeutic advances for treating memory impairments in perinatal brain injuries with implications for cerebral palsy: a systematic review and meta-analysis of preclinical studies

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ABSTRACT

Cerebral palsy (CP) is a neurodevelopmental disorder caused by damage to the immature brain. CP is considered the main cause of physical disability in childhood. Studies have shown that memory function and emotional behaviour are significantly impaired in CP. Current thought is that interventions for neuromotor damaged play a prominent role, but neglects the memory acquisition problems that affect the functioning and quality of life of these children. This systematic review aims to map and analyse pre-clinical interventions used to treat memory formation problems resulting from CP. For this, a search was carried out in the Pubmed, Web of Science, Scopus and Lilacs databases. Then, eligibility, extraction date and evaluation of the methodological quality of the studies were determined. 52 studies were included in this review, and 27 were included in a meta-analysis. Assessing memory performance as a primary outcome, and structural and biochemical changes in the hippocampus as a secondary outcome. CP models were reported to be induced by hypoxia-ischemia, oxygen deprivation and liposaccharide (LPS) exposure, resulting in impairments in the formation of short-term and long-term memory in adult life. A reduction in escape latency and dwell time were observed in the target quadrant as well as an increase in the time needed for the rodents to find the platform in the Morris Water Maze (MWM). Brain injuries during the perinatal period are considered an insult that negatively impacts hippocampus maturation and causes impairment in memory formation in adult life. Some studies reported that regions of the hippocampus such as the dentate gyrus and *cornu ammonis* 1 were impaired in CP, noting an increase in oxidative stress enzymes and pro-inflammatory cytokines, associated with a reduction in BDNF and neurogenesis levels. These were reported to cause a reduction in the number of neurons and the volume of the hippocampus, in addition to an increase in astrogliosis and apoptosis of neurons and difficulties in forming new memories similar to those that occur in children with CP. Interventions that reduced neuroinflammation and the presence of free radicals were highlighted as a therapy for the memory disturbance present in CP. Preclinical studies registered treatments with oxygen interventions, resveratrol and erythropoietin, which were able to reduce the damage to the hippocampus and promote improvements in memory and behaviour. In the meta-analysis of selected studies, we observed favorable results, through effect size, for the use of oxygen interventions (SDM -6.83 95% CI [-7.91, -5.75], Z = 12.38, p = 0.03; I² = 71%), erythropoietin (SDM -3.16 95% CI [-4.27, -2.05], Z = 5.58, p = 0.002; I² = 82%) and resveratrol (SDM -2.42 95% CI [-3.19, -1.66], Z = 6.21, p = 0.01; I² = 77%), stimulating plastic responses in the hippocampus and facilitating the memory formation, with these presenting positive effects in general (SDM -2.84 95% CI [-3.10, -2.59], Z = 22.00; p < 0.00001; I² = 92.9%). These studies demonstrate possible

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avenues of intervention for memory alterations in experimental models of early brain injuries, highlighting promising interventions that can facilitate the maturation of the hippocampus and memory formation and, consequently, minimize functional problems that arise during development.

1. Introduction

Cerebral palsy (CP) is a neurodevelopmental disorder with a number of functional repercussions in the organism (Pereira et al., 2021; Fragopoulou et al., 2019; Jacobsson and Hagberg, 2004). Due to the complexity of its aetiology, its causes are not fully understood. Brain injuries in the neonatal and perinatal period are known to contribute to CP development, including asphyxia, hypoxia-ischemia and infections (Fragopoulou et al., 2019; McIntyre et al., 2022; Jacobsson and Hagberg, 2004; Blair and Stanley, 2002). These trigger a cascade of functional consequences (Fragopoulou et al., 2019). Given the alterations and damages present in CP, it has been the subject of a number of studies: pre-clinical studies have pointed to a relationship between the lesions and brain alterations present in CP and their impact on behaviour, especially in locomotion (Pereira et al., 2021), where it is possible to observe a delay in the acquisition of motor skills (Krigger, 2006; Ward et al., 2006; Mockford and Caulton, 2010; Peterson et al., 2013; Coq et al., 2008) and in motor coordination and movement (Coq et al., 2008; Pereira et al., 2021), causing functional impairments of various aspects of locomotion (Pereira et al., 2021).

Thus, the CP is understood as a set of syndromes that cause impairments in the movement and posture of affected children (Marret et al., 2013; Rumajogee et al., 2016). Nevertheless, sensory-perceptual abnormalities are also found in association with CP, impairing cognitive abilities such as memory (Hirsh et al., 2010; Al-Nemr and Abdelazeim, 2017; Pirila et al., 2004; Pueyo et al., 2009; White and Christ, 2005). For this reason, a high prevalence of learning problems is observed, in approximately about 40% of children with CP (Himmelmann et al., 2006; Van Rooijen et al., 2015). Memory and fine motor movement impairments are associated with low school performance in children with CP (Van Rooijen et al., 2015).

The development of CP is multifactorial and may occur after brain lesions in prenatal, perinatal and postnatal periods (Graham et al., 2019). The main risk factors for the emergence of CP are closely linked to events that occur in the foetal and neonatal period, such as foetal growth restriction, multiple pregnancies, birth asphyxia and, above all, premature birth, which is still considered the main risk factor for the development of CP (Graham et al., 2019; Oskoui et al., 2013; Stavsky et al., 2017). Due to the number and complexity of events that can trigger the onset of CP, it is considered the most common cause of physical disability in childhood (Himmelmann, 2013; Yeargin-Allsopp et al., 2008), affecting about 3,4 per 1000 live births in regions from low-income countries and 1,5 per 1000 live births in regions from high-income countries (McIntyre et al., 2022), thus pointing to the relevance of obstetric care.

However, recent preclinical studies have pointed to impairments beyond the neuromotor sequelae, observing an increase in depressive and anxiogenic behaviours (Herrera et al., 2018; Granja et al., 2021) and a reduction in memory performance (Granja et al., 2021; Matsuda et al., 2021) in early brain lesion models. This difficulty in forming and retrieving new memories can be associated with reported damages in the brain areas related to emotional behaviour and memory, such as the frontal cortex and hippocampus (Granja et al., 2021; Visco et al., 2021; Basilious et al., 2014; Matsuda et al., 2021). The hippocampus can be understood as a structure sensitive to neuroinflammation and asphyxia in the perinatal period, showing a reduction in neurogenesis and a significant increase in neurodegeneration, accompanied by severe behavioural and memory alterations (Granja et al., 2021; Visco et al., 2021; Basilious et al., 2014; Matsuda et al., 2021). Among the most present alterations, there is damage to the *cornu ammonis* 1 (CA1) and 3 (CA3)

and the dentate gyrus (DG) of the hippocampus, reflected in changes in memory formation especially short-term spatial memory and reference memory (Matsuda et al., 2021; Takada et al., 2016). Also, in these previous studies, the literature reported that perinatal brain damage affected neurogenesis after birth in key brain regions such as the hippocampus and subventricular zone (Visco et al., 2021). For this reason, early brain injury affects hippocampal maturation and impairs memory formation.

The first years of life, this perinatal period, represent a crucial phase for development, with consequences on both the physiological systems and behaviour (Kelly et al., 2012; Anderson et al., 2011). The effects of hypothermia on memory are not yet fully understood, because memory have not yet matured in this early period; there are some disagreements about its long-term repercussion (Cainelli et al., 2021). In this context, various types of interventions are currently being studied to mitigate functional impairments in individuals with brain injuries, hypothermia is commonly used in children with brain injuries because can impede the extent of brain infarct (Wagner et al., 2002). Hypothermia can reduce oxidative stress and inhibit the release of pro-inflammatory cytokines after brain injury (Talma et al., 2016), as well as reduce neurodevelopmental damage in newborns (Azzopardi et al., 2014) and also reduce the risk of mortality in neonates, becoming an acute therapeutic strategy investigated in clinical and preclinical studies (Cainelli et al., 2021; Azzopardi et al., 2014; Shankaran et al., 2012).

In this context, despite advances in perinatal care to reduce mortality and functional consequences, there are still few therapeutic strategies that address the central problem of CP as a brain injury for clinical use. As well, there are reported few strategies to attenuate the damage and memory formation disturbances in subjects with brain lesions. Given the annual increase in cases of CP and brain lesions cases, we questioned: what are the treatment prospects for memory formation problems during lifespan in subjects with CP? Thus, the aim of this review is to map and analyse the effects of the main used in the treatment of memory formation problems and hippocampal damage present in adult animals that were subjected to brain damage during the perinatal period with implications for the development of cerebral palsy.

2. Materials and methods

2.1. Systematic review report and description of protocol

The present systematic review was carried out according to the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement for transparent reporting of systematic reviews and meta-analyses (Rethlefsen et al., 2021; Liberati et al., 2009). The protocol used for this systematic review was registered with the International Prospective Register of Systematic Review (PROSPERO) (Registration n. CRD42022298566).

2.2. Search strategy

The search in the scientific literature was carried out between November 2021 and January 2022 in the Medline/Pubmed databases (National Library of Medicine / Medical Literature Analysis and Retrieval System Online: 1943- Jan/2022), Web of Science 1991- Dec/2021), SCOPUS (1953-Jan/2022) and LILACS (Latin American and Caribbean Literature in Health Sciences: 1990-Dec/2021). Appropriate terms were used in each database to cover experimental models of cerebral palsy and interventions aimed at memory performance according to MeSH (Medical Subject Headings), DeCS Descriptors (Health Science

Descriptors) and appropriate keywords and synonyms for each database, using the following terms: cerebral palsy AND memory AND treatment (Table 1).

2.3. Eligibility

No linguistic preference or year of publication restrictions were applied to the database search. The selection of studies was in two phases: the first, by title and abstract. Where there was any doubt whether the method within the defined inclusion and exclusion criteria, the methodology section was read in full. The second phase consisted of a complete reading of the selected articles, being considered for inclusion in the present review according to the eligibility criteria. In addition, the reference lists of included articles were inspected and relevant articles were selected and submitted to the eligibility criteria. This process was carried out by two independent reviewers (Calado and Pereira). Any disagreement or conflict was resolved by discussion and consensus between the two reviewers and, when necessary, a third reviewer was consulted (Toscano).

The studies selected in this review carefully followed the inclusion criteria. The preclinical studies followed the following criteria: i) rodents that were submitted to any experimental model of CP in the prenatal, perinatal or postnatal period or rodents submitted to brain injuries in the prenatal, perinatal or postnatal period that could result in CP (up to day 10 of postnatal life); ii) studies that assessed memory through behavioural tests; iii) animals that had been exposed to any intervention with an effect on memory after the brain insult, where the type of intervention, exposure time and dosage were detailed; iv) studies that assessed the impact of brain injury and intervention on the hippocampus and the method used to assess; v) studies that had a control group in their experimental design. On the other hand, the following exclusion criteria were adopted: i) non-original article; ii) did not use a cerebral palsy model; iii) did not assess memory; iv) used genetically modified animal species; v) reported brain damage in adult animals; vi) in vitro studies; vii) did not assess memory; viii) did not assess the hippocampus; ix) reported intervention before brain injury; x) did not report the age of the animals when they were submitted to brain injury; (Table 2 and Fig. 1). Discrepancies in the inclusion of both preclinical studies were resolved after consensus between the two authors (Calado and Pereira) or were referred for evaluation by a third reviewer (Toscano).

Table 1
Standard terms used in the search strategy.

Search strategy		
Component		Terms/Boolean operators
Cerebral palsy	OR	(Perinatal asphyxia) OR (anoxia) OR (hypoxic-ischemic) OR (ischemic) OR (hypoxic) OR (lipopolysaccharide maternal exposure) OR (brain paralysis) OR (encephalopathy infantile) OR (neonatal stroke) OR (white matter damage) OR (encephalopathy) OR (brain inflammatory)
AND Memory	OR	(Memory disorders) OR (memory impairment) OR (cognitive performance) OR (neurobehavior) OR (cognition) OR (executive function) OR (system executive)
AND Treatment	OR	(behaviour therapies) OR (cognition therapies) OR (neuromodulation) OR (cognitive technique) OR (non-invasive techniques) OR (invasive techniques) OR (electrical stimulation) OR (direct current stimulation) OR (transcranial magnetic stimulation) OR (neurofeedback) OR (attenuates) OR (drug) OR (pharmacological treatment) OR (nutritional intervention)
AND NOT review	OR	(meta-analysis) OR (case study) OR (case report) OR (Alzheimer) OR (Alzheimer's disease)

Note: the terms used in the search strategy may have varied according to the specific needs of each database.

Table 2
Inclusion and exclusion criteria in pre-clinical studies.

Inclusion criteria	Exclusion criteria
Participants:	Participants:
o Animals.	o Genetically modified animal species;
Exposure:	o Non-rodents;
o Animal model of brain injury with implications for the development of cerebral palsy and the treatment of memory disorders resulting from the injury.	Exposure:
Control:	o Brain injury after the gestational, perinatal, or neonatal period;
o Sham	o Intervention started before brain injury;
Outcomes:	o Unclear treatment protocol;
o memory performance;	o Studies that do not report the age of rodents during brain injury induction.
o Changes in the hippocampus.	o Control:
Study type:	o Studies without a control group
o Original data;	Outcomes:
o Full-text was available.	o Studies that do not mention the test used to assess memory;
	o In vitro evaluation of the hippocampus.
	Study type:
	o Non-original data (e.g., reviews, editorial).

2.4. Data extraction

Data extraction from the studies selected was performed by two independent researchers (Calado and Pereira) using a specific form that was developed to collect the results relevant to the review. The following information for clinical trials was extracted from each study: i) surname of the first and second author; ii) year of publication; iii) information regarding subjects in preclinical studies (species, sex and number of animals per group); iv) brain injury with implications for CP (the date the injury occurred); v) intervention used (dosage, treatment period and route of administration); vi) memory assessment as a primary outcome (type of memory assessed, method used and age during the task); vii) repercussions of interventions on the hippocampus and technique used to assess structural and biochemical parameters as a secondary outcome; viii) results for each comparison between control and exposed, extracting the mean value and standard deviation; ix) statistical method used and descriptive results, as a secondary result. All characteristics of the included studies are summarized in Table 3.

2.5. Assessment of methodological quality

Three researchers (Calado, Pereira and Souza) independently assessed the methodological quality of the studies included in this review using Syrcle's Risk of Bias (RoB) tool (Hooijmans et al., 2014a, 2014b). This tool is guided by the Cochrane Risk of Bias tool, adjusted for aspects of bias that play a specific role in animal studies (Hooijmans et al., 2014a, 2014b). This tool is composed of 10 items that assess selection bias, performance bias, detection bias, attrition bias, reporting bias and other sources of bias, evaluating the following items: i) sequence generation; ii) baseline and concealment characteristics; iii) allocation concealment; iv) housing for random allocation of animals; v) blinding caregivers and researchers involved in the research; vi) random outcome assessment; vii) blinding of outcome advisor; viii) incomplete outcome data; ix) selective reporting of results; x) other sources of bias (Hooijmans et al., 2014a, 2014b).

A "yes" judgment indicates a low risk of bias; a "no" judgment indicates a high risk of bias; the "uncertain" judgment indicates that insufficient detail was reported to provide an adequate assessment of the

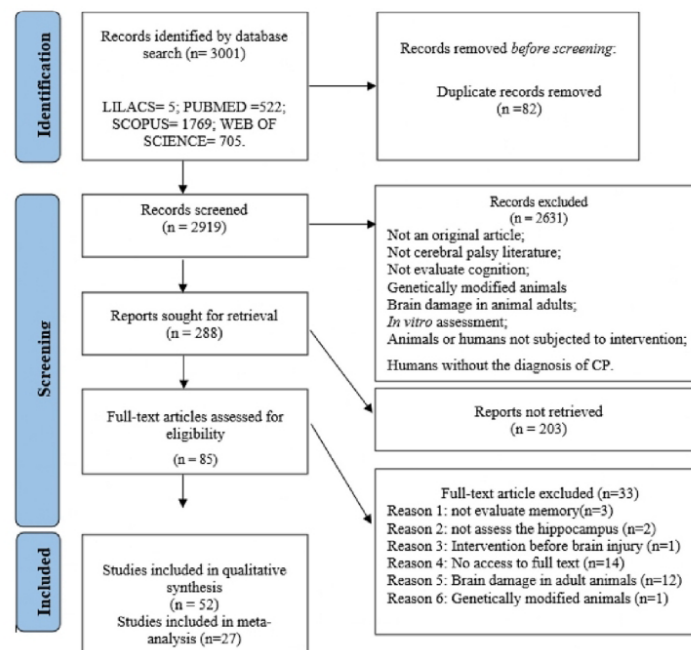


Fig. 1. PRISMA flow diagram of the study selection process.

risk of bias (Hooijmans et al., 2014a, 2014b). Conflicts and disagreements during the evaluation were resolved in a consensus among the three researchers (Calado, Pereira and Souza), in the absence of consensus, a fourth author was consulted (Toscano). RevMan v.5.3 was used to create the “summary risk of bias” and “risk of bias” graphs.

2.6. Statistical analysis

The software RevMan v.5.4 was used to calculate the meta-analytic comparisons for each behavioural parameter in the Morris water maze test, as assessed and reported in >3 studies. Cross-sectional comparisons between exposed and control animals assessed standardized mean differences (SMDs) calculated from mean and variance data computed with Cohen's d pooled effect sizes (ES) and 95% confidence intervals (95% CI). These were weighted for sample size, using a random-effects model (Hooijmans et al., 2014a, 2014b). Sensitivity analyses were conducted to test the effects of removing potentially incomparable studies. Incompatible studies were those with insufficiently matched exposed and control animals, factors that could have modified the relationship between Cerebral Palsy and behaviour (e.g. age analysis). These data are subsequently reported as “potentially incomparable”. The extent of heterogeneity between studies was inspected using I^2 , with I^2 values exceeding 60% indicating important heterogeneity (Hooijmans et al., 2014a, 2014b). Potential sources of statistical heterogeneity were explored; depending on sufficient data availability, variability, and the number of studies, meta-regression was planned. In comparisons that contained at least ten studies, the likelihood of publication bias was also assessed using the observation of funnel plots. Thus, a subgroup analysis was performed referring to different models of CP related to each outcome in the Morris water maze test: escape latency and time spent in the target quadrant.

3. Results

3.1. Selection of studies

Initially, the search in the databases with the appropriate terms found a total of 3001 articles subject to analysis; 82 duplicate studies were found, which were immediately removed. After that, 2919 studies were left to be analysed according to the screening eligibility criteria by reading the title and abstract of the article. As a result, 2631 studies presented one or more exclusion criteria, leaving 288 articles for full-text reading. After reading the full text, we detected 33 studies that had one or more exclusion criteria, such as not evaluating memory through tests, not evaluating the effects of the insult on the hippocampus, the intervention starting before the brain injury or because we did not have access to the full text. For this reason, 52 studies were included in the final qualitative analysis, and 27 were included in the meta-analysis. (Fig. 1).

3.2. Characteristics of studies included

A systematic synthesis of the characteristics of the 52 studies included is presented in Table 3. Predominantly, of these 52 studies, 28 used Sprague Dawley rats (Berger et al., 2019; Chen et al., 2021; Cho et al., 2018; Fan et al., 2008; Gonzalez et al., 2009; Gao et al., 2020; Gou et al., 2020; Holubiec et al., 2018; Huang et al., 2021; Jung and Kim, 2017; Kim et al., 2017; Li et al., 2019b; Liu et al., 2013; Lowe et al., 2017; Niu et al., 2021; Pan et al., 2012; Pak et al., 2018; Ren et al., 2017; Sampath et al., 2020; Sun et al., 2014; Wagner et al., 2002; Wang et al., 2002; Wei et al., 2015; Wei et al., 2017; Xiao et al., 2016; Xu et al., 2015; Zhang et al., 2014; Zhao et al., 2014), 16 used Wistar rats (Dell'Anna et al., 1997; Farfán et al., 2020; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Karalis et al., 2011; Kumral et al., 2004; Iuvone et al.,

Table 3
Characteristics of included rodent studies.

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
H-I	Berger et al., 2019	Sprague Dawley rats Both sexes CP I: P7	i) HI + Mel; ii) Sham + Mel; iii) HI + DMSO; iv) Sham + DMSO; v) HI + PBS; vi) Sham + PBS. n = 6–11 animals per group	Intraperitoneal injections of melatonin (10 mg/kg) or DMSO at 3 time points: immediately, 6 h and 25 h after the hypoxia	i) Infarct volume at 1 day after HI ($P < 0.05$) in the HI + Mel group compared with the HI + DMSO group.	The novel object recognition test was performed at 12 and 41 days after HI i) Exploration time of the novel object in the HI group compared to the sham group. No significant differences were found in the HI + Mel group compared to the HI group Morris water maze test began 8 days after brain damage
Ischemia	Chen et al., 2014	Male ICR mice CP I: 20 ± 2 g	i) Sham; ii) Model control; iii) AS 20 mg/kg; iv) AS 40 mg/kg; v) AS 60 mg/kg. n = not specified	Mice were treated orally with 20, 40, or 60 mg/kg of Asiaticoside (AS) 24 h after the operation, and then once a day for a week. The sham-operated control group and model control group were treated with distilled water.	i) iNOS activity in AS-treated HI animals compared to HI and placebo-treated animals with 40 and 60 mg/kg AS ($p < 0.05$, $p < 0.01$) i) microglial overactivation and p38 MAPK phosphorylation observed in the HI + AS treatment group (20 mg/kg or 40 mg/kg or 60 mg/kg) compared to the model control group ($p < 0.05$, $p < 0.05$ and $p < 0.01$ respectively).	i) Escape latency in the HI + AS 60 mg/kg treatment group and HI + AS 40 mg/kg compared to the model control group ($p < 0.01$ and $0 < 0.01$, respectively)
H-I	Chen et al., 2015	Rats Both sexes CP I: P3	i) HI + PBS; ii) HI + OPC; iii) Sham + OPC; iv) Sham + PBS n = 6–8 animals per group	Transplantation of oligodendrocyte progenitor cells (OPC) in animals 2 h after brain damage, injected into the left lateral ventricles.	i) iNOS activity in AS-treated HI animals compared to HI and placebo-treated animals with 40 and 60 mg/kg AS ($p < 0.05$, $p < 0.01$) i) microglial overactivation and p38 MAPK phosphorylation observed in the HI + AS treatment group (20 mg/kg or 40 mg/kg or 60 mg/kg) compared to the model control group ($p < 0.05$, $p < 0.05$ and $p < 0.01$ respectively).	i) Escape latency in the HI + AS 60 mg/kg treatment group and HI + AS 40 mg/kg compared to the model control group ($p < 0.01$ and $0 < 0.01$, respectively)
Oxygen deprivation	Chen et al., 2021	Rats Both sexes CP I: P3	i) control saline; ii) Hypo + saline; iii) Hypo + Free-Dex; iv) Hypo + Dex n = 12 animals per group	Dexamethasone (25 µg/kg, i.p.) 30 min pre (Hypo+Pre-Dex group) or post (Hypo+Post-Dex group) hypoxia injected intraperitoneally.	i) Synapse density ($p < 0.05$) and i) PSD95 and synaptophysin protein expression ($p < 0.05$) in the Hypo + Dex group compared to Hypo + saline group. i) NOX2 protein expression ($p < 0.05$) and i) NF-κB activation and proinflammatory cytokines, including IL-1β, IL-6, and TNF-α ($p < 0.05$) in the Hypo + Dex group compared to Hypo + saline group.	Morris water maze at P31-P36, i) escape latency ($p < 0.05$), i) platform crossings ($p < 0.05$) and i) time spent in the platform quadrant ($p < 0.05$) in the Hypo + Dex group compared to Hypo + saline group.
LPS	Cho et al., 2018	Sprague-Dawley rat Both sexes CP I: Antenatal (maternal LPS injection at E15, E17 and E20)	i) Control; ii) Control TM; iii) CP; iv) CP + TM. n = 8 animals per group	Treadmill running: 5 weeks after birth, for 30 min, 5 times a week during 6 weeks.	i) cell proliferation in DG ($p < 0.05$), i) p-P3K/PDK ratio ($p < 0.05$), i) p-Akt/Akt ratio ($p < 0.05$) and i) Wnt-3 expression ($p < 0.05$) in CP + TM group compared to CP group.	Step-down avoidance task P35-P77, i) Step-down latency time ($p < 0.05$)
Anoxia	Dell'Anna et al., 1997	Wistar rats Both sexes CP I: P2	i) Sham Saline; ii) Anoxia Saline; iii) Sham ALC; iv) Anoxia ALC.	The animals received intraperitoneal injections of ALC (50 mg/kg) or saline (50 mg/kg) of a 0.5 ml volume, daily from P2 (1h after brain injury) until P60.	Histological analysis of the brain and hippocampus did not show changes that could be related to ALC treatment	The Maze Test at P30-P35 i) time to reach the food compared to the Anoxia Saline group ($p < 0.001$) Morris water maze at P50-P60

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Table 3 (continued)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
LPS	Fan et al., 2008	Sprague-Dawley rats Both sexes CP I: P5	i) Saline; ii) Saline + PBN; iii) LPS; iv) LPS + PBN (100 mg/kg) or vehicle alone was administered 5 min after the LPS injection n = 12 animals per group.		i) Enlargement of ventricles comparing the LPS + PBN treatment group with the LPS + Saline i) Neuronal loss; improved the associated neurological dysfunctions ($p < 0.05$) in the LPS + PBN treatment group compared to the LPS + Saline.	i) Escape latency ($p < 0.01$) in anoxia ALC compared to the Anoxia saline group. Passive avoidance test P20-P21 i) The number of shocks applied ($p < 0.05$) in the LPS + PBN treatment group with the LPS + Saline.
Oxygen deprivation	Fufin et al., 2020	Wistar rats Both sexes CP I: P0	i) control; ii) A; iii) A + DFX + MSC; iv) A + TNF-α + IFN-γ + MSC-S n = 5–8 animals per group	MSC-S, preconditioned with either deferoxamine (a hypoxia-mimetic) or TNF-α + IFN-γ (pro-inflammatory cytokines) intranasally administered 16 µl, 2 h post-asphyxia or in two doses, 2 h and 7 days post-asphyxia	i) GSSG/GSH Ratio in the hippocampus at P7 ($p < 0.005$) and P60 ($p < 0.0005$) Hippocampus at P7: i) cytoplasmic NRF2 protein levels ($p < 0.05$) i) nuclear NRF2 protein levels ($p < 0.005$) i) NRF2 effector NQO1 protein levels ($p < 0.05$) i) nuclear p65 protein levels ($p < 0.0001$) i) microglial primary ($p < 0.005$) and secondary ($p < 0.05$) process length in hippocampal CA1 region (only A + TNF-α + IFN-γ MSC-S versus A) i) cleaved-caspase-3 protein level	Novel object recognition test at P30-P31 i) Memory index ($p < 0.05$)
H-I	Gao et al., 2020	Sprague-Dawley rat Both sexes CP I: P7	i) C + S; ii) Hypo/Reox; iii) Hypo/Reox D1; iv) Hypo/Reox D2; v) Hypo/Reox D3 n = 34 animals per group	Dexamethasone was injected intraperitoneally with 25 mg/kg (D1), 50 mg/kg (D2), and 75 mg/kg (D3)	i) mean density and relative expression level of neuroglobin at 2, 24, 48, and 72 h after Hypo/Reox ($p < 0.05$) i) Nissl staining positive cells at 2 h and 24 h after Hypo/Reox in the hippocampal CA1 region ($p < 0.05$) i) apoptosis of hippocampal neurons and i) relative expression level of cytosolic Cyt-c, Apaf-1, and caspase-3 at 2 and 24 h after Hypo/Reox.	Morris water maze at P28-P33: i) escape latency i) platform crossings In Hypo/Reox D2 group and Hypo/Reox D3 group compared to Hypo/Reox ($p < 0.05$).
Ischemia	Gonzalez et al., 2009	Sprague Dawley rats Both sexes CP I: P10	i) MCAO + single dose vehicle; ii) MCAO + single dose EPO; iii) MCAO + single dose EPO; iv) MCAO + 3 dose EPO; v) Sham + single dose vehicle; vi) Sham + single dose EPO; vii) Sham + 3 dose vehicle; viii) Sham + 3 dose EPO n = 6–12 animals per group	Intraperitoneal injections of one dose (after dose vehicle), three doses (after injury, 24 h and 7 days after injury) of erythropoietin (5 U/g/kg) or vehicle (5 U/g/kg) according to the experimental group	i) Brain tissue loss in MCAO + 3 dose EPO ($p < 0.05$) compared to MCAO + single dose EPO and MCAO + single dose or three-dose vehicle groups	Morris water maze at P65-P70 i) escape latency ($p < 0.05$) in MCAO + single dose EPO and MCAO + 3 dose EPO groups compared to sham groups
H-I	Gou et al., 2020	Sprague-Dawley rats Both sexes CP I: P7	i) Sham; ii) HI; iii) HI + DMSO treatment; iv) HI + Mel (10 mg/kg) treatment; v) HI + Mel treatment + RSL3 treatment (5 mg/kg); vi) HI + Mel treatment + n = 587 animals in total	Rats with HI were treated with intraperitoneal injections of melatonin (10 mg/kg) after brain injury	i) NeuN + cell number ($p < 0.01$ and $p < 0.05$) in HI + Mel (10 mg/kg) and HI + Mel (5 mg/kg) group compared to the HI group.	i) Escape latency ($p < 0.05$) and i) platform crossing ($p < 0.05$) in HI + Mel (10 mg/kg) and HI + Mel (5 mg/kg) group compared to the HI group.
H-I	Greggio et al., 2011	Wistar rats Males	i) Sham operated; ii) HI + Vehicle; iii) HI + NAP treatment n = 587 animals in total	Intraperitoneal injection of the NAP solution was administered at 3 µg/g body weight (0.1	i) inhibits neuron ferroptosis in the hippocampus ($p < 0.05$) i) Reduced frequency and rate of DNA damage in the HI + NAP group compared to the HI Vehicle group ($p < 0.001$)	Morris water maze at P60-P64 i) Escape of latency ($p < 0.001$) and i) Time

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Table 3 (continued)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
		CP E: P7	n = 5–9 animals per group	mL) immediately (0 h) and 24 h after the HI insult.	↓ Oxidative stress markers such as lipid peroxidation (TBA) and glutathione (GSH) ($P < 0.001$) in the HI + NAP group compared to the HI Vehicle Group	spent in the target quadrant ($p < 0.05$) in the HI + NAP group compared to the HI Vehicle group.
H-I	Ortiz et al., 2017	Wistar rats Both sexes CP E: P7	i) Control + standard environment; ii) Control + EE; iii) Control + GCSF + EE; iv) HI + standard environment; v) HI + EE; vi) HI + GCSF + EE n = 7–9 animals per group	Rats submitted to the HI model were treated from P7 to P11 with GCSF (50 µg/kg daily) or EE at P21 or were treated with GCSF + EE	↓ Expression of BDNF in HI + EE ($p < 0.01$) and HI + GCSF + EE ($p < 0.05$) groups compared to control + EE group in the right hippocampus.	Morris water maze at P50–P55 ↑ Escape latency ($p < 0.05$), ↓ time to locate the platform ($P < 0.01$), and ↑ spent more time ($p < 0.01$) in the reference quadrant and HI + EE group compared to the HI + standard environment.
H-I	Halls et al., 2019	Wistar rats Male CP E: P7	i) Control; ii) Sham; iii) HI; iv) HI + PTX60; v) HI + PTX100. n = 11–20 animals per group.	Pentoxifylline immediately after and again 2 h after HI with 60 mg/kg/dose (HI + PTX60) or 100 mg/kg/dose (HI + PTX100)	↓ Relative weight of the right hippocampus in the HI group compared to the HI + PTX60 group ($p < 0.05$).	Morris water maze at P77 –P85 No difference was observed between control or sham groups and HI + PTX60 on escape latency, path length and time spent in target quadrant.
H-I	Holubiec et al., 2018	Male Sprague Dawley CP E: P7	i) Sham-Vehicle; ii) Sham-O2; iii) Sham-O10; iv) Sham-P2; v) Sham-P10; vi) AI-Vehicle; vii) AI-O2; viii) AI-O10; ix) AI-P2; x) AI-P10 (n = 9) n = 7–9 animals per group	An hour after the brain damage was finished pups were injected intraperitoneally with a DMSO solution or OEA (2 mg/kg or 10 mg/kg) or PEA (2 mg/kg or 10 mg/kg).	↓ The group administered with 10 mg/kg of PEA, showed a significantly higher number of Iba-1 positive cells in the CA1 hippocampal area compared with sham groups ($p < 0.05$) ↓ PEA treatment was able to prevent neuroinflammation and ↓ astrogliosis and preserve cognitive functions in the AI O2, AI O10, AI P2 and AI P10 groups compared to the AI-vehicle group ($p < 0.05$)	Object Recognition, Morris Water Maze and Passive Avoidance tests when rats were 30 days old ↓ HI was associated with a spatial reference memory deficit that was prevented by the treatment with PAE (10 mg/kg)
H-I	Huang et al., 2021	Sprague-Dawley rat Male CP E: P7	i) Sham; ii) HI; iii) HI + Dex; iv) HI + Dex + mimic NC. n = 15 animals per group	Immediately intraperitoneally injected with 0.1 mg/kg Dex after HI induction (HI + Dex).	↓ hippocampal tissue and ↓ pro-inflammatory cytokines in the HI group compared to the Sham group ($p < 0.05$).	Water maze test ↓ escape latency and ↓ platform crossing
Oxygen deprivation	Iuvone et al., 1996	Wistar rats Both sexes CP E: P2	i) Sham; ii) Sham + anoxia; iii) Sham + treatment; iv) Anoxia + EE treatment n = 6 animals per group	Pups were weaned at P21 and, housed by the experimental group, in simple cages or with an enriched environment until P60.	↓ IL-6, IL-8 + TNF-α in HI + Dex and HI + Dex + mimic NC groups compared to HI ($P < 0.05$) ↓ expression of cytosolic proteins such as the calcium binding protein parvalbumin in the hippocampus ($p > 0.05$) in the group Anoxia+EE ($p < 0.03$) compared to Sham + anoxia group.	Water maze test at P58–P60 ↓ platform crossing velocity was significantly increased in the enriched treatment group ($p < 0.03$) compared to the Sham + anoxia group.
LPS	Jung and Kim, 2017	Sprague-Dawley rat Male CP E: Autometal (maternal LPS injection at E15, E17 and E20)	i) Control; ii) TM; iii) CP; iv) CP + TM; v) CP Treadmill running: 5 weeks after birth, for 30 min, 5 times a week during 4 weeks. n = 8 animals per group		↑ cell proliferation in DG, ↑ Synapsin I, ↑ PSD-95, ↑ p-P3K, ↑ p-Akt time and ↓ GSK-3β in CP + TM and CP + TMx group compared to the CP group ($p < 0.05$)	Step-down avoidance task (Short-term memory) ↑ Step-down latency time (CP + TM and CP + TM + SR versus CP, $p < 0.05$)

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Table 3 (continued)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
H-I	Karalis et al., 2011	Wistar rats Both sexes CP E: P7	i) Sham-operated; ii) HI + Resveratrol-treated; iii) HI-treated-vehicle n = 15–10 animals per group	Rats submitted to the HI model treated immediately after hypoxia with RES (90 mg/kg).	↓ Extension of injury observed in the ipsilateral cerebral hemisphere of resveratrol-treated rats compared to HI-treated rats ($p < 0.05$)	Morris water maze from P51 to P55 ↑ Escape latency ($p = 0.016$; $p = 0$; $p = 0.012$), and ↑ swimming time to locate the platform in CP animals ($p < 0.01$) in compared to control animals.
Ischemia	Kim et al., 2006	Male ICR mice CP E: 25–30 g	i) Sham; ii) Ischemia + Oroxynlin treatment; iii) Ischemia control n = 8 animals per group	Mice were treated orally with 1.25, 2.5, or 5 mg/kg of oroxynlin A 60 min after reperfusion, and then once a day for a week. The last treatment was completed 1 h before each test	↓ Cresyl violet from CA1 and DG indicated loss of cells in the CP group compared to the sham animals ($p < 0.05$), and reduction in the number of pyramidal cells of CA1 ($p < 0.01$) ↓ Number of cells in DG and CA1 in the Ischemia + Oroxynlin group compared to the Ischemia control group ($p < 0.05$)	Y-maze task ↓ spontaneous alternation ($p < 0.05$) in the ischemia group compared to the sham group. Morris Water Maze ↑ Escape latency and the time searching the platform ($p < 0.05$) in the ischemia group compared to the sham group. [Escape latency and the time searching the platform ($p < 0.05$) in the Ischemia + Oroxynlin group compared to the Ischemia control group ($p < 0.05$)
H-I	Kim et al., 2017	Sprague Dawley rats Both sexes CP E: P7	i) Sham; ii) HI + Saline; iii) HI + LCM pre-treatment; iv) HI + LCM treatment n = 13–25 animals per group	Animals received treatment with Icosamide (50, 100, 200, and 300 mg/kg) in two doses administered with an orogastric tube immediately, before and after brain injury or immediately after brain injury and two hours after brain injury.	↓ Infarcted areas found in several brain regions including the hippocampus ($p < 0.005$) in the HI + LCM treatment compared to the HI + saline group.	Morris water maze at P56–P61 ↑ Escape latency ($p < 0.001$) in the HI + LCM treatment compared to the HI + saline group.
H-I	Kumral et al., 2004	Wistar rats Both sexes CP E: P10	i) HI + EPO; ii) HI + Saline; iii) Sham operated; iv) Normal control n = 7 animals per group	After HI or after the sham operation, each animal received a single intraperitoneal injection of EPO (1000 unit/kg) or saline (1000 unit/kg) according to the experimental group.	↑ treatment with EPO significantly protected CA1 neurons against neonatal HI insult as compared to the HI + Saline group ($p < 0.05$)	Morris water maze at P28–P32 and P147–P152 ↓ escape latency ($p < 0.05$) and ↑ time spent in the target quadrant ($p < 0.05$) in the HI + EPO group compared to the HI + saline group.
H-I	Li et al., 2019b	Sprague Dawley rats Males CP E: P7	i) Sham; ii) CP; iii) CP + vitB1 and vitB2-HA n = 6 animals per group	The animals were treated with Vitamin B1 (300 mg) and B12 (1 mg) injected for 0.1 ml in one acupoint one time per day and lasted for 5d in each course.	↑ increase expression of MALAT1, miR-1, and BDNF in the hippocampus and ↓ The PI3K and Akt phosphorylation was enhanced in the CP + VitB1 + VitB2-HA compared to the CP group.	Step-down avoidance task ↓ Latency to fall in seconds ($p < 0.05$) in the CP + VitB1 + VitB2-HA compared to the CP group.
H-I	Li et al., 2019a; Li et al., 2019	CS7BL/6 mice Both sexes CP E: P7	i) Control; ii) HI model; iii) HI + RES 10 mg/kg; iv) HI + RES 40 mg/kg n = 4–10 animals per group	Treatment mice received high (40 mg/kg) or low (10 mg/kg) Resveratrol. HI model and control group received an equal volume vehicle.	↑ Proliferation of neural stem cells and increase neuronal differentiation in the hippocampal DG region ($p < 0.01$) in the HI-RES 10 mg/kg and HI + RES 40 mg/kg group compared to the HI model group.	↑ Step-down latency time ($p < 0.05$ in the CP + VitB1 + VitB2-HA compared to the CP group). Morris water maze at P28–P33, approximately. ↓ Receiving groups both RES dosages showed remarkable shorter escape latency

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Table 3 (continued)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
H-I	Liu et al., 2013	Sprague-Dawley rats Both sexes CP I: P7	i) HI with HBO treatment; ii) HI; iii) Sham. n = 36 animals per group	At 60 min after the end of hypoxia, pups in the HBO-treated group were placed in the HBO chamber (100% oxygen, while under 2.5 absolute atmosphere) for 90 min and returned to their cages after treatment.	i) Extent of brain damage was observed ($p < 0.001$) in the HI + HBO group compared to the HI group. i) Infarcted areas in several brain regions of 24.50% and 29.83% in the cerebral cortex and the hippocampus ($p < 0.01$), in the HI + HBO, the group compared to the HI group.	compared with the HI model group ($p < 0.001$). Morris water maze test from P56 to P60 ↑ escape latency and crossing platform in the HI group.
H-I	Lowe et al., 2017	Sprague-Dawley rats Both sexes CP I: P7	i) Sham; ii) HI + Hypothermia with saline; iii) HI + Hypothermia + NAC; iv) HI + Hypothermia + NAC + ViD n = 10–12 per sex per group	Hypothermia started together with the application of NAC (50 mg/kg/day) OR ViD (0.1 µg/kg/day) or NAC (50 mg/kg/day) and ViD (0.1 µg/kg/day) 1 h after injury brain and at P8-P14 by intraperitoneal injections and continued by gavage at P15-P21.	i) CYP24A1 increased in both hippocampi after HNAC + ViD treatment in females ($p = 0.03$) compared to HI + hypothermia in the saline group. i) In males, CYP24A1 protein levels decreased after treatment with HNAC + ViD in the ipsilateral hippocampus ($p < 0.029$) compared to HI + hypothermia in the saline group.	↑ escape latency and crossing platform in the HI + HBO group ($P < 0.01$) compared to the HI group. Morris water maze at P35-P48 i) time to locate the platform, i) swimming speed ($p < 0.001$) and i) distance traveled in the hypothermia + NAC + ViD group compared to Hypothermia with saline group ($p < 0.05$)
H-I	Miguel et al., 2020	Wistar rats Male CP I: P7	i) CS; ii) C + MPH; iii) HI + S; iv) HI + MPH n = 11–13 animals per group	Methylphenidate (MPH) was injected intraperitoneally (dose of 2.5 mg/kg, volume of 1 mL/kg) 30 min prior to each behavioural session (from P30 to P45).	i) BDNF levels in the hippocampus at P45 in the HI + MPH group compared to CS group ($p < 0.05$).	Novel-object recognition and Morris water maze between P30 and P45: No difference between groups was observed in the assessed parameters
Oxygen deprivation	Morales et al., 2009	Wistar rats Both sexes CP I: P0	i) Control Vehicle; ii) Control Nicotinamide; iii) Asphyxia vehicle; iv) Asphyxia Nicotinamide n = 4–9 animals per group.	Nicotinamide (0.8 mmol/kg, i.p. (100 mg/kg)) or vehicle administered to pups exposed to asphyxia or Caesarean section, with the following administration schedule: intraperitoneal injections at 24 h, 48 h, and 72 h after brain injury.	i) the number of apoptotic nuclei. ($p > 0.05$) in the Asphyxia nicotinamide group compared to the Asphyxia vehicle group.	↑ exploration of the new object compared to the Asphyxia Vehicle group ($p < 0.05$) Y Maze test no effect was observed on spontaneous behavioural alternation in all groups.
H-I	Morin et al., 2017	C57BL6/JNrl Mice Male CP I: P9	i) Sham; ii) HI-S; iii) HI-C3a. n = 18–19 animals per group.	C3a 8 µl, i.e. 1.6 pmol (4 µl /nostril; corresponding to ca. 2.56 µg/kg body weight) was given intranasally 1 h after HI induction every 24 h for three days (P9, P10, and P11)	i) astrocyte activation in CA1 + DG of the dorsal hippocampus at P55 in HI-C3a compared to the HI-S group ($p < 0.05$)	Object recognition test at P53-P54: ↑ time exploring the novel object ($p < 0.01$) ↑ total object exploration time ($p < 0.05$)
H-I	Mori et al., 2015	Wistar rats Male CP I: P7	i) Sham; ii) HI; iii) HI + S; iv) HI + SDF-1α 60; v) HI + SDF-1α 600 n = 12–15 animals per group.	Intracranial injections stromal cell-derived factor-1α (SDF-1α) in 0.1% 60 or 600 µg/kg	No difference between Sham and HI-C3a. Total area of the infarct in the hippocampus was high in the HI group (64.1% ± 2.9%) and was not affected by 60 or 600 µg/kg SDF-1α (66.2% ± 3.0%).	Morris water maze at P18-P22: ↑ escape latency ↑ platform crossings (HI + SDF-1α 600 versus HI + S $p < 0.05$), approximately
H-I	Mishima et al., 2004	Wistar rats Both sexes CP I: P7	i) sham-control; ii) normothermic; iii) hypothermic; iv) hyperthermic n = 3–5 animals per group	The HI group was further divided into three groups by the chamber temperature: hypothermic group (27 °C), hyperthermic group (37 °C), and normothermic control group (33 °C) during 2 h of hypoxia.	i) in each section of ipsilateral hemispheres, the normothermic group showed a decrease in each area in the 18th week following HI insult, and hyperthermia significantly increased the brain injury area in all sections compared with the normothermic group ($p < 0.01$)	↑ HI insult (normothermic group) severely impaired spatial learning, and hyperthermia reduced the spatial learning in the water maze test, increasing the platform crossing ($p < 0.01$). Morris Water Maze P12-P17,
H-I	Niu et al., 2021	Sprague-Dawley rat Male CP I: P7	i) Sham; ii) HI; iii) HI + Tuina n = 10 animals per group	Spinal Tuina: On the governor vessel, bladder meridian and acupoints Bailu, Yintang, Shenting, Shimencong, Quchi, Waiguan, Hegu, Yanglingquan, Zusanli, Sanyinjiao, and	i) expression of NLRP3 (no difference between Sham and HI-C3a) i) cleaved caspase-1, i) IL-1β, i) IL-18, and i) GSDMD ($p < 0.05$)	Morris Water Maze at P50 ↑ time finding the platform ($p < 0.05$) and ↑ time spent in the target quadrant ($p < 0.05$) (continued on next page)

Table 3 (continued)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
H-I	Odorcyk et al., 2016	Wistar rat Both sexes CP I: P7	i) Sham; ii) HI + S; iii) HI + V; iv) HI + HqAE n = 10–15 animals per group	Taichong. Once a day from P7 to P49 and lasted for approximately 20 min each time. Huperzia quadrifaria alkaloid extract (HqAE) was injected intraperitoneally with 10 mg/kg administered 1, 24, 48 and 72 h after HI	i) AChE Activity in the HI group i) The number of apoptotic cells in the HI + HqAE group.	in the HI + Tuina group compared to the HI group. Morris Water Maze (between P30 and P45) ↑ time spent in finding the platform ($p < 0.05$) Step down inhibitory avoidance (between P30 and P45) ↑ Step-down latency time (HI + HqAE Short-term test (Central bar) and long-term test (Right bar) compared to the training day, $p < 0.05$) Passive avoidance test (P45): ↑ entry latency in the HI + TM and HI + EA + TM groups compared to the HI group ($p < 0.05$)
H-I	Pak et al., 2018	Sprague-Dawley rat Male CP I: P7	i) C; ii) HI; iii) HI + EA; iv) HI + TM; v) HI + EA + TM n = 10 per group	Electroacupuncture (EA): Electrical stimulation (2 Hz, 1 mA) at two acupoints, Baihui (GV20) and Zusanli (ST36) at a frequency of 2 Hz and an intensity of 1 mA for 20 min. Treadmill exercise: running at 5 m/min for 10 min in the first week and 15 min in the second week. In the third week, they ran at 5 m/min for 5 min, then increased to 7 m/min for 10 min and returned to 5 m/min for 5 min. 5 days per week. EA and TM were performed from 3 to 5 weeks after HI induction.	Electroacupuncture (EA): Electrical stimulation (2 Hz, 1 mA) at two acupoints, Baihui (GV20) and Zusanli (ST36) at a frequency of 2 Hz and an intensity of 1 mA for 20 min. Treadmill exercise: running at 5 m/min for 10 min in the first week and 15 min in the second week. In the third week, they ran at 5 m/min for 5 min, then increased to 7 m/min for 10 min and returned to 5 m/min for 5 min. 5 days per week. EA and TM were performed from 3 to 5 weeks after HI induction.	↑ time finding the platform ($p < 0.05$) and ↑ time spent in the platform quadrant ($p < 0.05$) in HI + SP group compared to the HI + Vehicle group. Morris water maze at P35-P40, approximately. i) Search time ($p < 0.01$), i) platform search time, ($p < 0.001$), i) platform crossing, and time in the target quadrant ($p < 0.05$) in the RES treatment group compared to HI + vehicle group.
H-I	Pan et al., 2012	Sprague-Dawley rats Both sexes CP I: P7	i) Sham; ii) HI + Vehicle; iii) HI + SP n = 3–9 animals per group	The animals received intraperitoneal injections of SP or vehicle in a single dose at 5 min after brain injury in a dose of 500 mg/kg	↑ hippocampal tissue compared to the HI Vehicle group 48 h and 7 weeks after injury ($p < 0.001$)	Morris water maze at P56-P60, approximately ↑ latency time ($p < 0.05$) and ↑ time spent in the platform quadrant ($p < 0.05$) in HI + SP group compared to the HI + Vehicle group.
H-I	Peng et al., 2022	Mice Both sexes CP I: P7	i) Sham; ii) HI + PBS; iii) HI + RES n = 8 animals per group	Mice were treated with RES (100 mg/kg) immediately after hypoxia and 24 and 48 h after the insult.	↑ the morphology and staining of hippocampal neurons in the RES treatment group ($p < 0.05$) compared to the HI + PBS group. ↑ the number of neuronal dendrites, the density of dendritic spines, and the percentage of mushroom-shaped spines ($p < 0.05$) in the RES treatment group.	Morris water maze at P35-P40, approximately. i) Search time ($p < 0.01$), i) platform search time, ($p < 0.001$), i) platform crossing, and time in the target quadrant ($p < 0.05$) in the RES treatment group compared to HI + vehicle group.
H-I	Potter et al., 2018	Wistar rats Male CP I: P6	i) Sham saline normothermic; ii) Sham saline hypothermic; iii) Sham caffeine normothermic; iv) Sham caffeine hypothermic; v) HI saline normothermic; vi) HI caffeine normothermic; vii) HI saline hypothermic; viii) HI caffeine hypothermic n = 6–10 animals per group.	After brain injury, the animals received caffeine citrate (20 mg/kg) or saline through intraperitoneal injections. After that, the animals were placed in normothermic or hypothermic baths for 180 min according to experimental group.	For Caffeine Treatment, right cortices and hippocampal volumes for HI Caf did not differ from either Sham or HI groups, indicating no differences between scores ($p > 0.05$).	Morris water maze at P90 i) performance in hypothermic as compared to normothermic shams, regardless of caffeine status ($p < 0.05$). No group differences (Hypothermia/Caffeine) effects were seen for the water escape task ($p = 0.05$) Data from the combined (Caffeine + Hypothermia aka "CafCool") groups could not be interpreted, and those findings are not included. (continued on next page)

Table 3 (continued)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPOCAMPAL OUTCOMES	MEMORY OUTCOMES
Oxygen deprivation	Preethi et al., 2006	C57/BL6J mice Both sexes CP I: P7 or P8	i) sham; ii) CP + Re-O ₂ ; iii) CP+ Re-Air n = 112 animals in total	Immediately after hypoxic exposure, pups were exposed either to room air (RA) or 100% O ₂ for 30 min at 25 °C and then returned to their dams.	↓ loss of the hippocampal tissue volume ($p < 0.01$) in CP+ Re-O ₂ compared to CP + Re-Air. ↓ Hippocampal atrophy in Re-Air in granular zones CA1 – CA4, compared to Re-O ₂ mice, significantly better preserved in Re-O ₂ mice compared to Re-Air counterpart.	Morris water maze at P33-P36, approximately. ↓ Navigational learning ($p < 0.01$), spatial memory ($p < 0.0001$), and spatial orientation skills ($p < 0.01$) were significantly better preserved in Re-O ₂ mice compared to Re-Air counterpart.
H-I	Ren et al., 2017	Sprague Dawley rats Both sexes CP I: P2	i) Sham; ii) HI; iii) HI-EPO (immediately); iv) HI + EPO (48h after brain injury) n = 12–19 animals per group	Immediately or 48 h after brain injury, the animals received a single dose of EPO (5000 U/kg) or saline (0.1 ml/kg) according to the experimental group.	↓ significant reduction of MBP expression in the HI-injured brain at P9 ($p = 0.006$) and P16 ($p = 0.01$) compared with the sham group. No significant difference was found in the two EPO groups compared with the sham group at both P9 and P16 ($p > 0.05$). ↓ The hippocampus in the HI group when compared to the others ($p < 0.05$) ↓ Cleaved caspase 3 expression in the hippocampus in the HI and HI + Allopurinol group. When sex was considered, there were no differences in the hippocampal volume between HIA, HH, and HHIA among the females ($p = 0.398$; $p = 1$; $p = 1$)	Morris water maze at P33-P37, approximately ↓ platform search time in HI + EPO treatment immediately or 48 h after brain injury ($p = 0.00$). Morris Water maze at P25-P35, approximately. ↓ All groups acquired the task and improved their performance over time ($p < 0.0001$), but with significant differences in the learning process, with the HI group being the worst. ↓ In females, global learning was also different between groups ($p = 0.001$). Control, HH ($p = 0.999$), HHIA ($p = 0.991$), and HIA ($p = 0.719$) groups showed normal learning performance. Novel object recognition (P45): ↓ Discrimination index in the HI-3 L group compared to the HI group ($p < 0.01$)
H-I	Rodrigues-Fajul et al., 2017	Wistar rats Both sexes CP I: P10	i) Sham intervention (Control); ii) HI; iii) HI + Dual therapy (hypothermia and allopurinol; HHIA) n = 7–8 animals per group.	All treatment groups received a single intraperitoneal injection of allopurinol at 135 mg/kg (volume 0.01 ml/g) or saline, 15 min after hypoxia, depending on the randomization, before beginning hypothermia or normothermia protocol.	Magnetic resonance imaging (P7) ↓ ipsilateral ADC in the hippocampus (HI-L versus HI on P7, $p < 0.01$; HI-3 L versus HI on P7, P8 and P9, $p < 0.05$; HI-3 L versus HI-L on P8 and P9, $p < 0.05$) ↓ tissue hippocampal in the HI group ($p < 0.05$) compared to the sham vehicle group. ↓ tissue hippocampal and cortical in the HI-flupirtine group ($p < 0.05$) compared to the HI group ($p < 0.01$). ↓ BDNF expression in the hippocampus of the rats in the HI + Polydatin group ($p < 0.05$) compared to the HI group in P10 and P21. At 24 h after the HI induction, there was no significant difference in BDNF expression in the left hippocampal CA1 ($P > 0.05$)	Morris water maze at P116-P138 ↓ The performance of sham rats in the HI + Flupirtine group on all trial days was very similar to sham rats treated with vehicle ($p < 0.05$) Y-maze learning test at P17 ↓ reaction time in HI + Polydatin and Sham group compared to the HI group ($P < 0.01$) ↓ correct avoidance rate in the rats in the Polydatin group, compared to the HI group ($P < 0.01$).
H-I	Roumes et al., 2020	Wistar rats Both sexes CP I: P7	i) Sham; ii) HI; iii) HI-L; iv) HI-3 L n = 10–17 animals per group.	Intraperitoneal injection(s) of lactate (40 μmol) after hypoxia (HI-L group) or three consecutive (150 min, 24 h and 48 h post-HI event, HI-3 L group)	↓ ipsilateral ADC in the hippocampus (HI-L versus HI on P7, $p < 0.01$; HI-3 L versus HI on P7, P8 and P9, $p < 0.05$; HI-3 L versus HI-L on P8 and P9, $p < 0.05$) ↓ tissue hippocampal in the HI group ($p < 0.05$) compared to the sham vehicle group. ↓ tissue hippocampal and cortical in the HI-flupirtine group ($p < 0.05$) compared to the HI group ($p < 0.01$). ↓ BDNF expression in the hippocampus of the rats in the HI + Polydatin group ($p < 0.05$) compared to the HI group in P10 and P21. At 24 h after the HI induction, there was no significant difference in BDNF expression in the left hippocampal CA1 ($P > 0.05$)	Morris water maze at P116-P138 ↓ The performance of sham rats in the HI + Flupirtine group on all trial days was very similar to sham rats treated with vehicle ($p < 0.05$) Y-maze learning test at P17 ↓ reaction time in HI + Polydatin and Sham group compared to the HI group ($P < 0.01$) ↓ correct avoidance rate in the rats in the Polydatin group, compared to the HI group ($P < 0.01$).
H-I	Sampath et al., 2020	Sprague-Dawley rats Male CP I: P7	i) Sham + Vehicle; ii) Sham + Flupirtine; iii) HI + Vehicle; iv) HI + Flupirtine. n = 5–15 animals per group.	Pups were administered a single dose of 25 mg/kg flupirtine maleate or an equivalent volume of vehicle every 24 h for 4 days after HI induction (total 4 doses).	↓ tissue hippocampal in the HI group ($p < 0.05$) compared to the sham vehicle group. ↓ tissue hippocampal and cortical in the HI-flupirtine group ($p < 0.05$) compared to the HI group ($p < 0.01$). ↓ BDNF expression in the hippocampus of the rats in the HI + Polydatin group ($p < 0.05$) compared to the HI group in P10 and P21. At 24 h after the HI induction, there was no significant difference in BDNF expression in the left hippocampal CA1 ($P > 0.05$)	Morris water maze at P116-P138 ↓ The performance of sham rats in the HI + Flupirtine group on all trial days was very similar to sham rats treated with vehicle ($p < 0.05$) Y-maze learning test at P17 ↓ reaction time in HI + Polydatin and Sham group compared to the HI group ($P < 0.01$) ↓ correct avoidance rate in the rats in the Polydatin group, compared to the HI group ($P < 0.01$).
H-I	Sun et al., 2014	Sprague-Dawley rats Both sexes CP I: P7	i) Sham-operated (SO); ii) HI; iii) HI + Polydatin n = 40–58 animals per group	Polydatin dry powder was dissolved in physiological saline and injected intraperitoneally into the rats from the Polydatin group (10 mg/kg body weight) once a day for 10 consecutive days.	↓ BDNF expression in the hippocampus of the rats in the HI + Polydatin group ($p < 0.05$) compared to the HI group in P10 and P21. At 24 h after the HI induction, there was no significant difference in BDNF expression in the left hippocampal CA1 ($P > 0.05$)	Morris water maze at P116-P138 ↓ The performance of sham rats in the HI + Flupirtine group on all trial days was very similar to sham rats treated with vehicle ($p < 0.05$) Y-maze learning test at P17 ↓ reaction time in HI + Polydatin and Sham group compared to the HI group ($P < 0.01$) ↓ correct avoidance rate in the rats in the Polydatin group, compared to the HI group ($P < 0.01$).

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Table 3 (continued)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPOCAMPAL OUTCOMES	MEMORY OUTCOMES
H-I	Wagner et al., 2002	Sprague-Dawley rats Both sexes CP I: P7	i) HI + hypothermia; ii) HI + normothermia; iii) Control + hypothermia; iv) Control + normothermia. n = 14–17 animals per group.	Two hours after the HI insult, one group of animals was maintained at 37 °C (normothermic temperature) measured rectally for another 26 h, whereas the second group of animals was cooled to 30 °C (hypothermic temperature) measured rectally and kept at this temperature for 26 h.	↓ size of cerebral infarction by 23% at 6 weeks after the insult in the HI + Hypothermia compared to the HI + normothermia group ($p < 0.05$).	Morris water maze at P42-P47, approximately. ↓ escape latency in HI + Hypothermia compared to the HI + normothermia group ($p < 0.05$).
H-I	Wang et al., 2002	Sprague-Dawley rats Both sexes CP I: P7	i) HI; ii) HI + Saline-treated; iii) HI + HupA 0.05; iv) HI + HupA 0.1 mg/kg. n = 10–12 animals per group	Daily i.p. HupA (0.05 mg/kg or 0.1 mg/kg) or saline started on P7 immediately after hypoxia-ischemia and terminated before the day of sacrifice (P42).	↓ hippocampal atrophy in HI + HupA 0.1 mg/kg group compared to the HI group ($p < 0.01$).	Morris water maze at P36-P41 ↓ escape latency in HI + HupA 0.1 mg/kg group compared to the HI group ($p < 0.01$). Morris Water Maze at P36-P41
H-I	Wang et al., 2021	Rats Both sexes CP I: P7	i) Sham; ii) HI; iii) HI + Sevoflurane n = 18 animals per group	Neonatal rats immediately inhaled 2% sevoflurane and received 30% O ₂ and 70% N ₂ humidified mixed gas for 30 min after induction of HI.	↓ The neuronal density in the HI group compared to the Sham group ($p < 0.001$) and inflammation markers in the HI + Sevoflurane group compared to the HI group ($p < 0.001$) ↓ The protein levels of G9a and H3K9me2 in the HI group compared to the Sham group ($p < 0.05$) and ↓ in HI + Sevoflurane group ($p < 0.001$)	↓ escape latency in HI + Sevoflurane group compared to the HI group ($p < 0.001$). ↓ escape latency in HI + Sevoflurane group compared to the HI group ($p < 0.001$).
H-I	Wei et al., 2015	Sprague-Dawley rats Both sexes CP I: P7	i) Sham; ii) HI; iii) HI + HBO n = 40 animals per group	Rats were put into the HBO chamber within 15–30 min after brain injury and spent 15 min in the chamber with pure oxygen. HBO therapy performed once daily for 7 days.	↓ The lesions in CA1, CA3 and DG ($p < 0.05$) in the HI + HBO group compared to the HI group. ↓ Nestin and BrdU positive cells in the HBO group compared to HI and Sham group ($p < 0.01$)	Morris water maze at P37-P43 ↓ platform crossing in the HI + HBO group compared to the HI group. ($p < 0.05$). No differences were found between the 1+ HBO group and the Sham group ($p > 0.05$). Morris water maze at P30-P35
H-I	Wei et al., 2017	Sprague-Dawley rats Both sexes CP I: P7	i) Sham; ii) HI; iii) HI + HBO; iv) HI + NGF n = 8 animals per group	Animals were exposed to a hyperbaric oxygen chamber after brain injury and/or received intraperitoneal injections of 0.5 μg NGF for each rat after 15–30 min of brain damage for 3 days.	↓ Brain and hippocampus tissue in all experimental groups compared to the HI group	↓ escape latency in all groups compared to sham group ($p < 0.05$)
Oxygen deprivation	Xiao et al., 2016	Sprague-Dawley rats Male CP I: P0	i) S; ii) Hy; iii) Hy + PPADS; iv) Hy + TNP-ATP n = 17 per group	A single dose of PPADS dissolved in saline (5, 10, 20, or 40 mg/kg) or TNP-ATP dissolved in saline (1, 2, 4, or 8 mg/kg) was injected intraperitoneally (i.p.) 2 h after the hypoxia exposure.	↓ IL-1b expression (Hy + TNP-ATP versus Hy 1 day after HY, $p < 0.05$) ↓ glutamate level (Hy + TNP-ATP versus Hy 4 h and 1 day after HY, $p < 0.05$) ↓ EAAT2 (Hy + PPADS versus Hy 1 day after HY, $p < 0.05$)	Y-Maze at P30: ↓ number of days needed to reach the learning criterion, with a peak effect at 10 mg/kg for PPADS and 2 mg/kg for TNP-ATP ($p < 0.01$) Morris water maze at P45: ↓ platform crossings ($p < 0.05$) and ↓ time spent in the target quadrant Hy + TNP-ATP 2 mg/kg versus Hy, $p < 0.05$) Morris Water Maze P90, approximately ↓ time spent in finding the platform and ↓ platform crossing times ($p < 0.05$)
Oxygen deprivation	Xu et al., 2015	Sprague-Dawley rats Male CP I: P0	i) C; ii) P; iii) PA + 0.33 MCP; iv) PA + 1.0 MCP; v) PA + 3.0 MCP. n = 15 animals per group	Marine collagen peptides (MCPs) intragastrically administered with 0.33 g/kg, 1.0 g/kg and 3.0 g/kg body weight MCPs from postnatal day 0 (P0) till the age of 90-days.	↓ neuron count in the hippocampal CA1 region at 3 months of age (PA + 1.0 MCP and PA + 3.0 MCP versus PA, $p < 0.01$) ↓ hippocampal AChE activity (PA + 1.0 MCP and PA + 3.0 MCP versus PA, $p < 0.01$)	Morris Water Maze P90, approximately ↓ platform crossing times ($p < 0.05$)

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Table 3 (continued)

CP MODEL	AUTHOR (YEAR)	STRAIN	EXPERIMENTAL GROUPS (n)	INTERVENTION	HIPPOCAMPAL OUTCOMES	MEMORY OUTCOMES
H-I	Zhang et al., 2014	Sprague Dawley rats Both sexes CP I: P7	i) HI; ii) Sham; iii) HI + Carnosine; iv) Normal n = 10 animals per group	The carnosine treatment group received an intraperitoneal injection of 250 mg/kg of carnosine at 0 h, 24 h and 48 h after the end of the hypoxic episode.	↑ p-CREB/CREB ratio in hippocampus (PA + 1.0 MCP and PA + 3.0 MCP versus PA, $p < 0.01$; PA + 0.33 MCP versus PA, $p < 0.05$) ↑ BDNF expression (PA + 1.0 MCP and PA + 3.0 MCP versus PA, $p < 0.05$) ↓ The level of oxidative stress markers ($p < 0.05$) in the HI + Carnosine compared to the HI group. ↓ Several TUNEL-positive cells in the CA1 region of the hippocampus and cortex ($p < 0.05$) in the HI + Carnosine compared to the HI group.	Morris water maze at P28-P33 ↓ swimming time to find the platform ($p < 0.05$) in the HI + Carnosine compared to the HI group.
H-I	Zhao et al., 2014	Sprague-Dawley rats Both rats CP I: P7	i) Control; ii) Sham + NS + 1.5% isoflurane posttreatment; iii) HI + NS; iv) HI + NS + ISO v) HI + Atr; vi) HI + Atr + ISO vii) HI + CaA; viii) HI + CaA + ISO n = 45 animals per group	NS, Atr or CaA were injected into the lateral cerebral ventricle immediately after HI.	↓ Weight and neuronal density in the CA3 hippocampus ($P < 0.05$) in the HI group compared to the sham group. ↓ brain loss was reduced by isoflurane postconditioning. CaA injection in HI + NS + ISO and HI + CaA + ISO compared to the HI group ($p < 0.05$)	Morris water maze at P36-P41, approximately ↓ escape latency in all groups received ISO treatment ($p < 0.05$) compared to the HI group.

Abbreviations: Acetylcholinesterase (AChE); Acetyl-L-carnitine (ALC); Alpha-phenyl-n-tert-butyl-nitron (PBN); Apparent Diffusion Coefficient (ADC); Apoptotic Protease activating factor-1 (Apaf-1); Asiaticoside (AS); ATP analogue 20,30-0-(2,4,6-trinitrophenyl)-adenosine 50-triphosphate (TNP-ATP); Anoxia-ischemia (AI); Brain-derived neurotrophic factor (BDNF); cAMP Response Element Binding Protein (CREB); Cerebral Palsy (CP); Cerebral Palsy Induction (CP I); Choice Reaction Time (CRT); Cytosolic cytochrome c (Cyt-c); Dentate gyrus (DG); Dexmedetomidine (DEX); Dimethyl Sulfoxide (DMSO); Embryonic day (E); Enriched Environment (EE); Erythropoietin (EPO); Excitatory amino acid transporter 2 (EAAT2); Gasdermin-D (GSDMD); Glycogen synthase kinase-3 β (GSK-3 β); Granulocyte colony-stimulating factor (G-CSF); Hydro-acupuncture (HA); Hyperbaric oxygen (HBO); Hypoxia/Reoxygenation (Hypo/Reox); Human adipose mesenchymal stem cells (MSC-S); Huperzine A (HupA); Inducible Nitric Oxide Synthase (iNOS); Interleukin-1 beta (IL-1 β); Interleukin-6 (IL-6); Interleukin-18 (IL-18); isoflurane (ISO); Lipopolysaccharide (LPS); Maternal lipopolysaccharide (LPS); melatonin (Mel); Minute (min); Mtp inhibitor Cyclosporin A (CaA); Myelin basic protein (MBP); N-acetylcystein (NAC); NADPH oxidase 2 (NOX2); Neural Stem Cells (NSC); Normal saline (NS); Nuclear Erythroid 2-Related Factor 2 (NRF2); Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B); number of animals per group (n); Oleylethanolamide (OEA); Oligodendrocyte Progenitor Cell (OPC); Opener Atractyliside (Atr); Oxidized Glutathione (GSSG); Palmitoylethanolamide (PEA); Pentoxifylline (PT); Phosphorylated Akt (p-Akt); Phosphorylated CREB (p-CREB); phosphorylated Glycogen synthase kinase-3 β (p-GSK-3 β); pyridoxal phosphate-6-azophenyl-20,40-disulphonic acid (PPADS); phosphatidylinositol 3-kinase (PI3K); Phosphorylated PI3K (p-PI3K); Postnatal day (P); Postsynaptic density protein 95 (PSD95); Protein Kinase B (Akt); Resveratrol (RES); Saline (S); Sodium pyruvate (SP); Stromal cell-derived; Traditional Chinese medicine (TCM); Transplantation of oligodendrocyte progenitor cells (OPC); Treadmill exercise (TM); Tumor Necrosis Factor- α (TNF- α); V: Vehicle (V); Vitamin B (VitB); Vitamin D (VitD); 4-hydroxynonenal (4-HNE); 8-amino-acid NAP (NAP).

Symbols: ↑- Increased parameter; ↓- Reduced parameter.

1996; Miguel et al., 2020; Mishima et al., 2004; Morales et al., 2009; Mori et al., 2015; Odorczyk et al., 2016; Potter et al., 2018; Rodríguez-Fanjul et al., 2017; Roumes et al., 2020), 3 used C57BL/6 mice (Li et al., 2019a; Morán et al., 2017; Presti et al., 2006), 2 studies used ICR mice (Chen et al., 2014; Kim et al., 2006), 2 studies did not specify the strain of mice (Chen et al., 2015; Wang et al., 2021) and 1 study did not specify the mice lineage (Peng et al., 2022).

13 studies used only males (Chen et al., 2014; Greggio et al., 2011; Halis et al., 2019; Holubiec et al., 2018; Huang et al., 2021; Kim et al., 2006; Li et al., 2019b; Miguel et al., 2020; Morán et al., 2017; Niu et al., 2021; Potter et al., 2018; Sampath et al., 2020; Xu et al., 2015) while 39 used rodents of both sexes (Berger et al., 2019; Chen et al., 2015; Chen et al., 2021; Cho et al., 2018; Dell'Anna et al., 1997; Fan et al., 2008; Farfán et al., 2020; Gao et al., 2020; Gonzalez et al., 2009; Gou et al., 2020; Griva et al., 2017; Jung and Kim, 2017; Karalis et al., 2011; Kim et al., 2017; Kumral et al., 2004; Li et al., 2019a; Liu et al., 2013; Lowe et al., 2017; Luvone et al., 1996; Morales et al., 2009; Mishima et al., 2004; Mori et al., 2015; Odorczyk et al., 2016; Pan et al., 2012; Pak et al., 2018; Peng et al., 2022; Presti et al., 2006; Ren et al., 2017; Rodríguez-Fanjul et al., 2017; Roumes et al., 2020; Sun et al., 2014; Wagner et al., 2002; Wang et al., 2002; Wang et al., 2021; Wei et al., 2015; Wei et al., 2017; Zhao et al., 2014; Zhao et al., 2014; Zhao et al., 2014). All characteristics of the included studies are summarized in Table 3.

3.2.1. CP experimental models and phenotypic characteristics

The experimental models of CP were based on brain lesions in the perinatal period up to the 10th day of postnatal life that might reproduce the CP phenotype in animals. The vast majority of studies used the hypoxia-ischemia model through occlusion of the carotid artery between P3-P7, with 41 studies (Berger et al., 2019; Chen et al., 2014; Chen et al., 2015; Gao et al., 2020; Gonzalez et al., 2009; Gou et al., 2020; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Holubiec et al., 2018; Huang et al., 2021; Karalis et al., 2011; Kim et al., 2006; Kim et al., 2017; Kumral et al., 2004; Li et al., 2019b; Li et al., 2019a; Liu et al., 2013; Lowe et al., 2017; Morán et al., 2017; Mori et al., 2015; Miguel et al., 2020; Mishima et al., 2004; Niu et al., 2021; Odorczyk et al., 2016; Pan et al., 2012; Pak et al., 2018; Peng et al., 2022; Potter et al., 2018; Ren et al., 2017; Rodríguez-Fanjul et al., 2017; Roumes et al., 2020; Sampath et al., 2020; Sun et al., 2014; Wagner et al., 2002; Wang et al., 2002; Wang et al., 2021; Wei et al., 2015; Wei et al., 2017; Zhao et al., 2014; Zhao et al., 2014). 3 studies used exposure to lipopolysaccharide at the end of pregnancy or within the first 48 h after birth (Cho et al., 2018; Fan et al., 2008; Jung and Kim, 2017), 6 used oxygen deprivation, inducing delivery by surgery and exposing the pups to nitrogen to induce asphyxia or asphyxia occurred within the first 48 h of full-term birth (Chen et al., 2021; Dell'Anna et al., 1997; Luvone et al., 1996; Presti et al., 2006; Xiao et al., 2016; Xu et al., 2015), and 2 used oxygen deprivation on the last day of pregnancy (Farfán et al., 2020; Morales et al., 2009).

Results reported on movement, coordination or posture reported by type of brain injury were summarized to verify the cerebral palsy phenotype in perinatal brain injury models. Of these 52 included studies, only 20 evaluated or reported these outcomes. 16 models used hypoxia-ischemia and observed a reduction in the fall latency time in the rotarod test or rope suspension test (Griva et al., 2017; Karalis et al., 2011; Li et al., 2019b; Liu et al., 2013; Lowe et al., 2017; Mori et al., 2015; Pak et al., 2018; Potter et al., 2018; Wagner et al., 2002), a decrease in the distance traveled in the open field test (Holubiec et al., 2018; Kim et al., 2017; Li et al., 2019a), an increase in time to cross the bar in the Balance Beam Test (Niu et al., 2021), a reduction in paw pressure in the catwalk test (Pak et al., 2018), a reduction in strength in the grip test (Sampath et al., 2020) and reduced sensorimotor activity in the foot-fault test (Pan et al., 2012; Wei et al., 2015) and one study found no significant differences (Zhao et al., 2014). While 2 studies used oxygen deprivation and observed a reduction in the distance traveled in the open field test (Luvone et al., 1996; Xu et al., 2015). Of the studies

that used exposure to LPS, only 1 reported a reduction in the fall latency time in the rotarod test (Jung and Kim, 2017). None study reported postural changes in their results.

3.2.2. Disturbances in memory formation present in CP models and forms of evaluation

The animals submitted to the experimental models of CP showed various types of memory and behavioural alterations arising from CP in adulthood, exhibited by a poorer performance when compared with the control and/or sham group in tests that evaluated different characteristics of memory. Sprague Dawley rats at 12 and 41 days after brain injury showed reduced exploitation of objects used in the novel object recognition test (NORT) (Berger et al., 2019), as well as a preference for the old object (Li et al., 2019a). These are parameters typical of animals with some memory formation disturbance. The Y-maze demonstrated reduced spontaneous alternation in CP animals (Kim et al., 2006), indicating the presence of deficits in spatial memory in CP. The step-down passive avoidance test, which consists of a tool used to assess memory through aversive electrical stimuli, demonstrated that the animals in the CP group had a poorer performance, showing a reduction in step-down latency and a greater number of errors (Chen et al., 2014; Li et al., 2019b; Liu et al., 2013), demonstrating impairments in memory formation and learning.

The Morris water maze (MWM) test was used to assess the memory performance of the animals, noting that animals with CP needed a longer time to locate the platform (Chen et al., 2014; Griva et al., 2017; Halis et al., 2019; Halis et al., 2019; Karalis et al., 2011; Li et al., 2019a; Lowe et al., 2017), and had a higher mean escape latency (Chen et al., 2015; Gao et al., 2020; Sampath et al., 2020; Gonzalez et al., 2009; Gou et al., 2020; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Karalis et al., 2011; Kim et al., 2006; Kumral et al., 2004; Liu et al., 2013; Lowe et al., 2017). In addition, they took more time in the task of finding the target quadrant (Greggio et al., 2011; Karalis et al., 2011) and presented fewer entries and less time spent in the target quadrant (Chen et al., 2015; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Holubiec et al., 2018; Kim et al., 2006; Kumral et al., 2004). In addition, these animals spent a shorter time at the platform crossing in the MWM test (Chen et al., 2015; Gao et al., 2020; Gou et al., 2020; Holubiec et al., 2018; Huang et al., 2021; Liu et al., 2013) and decreased exploration of the new object in the NORT (Morales et al., 2009), indicating severe disturbance of memory formation in adult animals that suffered brain damage in the perinatal period.

3.2.3. Hippocampus damages present in CP models and forms of evaluation

Magnetic resonance imaging studies of the brains of rats with CP found severe impairments in the morphology of the nervous system, observing a reduction in grey matter volume on the side ipsilateral to the brain lesion and the hippocampus of Sprague Dawley rats 41 days after HI (Berger et al., 2019). Corroborating this, a reduction in the absolute weight of the dissected hippocampus of animals with CP was observed in Wistar rats on the 81th post-natal day (Halis et al., 2019). Histological evaluations with HE point in the same direction, where a reduction in hippocampal tissue was noted (Berger et al., 2019; Gao et al., 2020; Huang et al., 2021), where the dorsal region was severely affected (Griva et al., 2017).

Experimental assays with PCR, Western blot and ELISA analyses found a significant expression of pro-inflammatory cytokines in the brain of CP animals, the most present being IL-1 β , IL-6, IL-8 and TNF- α (Chen et al., 2014; Huang et al., 2021) through an increase in the expression of cytosolic Cyt-c, apoptotic protease activating factor-1 (Apaf-1) and caspase-3 in the hippocampus (Gao et al., 2020; Liu et al., 2013) and a reduction in the expression of MiR-29a-3p, MALAT1, miR-1, and BDNF (Huang et al., 2021; Li et al., 2019b). Biomarkers of oxidative stress were also significantly present after brain injury, noting an increased expression of nitric oxide (NO) and nitric oxide synthase (iNOS) activity, TBARS and a reduction in GSH in the hippocampus of

animals with CP (Chen et al., 2014; Greggio et al., 2011). The selected studies reported an increase in the expression of caspase-3 and NeuN+ cells in the dentate gyrus, indicating an increase in apoptotic neurons after brain injury (Chen et al., 2015; Liu et al., 2013). Analysis by the TUNEL method showed a high level of apoptosis in the hippocampal neurons of the animals with CP (Gao et al., 2020; Huang et al., 2021; Liu et al., 2013). In the same vein, histological images of the hippocampus by the crystal violet technique demonstrated a reduction in the number of neurons and an increase in dark and deformed nuclei in the CP (Gao et al., 2020; Gonzalez et al., 2009; Kim et al., 2006; Kim et al., 2017; Kumral et al., 2004).

Immunohistochemistry and immunofluorescence also revealed a lower number and less density of the NeuN+ and BrdU+ cells in the CP group (Gao et al., 2020; Li et al., 2019a), in addition to a reduction in BDNF and synaptophysin (SYN) levels (Griva et al., 2017; Kim et al., 2006), revealing impairments in neurogenesis and neural plasticity present in CP. In addition to an increase in the number of Glial fibrillary acidic protein (GFAP) positive cells in CA1, CA3 and DG (Holubiec et al., 2018), indicating the presence of astrogliosis in CP. Synaptic alterations were also found: a reduction in the number of synapses and an enlargement of the synaptic cleft in the CA1 of the hippocampus was observed through transmission Electron Microscopy (TEM) (Chen et al., 2021), showing that brain injury in the critical period alters the maturation of the hippocampus and consequently impacts the memory formation in adulthood.

3.3. Therapeutic perspectives on memory disturbances and hippocampal damage present in CP models

The experimental models of CP caused alterations in the morphology, functioning and connection of the hippocampus cells, affecting their microarchitecture and causing severe memory implications in the short and long term. To define this problem, this study investigated possible invasive or non-invasive interventions for memory formation disturbances and hippocampal damage present in brain lesions with implications for CP. For the treatment of memory formation alterations and hippocampal damage present in CP, 4 studies used resveratrol or resveratrol precursors as an intervention (Karalis et al., 2011; Li et al., 2019a; Peng et al., 2022; Sun et al., 2014). Treatment with erythropoietin, a drug with anti-inflammatory and antioxidant action, also occupied a prominent place in this review, investigated in 3 studies (Kumral et al., 2004; Gonzalez et al., 2009; Ren et al., 2017). Supplementation with group vitamins was also highlighted in the treatment of memory alterations in CP, vitamins B1 and B2 (Li et al., 2019b), B3 (Morales et al., 2009) and D (Lowe et al., 2017). Physical exercise was also evaluated as a therapeutic strategy in 4 studies (Chen et al., 2021; Farfán et al., 2020; Jung and Kim, 2017; Pak et al., 2018). 3 other studies investigated the repercussions of cell transplant treatment (Chen et al., 2015; Morán et al., 2017; Mori et al., 2015).

Herbal medicine or natural compounds treatment was evaluated for the treatment of CP memory formation disturbances in CP in 3 studies (Odrocyk et al., 2016; Roumes et al., 2020; Xu et al., 2015). Another 3 studies looked at the effects of sedative drugs to treat brain injury (Chen et al., 2021; Gao et al., 2020; Huang et al., 2021). 4 studies investigated the effects of hypothermia or hyperthermia (Mishima et al., 2004; Potter Pak et al., 2018; Rodríguez-Fanjul et al., 2017; Wagner et al., 2002). Oxygen therapy was also evaluated in 4 studies (Liu et al., 2013; Presti et al., 2006; Wei et al., 2015; Wei et al., 2017). Another 5 studies looked at the effects of antioxidant drugs such as carnitine (Zhang et al., 2014), NAPS (Greggio et al., 2011), sodium pyruvate (Pan et al., 2012) and acetyl-L-carnitine (Dell'Anna et al., 1997). Four other studies looked at the effects of anti-inflammatory drugs such as flupirtine maleate (Sam-path et al., 2020), isoflurane (Zhao et al., 2014), sevoflurane (Wang et al., 2021), palmitoylethanolamide (Holubiec et al., 2018) and alpha-Phenyl-n-tert-butyl-nitron (Fan et al., 2008). Two other studies have investigated the effects of melatonin treatment (Berger et al., 2019; Gou

et al., 2020). The effects of housing in an enriched environment after brain injury were also evaluated in 2 studies (Griva et al., 2017; Luvonne and Dell'Anna, 1996). Two other studies used herbal medicine with the treatment of asiaticoside (Chen et al., 2014) and huperzine A (Wang et al., 2002). One study used techniques based on Chinese medicine (Niu et al., 2021), 1 study investigated the use of pentoxifylline a peripheral vessel dilator (Halis et al., 2019), 1 article used oroxylin, a flavonoid, and another study evaluated the effects of the use of methylphenidate (Miguel et al., 2020). Other interventions were also evaluated such as TNP-ATP treatment (Xiao et al., 2016).

The question of memory outcome was analysed to elucidate the effect of each intervention on the CP model. In this review, we performed a meta-analysis that included HI-type brain injury or oxygen deprivation in the period from P0 to P10. Related to memory performance, the results of animals submitted to the MWM test from P28 to P85 were included. Thus, we grouped the studies according to the type of intervention by significant effect size in each subgroup with respect to latency escape in MWM analysis (SDM -2.84 95% CI [-3.10, -2.59], $Z = 22.00$; $p < 0.00001$; $I^2 = 92.9\%$) (Figs. 2 and 3). Among the interventions found, the following stand out: i) oxygen interventions (SDM -6.83 95% CI [-7.91, -5.75], $Z = 12.38$, $p = 0.03$; $I^2 = 71\%$); ii) early administration of resveratrol (SDM -2.42 95% CI [-3.19, -1.66], $Z = 6.21$, $p = 0.01$; $I^2 = 77\%$) and iii) erythropoietin interventions (SDM -3.16 95% CI [-4.27, -2.05], $Z = 5.58$, $p = 0.002$; $I^2 = 82\%$). For the meta-analysis of the length of stay in the target quadrant in the MWM, it was not possible to observe a significant effect size of the interventions compared to the control group (SDM 2.72 95% CI [1.84, 3.60], $Z = 6.05$; $p < 0.00001$; $I^2 = 82\%$) (Figs. 4 and 5). Thus, despite the heterogeneity among the studies, our meta-analysis of latency escape in MWM indicated that there are currently several promising interventions able to reduce impairments in memory formation in the CP model. In addition, a complementary meta-analysis was performed using the results of studies that showed the CP phenotype in an animal model associated with impairments in memory formation in the Morris water maze test (Figs. 6, 7, 8, and 9). These interventions will be detailed below. In addition, a graphic abstract has been added describing the main findings of this study for better understanding (Fig. 10).

3.3.1. Drugs with antioxidant action

A prominent place in the literature has been given to treatment with drugs with antioxidant action towards reducing the memory formation disturbance present in CP, given their ability to eliminate free radicals and exert a neuroprotective effect in the brain (Gonzalez and Ferriero, 2009). Treatment with 8-amino-acid (NAP) increased GSH levels and reduced TBARS levels, resulting in an improvement in memory performance in the MWM, reducing escape latency and time to find the target quadrant, in addition to promoting an increase in time spent in the target quadrant (Greggio et al., 2011). Treatment of the CP group with carnitine (250 mg/kg) was able to reduce the number of cells undergoing apoptosis and the expression of caspase-3. The levels of 8-iso-PGF2a, an important biomarker of oxidative stress, decreased in the CA1 field of the hippocampus in the CP group (Zhang et al., 2014).

Oxygen deprivation during the neonatal period, which can result in the development of CP, promotes a number of toxic events in the brain (McQuillen and Ferriero, 2004). Treatment with melatonin has been reported to be a tool in reducing damage to the nervous system and the memory formation problems present in CP. In this case, melatonin is characterized by being a neurohormone with neuroprotective effects (Berger et al., 2019), making it possible to detect a reduction in the size of the cerebral infarction in the hippocampus, an increase in the number of NeuN neurons, an increase in the expression of Gpx4 and a reduction in the expression of 4-HNE (Berger et al., 2019; Gou et al., 2020). Treatment with sodium pyruvate, in another study, promoted the preservation of hippocampal tissue, reducing the size of cerebral infarction in HI (Pan et al., 2012). Treatment with acetyl-L-carnitine (ALC) and huperzine-a, on the other hand, improved the performance of

the animals in the MWM, but the histological analyses did not show significant improvements in the hippocampus resulting from this treatment (Dell'Anna et al., 1997; Wang et al., 2021). Flavonoids were reported to have therapeutic potential for CP, noting a regulation of microglial cells that are disorganized in the hippocampus of animals with CP, in addition to greater survival of neurons in CA1 after brain injury (Kim et al., 2006).

Resveratrol, a polyphenol, has been suggested as a promising tool in the treatment of some brain injuries due to its strong antioxidant capacity. Regarding its use in CP disorders, an increase in the number of neurons and neuronal dendrites was observed, in addition to an increase in the density of dendritic spines and recuperation of the hippocampal neurons from the insult (Peng et al., 2022). In addition, there was increased SIRT1 levels and reduced brain lesion on the ipsilateral side to the lesion in the hippocampus (Peng et al., 2022; Karalis et al., 2011). An increase in cell proliferation and differentiation is also observed in the DG of the hippocampus in treatment with resveratrol (Li et al., 2019). A precursor to resveratrol, polydatin, increased BDNF levels in the hippocampal CA1, increasing brain plasticity (Sun et al., 2014). Regardless of dosage, this protective effect on the hippocampus resulted in some of the following on the MWM test: a reduction in the time the CP animals exposed to treatment took to find the platform (Peng et al., 2022; Karalis et al., 2011); an increase in the number of platform crossings (Peng et al., 2022); an increase of time spent in the target quadrant (Li et al., 2019); reduction in escape latency (Peng et al., 2022; Li et al., 2019; Karalis et al., 2011). On the maze-y test, there was increased alternation in animals treated with polydatin (Sun et al., 2014). In our meta-analysis, we observed significant effect size from the use of resveratrol (SDM -2.42 95% CI [-3.19, -1.66], $Z = 6.21$, $p = 0.01$; $I^2 = 77\%$) with reduced escape latency in the MWM test. These demonstrate promising effects of resveratrol use on CP memory formation disturbance.

The attenuation of damage to the nervous system, especially in the hippocampus, through the reduction of free radicals and toxic events, triggered behavioural changes that corresponded to improvements in the memory performance of the animals, through the reduction in escape latency (Griggio et al., 2011; Zhang et al., 2014; Dell'Anna et al., 1997; Gou et al., 2020; Wang et al., 2021; Kim et al., 2006), a decrease in the time to find the platform (Dell'Anna et al., 1997; Kim et al., 2006), a reduction in platform crossing (Gou et al., 2020) and a decrease in finding and an increase in permanence in the target quadrant (Griggio et al., 2011) in the MWM test. Regarding recognition memory, there was an increase in the exploration of the new object (Berger et al., 2019).

3.3.2. Drugs with anti-inflammatory action

Animals with CP showed an increase in inflammatory cytokines, indicating neuroinflammation, in addition to the presence of astrogliosis and increased Peroxisome Proliferator-Activated Receptor Alpha expression in the hippocampus (Holubiec et al., 2018). These changes are related to the memory formation disturbance found in CP (Jiang et al., 2018). From this perspective, interventions that aim to minimize neuroinflammation and its consequences become a target in the treatment of secondary disorders of CP, such as memory alteration. Treatment with palmitoylethanolamide 10 mg/kg reduced the number of Glial fibrillary acidic protein (GFAP) positive cells in hippocampal areas CA1, CA3 and DG, as well as a decrease in ionized calcium-binding adapter molecule cells in the CP group (Holubiec et al., 2018). However, treatment with flupirtine maleate or isoflurane or *a*-Phenyl-*n*-tert-butyl-nitron, or erythropoietin exerted a neuroprotective factor by preventing the loss of hippocampal tissue and reducing the size of the infarct in the hippocampus of CP animals (Sampath et al., 2020; Zhao et al., 2014; Fan et al., 2008; Gonzalez et al., 2009).

Erythropoietin treatment promoted a greater protective effect on CA1 (Kumral et al., 2004) and reduced myelin basic protein (MBP) expression (Ren et al., 2017) in CP animals. Our meta-analysis showed that improvement in the memory performance of the animals showed that the use of erythropoietin was able to reduce the escape latency in

the MWM test in CP animals (SDM 5.2% 95% CI [-4.27, -2.05], $Z = 5.58$, $p = 0.002$; $I^2 = 82\%$). Furthermore, *a*-Phenyl-*n*-tert-butyl-nitron prevented the enlargement of cerebral ventricles, improving the learning of rats (Fan et al., 2008), while pentoxifylline reduced the atrophy of the hippocampus present in CP, increasing the absolute weight (Halis et al., 2019). However, isoflurane attenuated the effects of inflammation and reduced the levels of G9a and H3K9me2 proteins that were elevated in CP (Wang et al., 2021). There was a reduction in glutamate levels and IL-1 β expression in adenosine 50-triphosphate (TNP-ATP) treatment in CP animals (Xiao et al., 2016).

The use of asiaticoside (AS) isolated from *Centella Asiatica* in CP significantly reduced microglial overactivation and phosphorylation of p38 MAPK in the hippocampus, and reduced markers of neuroinflammation in the hippocampus (Chen et al., 2014). Improvements with reduced neuroinflammation were reported to have therapeutic repercussions on behaviour, increasing cross-platform crossing (Holubiec et al., 2018; Xiao et al., 2016; Chen et al., 2014); reducing escape latency and time to find the platform (Zhao et al., 2014; Wang et al., 2021; Gonzalez et al., 2009; Kumral et al., 2004; Ren et al., 2017; Chen et al., 2014; Chen et al., 2014), decreasing swimming time (Zhao et al., 2014) and increasing time in the target quadrant (Gonzalez et al., 2009; Kumral et al., 2004; Xiao et al., 2016; Chen et al., 2014; Kumral et al., 2004; Xiao Takada et al., 2016; Chen et al., 2014; Chen et al., 2014) in the MWM test. Spatial memory in the maze-y test in the CP group was improved (Xiao et al., 2016).

3.3.3. Neurotransmitter reuptake inhibitors

Methylphenidate is a drug used in psychiatric disorders by inhibiting the reuptake of dopamine and norepinephrine. The use of methylphenidate in CP was able to stimulate an increase in BDNF levels, altering brain plasticity and promoting an attenuation of damage in the hippocampus. However, no differences were found in the assessment of memory function (Miguel et al., 2020).

3.3.4. Herbal medicine or natural compounds treatment

Nutrition is an essential part of brain and memory development, especially in neurodevelopmental disorders. Vitamin B1 and B2 supplementation increased the expression levels of BDNF and MALAT1, in addition to enhancing the phosphorylation of PI3K and Akt enzymes (Li et al., 2019b). While treatment with nicotinamide, a form of vitamin B3, reduced apoptosis in the hippocampus (Morales et al., 2009). In contrast, vitamin D treatment modulated the expression of CYP24A1, causing different effects in males and females (Lowe et al., 2017). Supplementation with several vitamins increased the latency time in the Step-down avoidance task (Li et al., 2019b), reduced the search time for the platform and increased swimming speed in the MWM (Lowe et al., 2017); vitamin supplementation increased exploration of the new object at NORT (Morales et al., 2009).

Other nutritional interventions, such as treatment with Huperzia quadrifariata alkaloid extract (HqAE), modulated the cholinergic system by reducing AChE activity, which was increased in animals with CP, and reduced the number of T-cells and apoptosis (Odojczyk et al., 2016). Supplementation with Marine collagen peptides (MCPs) also reduced AChE activity in addition to increasing the number of neurons and BDNF expression in the CA1 region (Xu et al., 2015). MRI images show that lactate reduced the volume of brain injury through metabolic changes in CP animals (Roumes et al., 2020). These studies provide evidence that nutrition is a key factor in memory formation, reducing the search time for the platform in the MWM test (Odojczyk et al., 2016; Xu et al., 2015) and increasing object discrimination and exploration time in the new object in the NORT of CP animals (Roumes et al., 2020).

3.3.5. Drugs with sedative action

Anaesthetics applied before or after brain injury have implications for the nervous system. Dexmedetomidine (DEX) treatment after brain injury attenuated hippocampal damage by regulating the number and

density of synapses, also preventing synaptic cleft enlargement and increasing the activity of the postsynaptic protein PSD-95 and the pre-synaptic protein synaptophysin and reducing the expression of NOX2 in microglia, of ROS and 4-HNE in the hippocampus (Chen et al., 2021). In addition to promoting the regulation of neuroglobin levels that were elevated in CP, it increased the number of neurons and reduced the number of cells with apoptosis (Gao et al., 2020; Huang et al., 2021). DEX treatment reduced IL-6, IL-8, and TNF- α levels and increased IL10 levels in the hippocampus of CP animals (Huang et al., 2021). These changes in the hippocampus resulted in changes in behaviour, such as a reduction in escape latency and an increase in platform crossing in the MWM test (Chen et al., 2021; Gao et al., 2020; Huang et al., 2021). Corroborating this, the meta-analysis findings highlight the effect size of sedatives on memory formation impairments present in CP (SDM -2.63 95% CI [-3.39, -1.88], $Z = 6.86$, $p = 0.0006$; $I^2 = 87\%$), demonstrating promising effects in reducing escape latency in the MWM test.

3.3.6. Treatment with cell implants

Treatment with transplantation of stem cells, mesenchymal cells or differentiated oligodendrocyte progenitor cells has demonstrated strong positive therapeutic evidence in brain injury and behavioural disorders present in CP. This was observed by an increase in the number of neurons in the subventricular zone of the hippocampus, and a reduction in apoptosis through decreased expression of caspase3 and NeuN in the hippocampus of CP animals (Chen et al., 2015). Intranasal administration of human adipose mesenchymal stem cells (MSC-S), preconditioned with either deferoxamine or TNF- α + IFN- γ , was reported to reduce the levels of cleaved caspase-3 proteins, cytoplasmic NRF2, nuclear NRF2, NRF2 effector NQO1 protein nuclear p65, promoting an increase in the length of the primary and secondary microglial process in the CA1 region of the hippocampus (Farfan et al., 2020). Intranasal administration of the peptide derived from complement activation C3a reduced the activation of astrocytes in the hippocampal areas CA1 and DG of CP animals (Morán et al., 2017). Infarct size was also reduced in the treatment with cells, this reduction observed in intracranial administration of the stromal cell-derived factor-1 α injections in the hippocampus of CP animals (Mori et al., 2015). This type of intervention was able to reduce latency escape (Chen et al., 2015; Mori et al., 2015), increase dwell time in the target quadrant (Chen et al., 2015) and increase platform crossing (Chen et al., 2015; Mori et al., 2015) in the MWM test of CP animals, as well as promote an increase in the exploration and recognition time of the new object in the NORT (Farfan et al., 2020; Morán et al., 2017).

3.3.7. Non-pharmacological interventions

Non-pharmacological interventions are also used to attenuate behavioural and memory disturbances resulting from brain injuries. An enriched environment is one of these therapeutic interventions. It is possible to observe by Cresyl Violet staining an increase in the number of neurons of CP rats housed in an enriched environment in the neonatal period of P5-P20 (Luvone and DellAnna, 1996), as well as a reduction in the size of the lesion in the hippocampus (Gonzalez et al., 2009). From these improvements in the hippocampus, behavioural changes were also observed in MWM, where animals showed better memory performance through a reduction in escape latency (Luvone and DellAnna, 1996; Griva et al., 2017), a decrease in time to find the target quadrant (Luvone and DellAnna, 1996) and to find the platform (Griva et al., 2017), and an increase in the number of entries in the target quadrant (Griva et al., 2017) in CP group.

Physical exercise was also able to promote improvements in motor and memory function in rats with CP. Treadmill running in CP rats was seen to facilitate neurogenesis and suppress apoptosis, increase cell proliferation in DG and synapsin levels (Cho et al., 2018; Jung and Kim, 2017), promoting therapeutic effects on memory through the PI3K-Akt-Wnt activation pathway, improving short-term memory by increasing the step-down latency time in the Step-down avoidance task (Cho et al.,

2018; Jung and Kim, 2017). Improvement in memory performance was also observed when physical exercise was associated with electroacupuncture (Pak et al., 2018) or with a physical therapy based on Chinese medicine, Tuina, which was able to reduce the expression of cleaved caspase-1, IL-18, IL-18 and cause a reduction in escape latency and an increase in dwell time in the target quadrant in MWM (Niu et al., 2021).

Hypothermia is a classic intervention in CP causing a reduction in brain damage (Mishima et al., 2004; Wagner et al., 2002; Wei et al., 2017; Rodríguez-Fanjul et al., 2017). However, one study reviewed did not observe significant differences between animals exposed to room temperature and those exposed to hypothermia in MWM (Mishima et al., 2004). In another experimental design, however, hypothermia showed behavioural deficits in the parameters evaluated in the MWM test (Potter et al., 2018). On the other hand, hyperbaric oxygenation soon after exposure was able to also reduce brain injury, in addition to decreasing the degeneration of the hippocampus fields CA1, CA2, CA3 and CA4 (Liu et al., 2013; Presti et al., 2006). An increase in the number of Bdnf-positive cells was noted, increasing cell proliferation (Wei et al., 2015). These improvements were observed through an increase in platform crossing and improved spatial learning (Wei et al., 2015; Mishima et al., 2004; Wagner et al., 2002). As seen in the meta-analysis, interventions with oxygen had the largest effect size in reducing the escape latency of animals with CP on the MWM test (SDM -6.83 95% CI [-7.91, -5.75], $Z = 12.38$, $p = 0.03$; $I^2 = 71\%$).

4. Study quality assessment

An evaluation of the methodological quality of the studies included in the review was performed using the SYRCLE risk of bias tool by three independent evaluators (Calado, Pereira and Souza). Item assessment of the baseline characteristics of the results of the experimental groups was similar for all 52 included studies in that they presented the participants' baseline characteristics. Regarding the random sequence generation item, 33 studies showed adequate performance (Berger et al., 2019; Chen et al., 2015; Chen et al., 2021; Dell'Anna et al., 1997; Farfan et al., 2020; Gao et al., 2020; Gonzalez et al., 2009; Gou et al., 2020; Greggio et al., 2011; Griva et al., 2017; Halis et al., 2019; Holubiec et al., 2018; Karalis et al., 2011; Kim et al., 2017; Li et al., 2019b; Li et al., 2013; Lowe et al., 2017; Luvone and DellAnna, 1996; Miguel et al., 2020; Morán et al., 2017; Niu et al., 2021; Odorczyk et al., 2016; Pan et al., 2012; Potter et al., 2018; Ren et al., 2017; Rodríguez-Fanjul et al., 2017; Sampath et al., 2020; Sun et al., 2014; Wang et al., 2002; Wei et al., 2015; Wei et al., 2017; Zhang et al., 2014; Zhao et al., 2014). Regarding allocation concealment, random housing and blinding of participants and personnel items, all studies showed a high risk of bias. With respect to the blinding of the participants, in the experimental studies of CP, this was not possible due to the phenotypic characteristics that enabled the identification of which animal was part of the group with CP or the control group.

19 studies presented adequate blinding of outcome assessment (Berger et al., 2019; Chen et al., 2015; Fan et al., 2008; Holubiec et al., 2018; Huang et al., 2021; Karalis et al., 2011; Kumral et al., 2004; Liu et al., 2013; Lowe et al., 2017; Miguel et al., 2020; Morán et al., 2017; Potter et al., 2018; Presti et al., 2006; Roumes et al., 2020; Sampath et al., 2020; Wang et al., 2002; Wang et al., 2021; Xiao et al., 2016; Zhao et al., 2014) and 15 studies presented adequate random outcome assessment (Chen et al., 2015; Gonzalez and Ferriero, 2009; Gou et al., 2020; Greggio et al., 2011; Holubiec et al., 2018; Kumral et al., 2004; Li et al., 2019b; Li et al., 2013; Lowe et al., 2017; Peng et al., 2022; Ren et al., 2017; Rodríguez-Fanjul et al., 2017; Wang et al., 2021; Xiao et al., 2016; Zhao et al., 2014). The reviewed studies were similar regarding the incomplete outcome data item, with only 3 studies showing a high risk of bias (Berger et al., 2019; Sun et al., 2014; Wagner et al., 2002). Selective reporting items were observed in only 4 studies that showed a high risk of bias in this evaluation item (Kim et al., 2017; Li et al., 2019b;

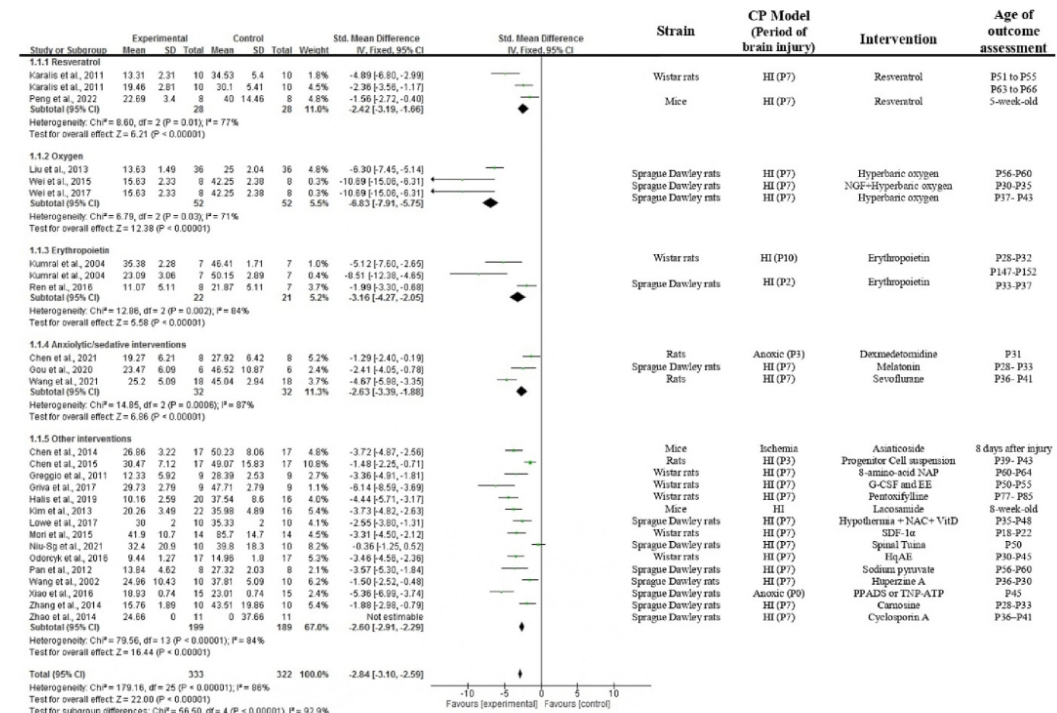


Fig. 2. Forest plot of included studies evaluating the escape latency in Morris water maze test (Subgroup: Intervention on models of CP). Horizontal lines represent the effect size $\pm$ the confidence interval (95%). The summary effect size is represented by the diamond. P: Postnatal day; HI: Hypoxic-ischemia; G-CSF: Granulocyte colony-stimulating factor; EE: Enriched environment; NAC: N-acetylcysteine; VitD: Vitamin; HqAE: Huperzia quadrifariata alkaloid extract; PPADS: pyridoxal phosphate-6 azophenyl-20,40-disulphonic acid; TNP-ATP: ATP analogue 20,30-0-(2,4,6-trinitrophenyl)-adenosine 50-triphosphate.

Morales et al., 2009; Pan et al., 2012). These results are presented in Fig. 11, which is a general summary of the results presented in percentages and the individual results of the evaluation of each article. These data indicate that consistency and methodological rigor were present in the experimental designs and the presentation of results in most of the included studies.

5. Discussion

This is the first study to systematically review and summarize promising treatment findings for memory formation alterations present in perinatal brain lesions with consequences for cerebral palsy, bringing new treatment perspectives that can be studied in the future as clinical interventions. We analysed how memory is affected and the morphological, biochemical and functional changes present in the hippocampus of animals submitted to early brain injuries associated with the aetiology of CP. Through behavioural parameters, we observed memory formation alteration in these models. Our analysis includes the main outcomes, the effects of interventions on the improvement of these damages. Through a qualitative analysis, associated with a meta-analysis, we observed the potential therapeutic effects of the use of oxygen interventions, resveratrol, anxiolytic/sedative interventions, erythropoietin and physical exercise on memory formation deficits and hippocampal damage present in CP through the reduction of free radicals and pro-inflammatory cytokines that trigger a cascade of neurotoxic events in the hippocampus.

Overall, the studies presented here indicate that spatial and recognition memory are impaired in CP, through increased escape latency, reduced permanence in the target quadrant and longer time to find the platform in the MWM test, reduced spontaneous switching in the Y-Maze test and T-Maze test, and decreased discrimination of new objects in the NORT. These memory alterations are related to the damage in the animals' hippocampus. A reduction in brain volume and relative weight was observed, while immunohistochemical markers and histological stains pointed to a reduction in the number of new neurons with an increase in astrogliosis, of oxidative stress enzymes and pro-inflammatory cytokines in regions such as CA1 and DG of the hippocampus. This set of adverse events can increase apoptosis and consequently the degradation of the hippocampus, signalling possible ways of understanding the memory formation disturbance present in perinatal brain lesions with consequences for CP. Thus, it can be understood that brain injury early in life promotes damage to the maturation of the hippocampus and consequently impairs memory formation in the adult life of animals. On the other hand, treatments that reduced neuro-inflammation and free radicals showed promising effects on memory, causing a protective effect on the hippocampus, preserving the number of neurons, increasing tissue volume and reducing astrogliosis, resulting in an improvement in the performance of animals on tests to assess memory.

It was possible to observe a heterogeneity of results independent of the model used in the hippocampus and the memory performance in animals with CP. A large number of studies reported a reduction in the

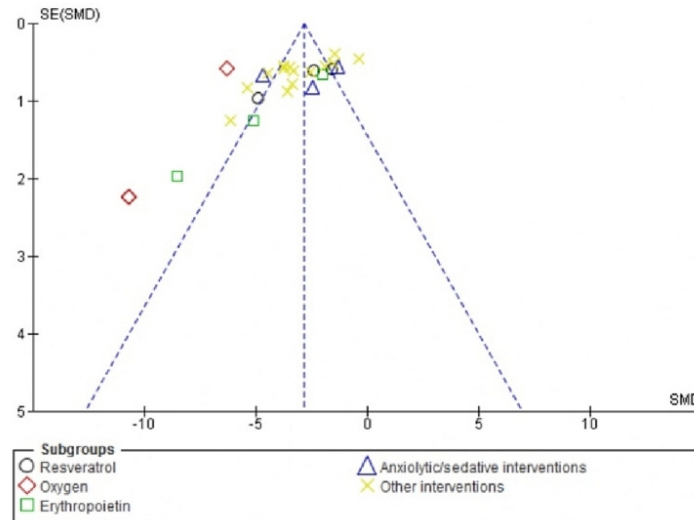


Fig. 3. Funnel plot of standardized mean differences (SMD) of the escape latency in the Morris water maze test. SE = standard error.

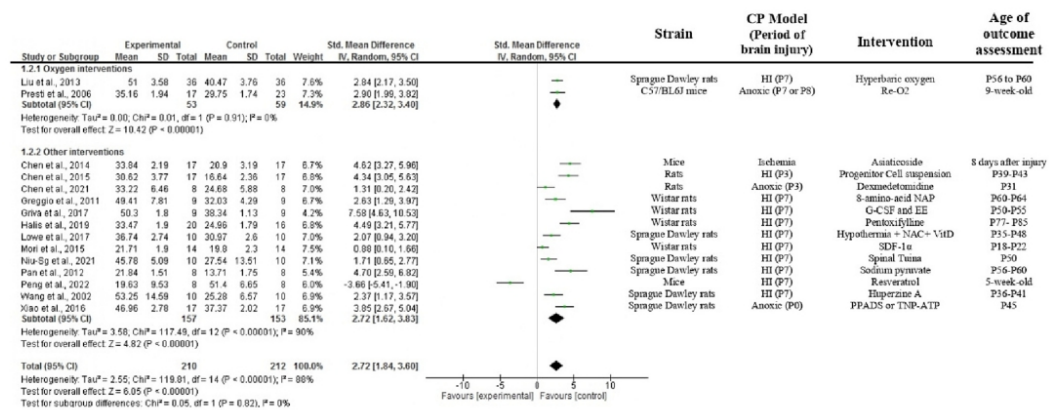


Fig. 4. Forest plot of included studies evaluating the time spent in the target quadrant in Morris water maze test (Subgroup: Intervention on models of CP). Horizontal lines represent the effect size $\pm$ the confidence interval (95%). The summary effect size is represented by the diamond. P: Postnatal day; HI: Hypoxic-ischemia; Re-O2: Reoxygenation; G-CSF: Granulocyte colony-stimulating factor; EE: Enriched environment; NAC: N-acetylcysteine; VitD: D Vitamin; PPADS: pyridoxal phosphate-6-azophenyl-20,40-disulphonic acid; TNP-ATP: ATP analogue 20,30-O- (2,4,6-trinitrophenol)-adenosine 50-triphosphate.

number of neurons and the volume of areas such as CA1 and DG and an increase in the number of glial cells in the hippocampus. In memory assessment tests on brain-injured subjects, a reduction was reported in target quadrant time and an increase in escape latency. Although CP is characterized by neuromotor and postural damaged, impairing motor skills (Kriger, 2006; Ward et al., 2006; Mockford and Caulton, 2010; Peterson et al., 2013; Coq et al., 2008; Pereira et al., 2021), many studies reported disturbances in memory and behaviour in experimental models of CP. In the present review, we observed that the hippocampus is a structure sensitive to oxygen deprivation in the prenatal, perinatal, or postnatal period (Matsuda et al., 2021) triggering a cascade of toxic events in the hippocampus, increasing levels of free radicals and inflammatory cytokines (Chen et al., 2014; Greggio et al., 2011; Huang

et al., 2021) and triggering a cascade of behavioural and memory changes (Greggio et al., 2011; Zhang et al., 2014; Dell'Anna et al., 1997; Gou et al., 2020; Wang et al., 2021; Kim et al., 2006; Peng et al., 2022).

Memory and learning are two processes that are closely linked. Learning is the process by which organisms acquire information, while memory can be defined as the ability to store this information (Bekinschtein et al., 2007). The formation of new memories involves a series of synaptic changes, in addition to changes in pre-existing proteins or the synthesis of new proteins in hippocampal neurons (Kandel, 2001). The formation of new memories causes changes in the brain, forming new synaptic connections, or strengthening connections related to the acquired information, which can be observed at the time of memory formation, known as engrams (Josselyn et al., 2015). For the proper

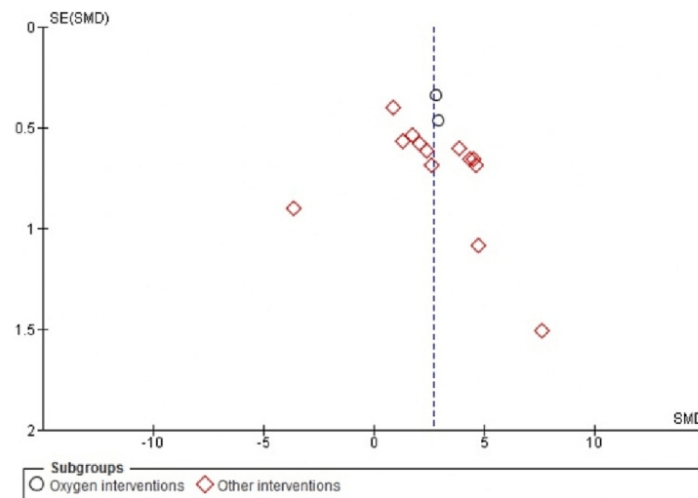


Fig. 5. Funnel plot of standardized mean differences (SMD) of the time spent in the target quadrant in Morris water maze test. SE = standard error.

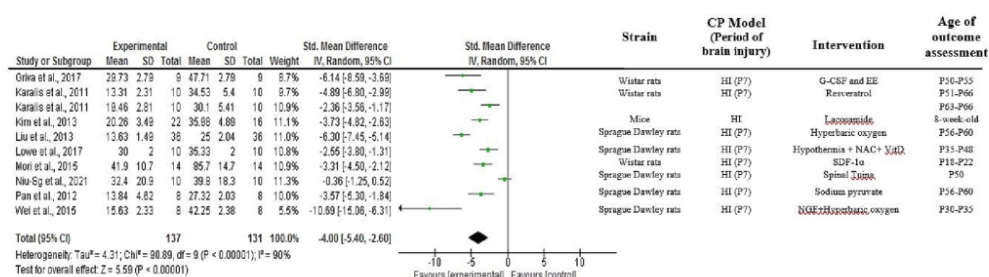


Fig. 6. Forest plot of included studies with CP experimental phenotype evaluating the escape latency in Morris water maze test. Horizontal lines represent the effect size $\pm$ the confidence interval (95%). The summary effect size is represented by the diamond. P: Postnatal day; HI: Hypoxic-ischemia; G-CSF: Granulocyte colony-stimulating factor; EE: Enriched environment; NAC: N-acetylcysteine; VitD: D Vitamin.

functioning of memory and learning, some essential steps are encoding, storing and recalling this information. A number of neural structures act on the proper functioning of memory, such as the amygdala in conditioned fear learning, the hippocampus in declarative memories, the caudate nucleus, medial septum and other cortical structures such as the prefrontal, entorhinal, parietal cortex (Izquierdo et al., 2013; Izquierdo et al., 1992). In our review, we studied the effects of perinatal brain injury models on memory and the hippocampus because of this relationship.

For the proper functioning of memory, certain proteins and molecules are essential, such as the brain-derived neurotrophic factor (BDNF) which is essential in neuronal plasticity in the growth of the dendritic spines of the hippocampus and in the storage of information, which is essential for the formation of long-term memories (Bekinschtein et al., 2007; Bekinschtein et al., 2008). In addition, other molecular bases of plasticity act in the hippocampus for memory formation where CREB levels participate in the transcription of long-term memories (Bevilaqua et al., 1999). The role of BDNF in the hippocampus is essential in the transformation of short-term memories into long-term, also acting on neurotransmission systems and synaptic plasticity. Some findings also

indicate that this protein actively participates in declarative memories, more specifically in memory recognition of objects (Gonzalez et al., 2019a; Gonzalez et al., 2019b). In our study, we identified declines in BDNF levels in the hippocampus of animals with perinatal brain lesions that may have implications for CP, explaining the memory formation disturbance in adult life that is associated with these lesions in the perinatal period.

Regardless of the experimental model used, by oxygen deprivation, maternal exposure to LPS, or occlusion of the carotid artery, an increase in the number of free radicals, pro-inflammatory cytokines, and glial cells was observed, while there was a reduction in the volume of the hippocampus associated with a memory formation deficit (Chen et al., 2014; Berger et al., 2019; Sampath et al., 2020; Zhao et al., 2014). In our study, we observed that this series of alterations in the hippocampus, caused by environmental insults in the critical period of development, is capable of causing repercussions on the behaviour of animals in adult life, with impairments in episodic memory being observed through poor performance in recognition objects tests and spatial location tests. Memory formation alteration can be understood as the proper functioning of the hippocampus, especially synapses, associated with

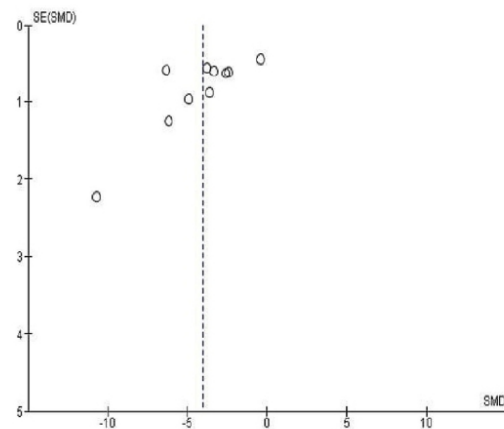


Fig. 7. Funnel plot of standardized mean differences (SMD) of the escape latency in Morris water maze test of included studies with CP experimental phenotype. *SE* = standard error.

memory (Zhang et al., 2015). Although memories are not reduced to the function of a single brain area, it is known that the hippocampus participates in the initiation of memories (Izquierdo et al., 2013; Izquierdo et al., 1992), which suggests why memories are impaired in hippocampal malformation.

Despite the scarcity of studies that evaluate memory in subjects diagnosed with CP, encouraging findings indicate that children with CP are at a higher risk of developing changes in memory and executive functions that can lead to impaired learning (Jenks et al., 2009; Schenker et al., 2005). Studies observe impaired learning in children with CP due to disturbance in memory formation and executive functions, compromising mathematical skills and school performance (Feldberg et al., 2021; Pereira et al., 2018; Stadskleiv et al., 2017). Recent clinical research has found changes in brain wave oscillations, such as alpha-beta, in cortical regions of adults with CP, for example in prefrontal and temporal areas, during the codification of information, associated with deficits in working memory (Hoffman et al., 2021). This suggests possible memory formation problems present during adulthood in subjects with CP as a result of dysfunctions in brain regions. In the same sense, we observed in our studies that the environmental insult in the perinatal period disrupts the functioning of the brain, promoting disturbances in the memory in rodents, in a similar way as observed in humans, making possible the investigation of interventions that can act in the brain injury and its consequences.

In our review, experimental models of perinatal brain injury with rodents proved to be an essential tool, within the limits of preclinical studies, enabling an understanding of the relationship between the extent of damage in the brain areas present in perinatal lesions with consequences for CP to the development of memory acquisition problems that can last into adulthood. This provides a tool for the investigation of interventions that act on the attenuation of neurological and memory sequelae, as used in our meta-analysis. Therapies that used oxygen, however, such as exposure to a hyperbaric oxygen chamber, had an excellent outcome in the treatment of memory formation deficits present in the brain-damaged model. Corroborating this, in our meta-analysis this intervention presented the best effect size among the analysed interventions (Figs. 2 and 3).

In many studies, excellent outcomes were observed in the exposure to an oxygen chamber before the first hour after brain injury, reported to reduce the size of the brain lesion and the degeneration of neurons in the CA1, CA2, and DG of the hippocampus (Liu et al., 2013; Wei et al., 2015; Wei et al., 2017). These interventions in the perinatal period had repercussions on permanent improvements in the animals, by an observed reduction in the escape latency at P30–35 (Wei et al., 2017) and at P37–43, the end of the adolescence period of the rodents of the Sprague-Dawley rats. In addition, improvements in this treatment were also observed in the perinatal period during the beginning of the adult life of these animals, with a reduction in the escape latency in P56–60 (Liu et al., 2013) and P60–P68 (Presti et al., 2006). This suggests that oxygen therapies are promising in the treatment of memory formation disturbance present in the perinatal brain lesions and that the treatment causes permanent repercussions on the organism. However, these

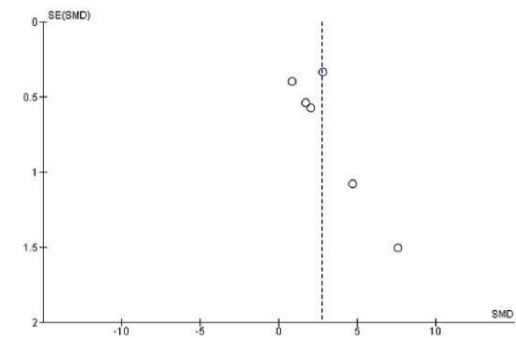


Fig. 9. Funnel plot of standardized mean differences (SMD) of the time spent in the target quadrant in Morris water maze test of included studies with CP experimental phenotype. *SE* = standard error.

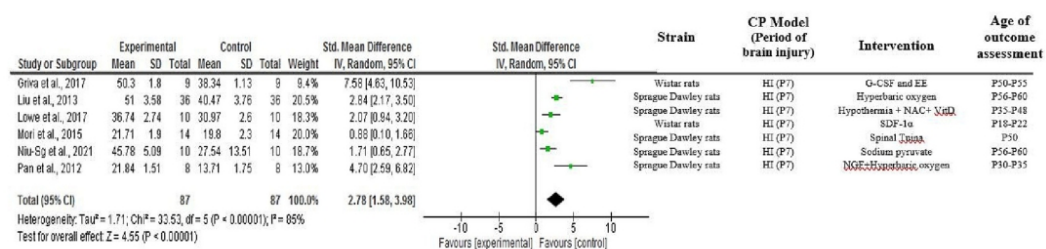


Fig. 8. Forest plot of included studies with CP experimental phenotype evaluating the time spent in the target quadrant in the Morris water maze test. Horizontal lines represent the effect size $\pm$ the confidence interval (95%). The summary effect size is represented by the diamond. P: Postnatal day; HI: Hypoxic-ischemia; G-CSF: Granulocyte colony-stimulating factor; EE: Enriched environment; NAC: N-acetylcysteine; VitD: D Vitamin.

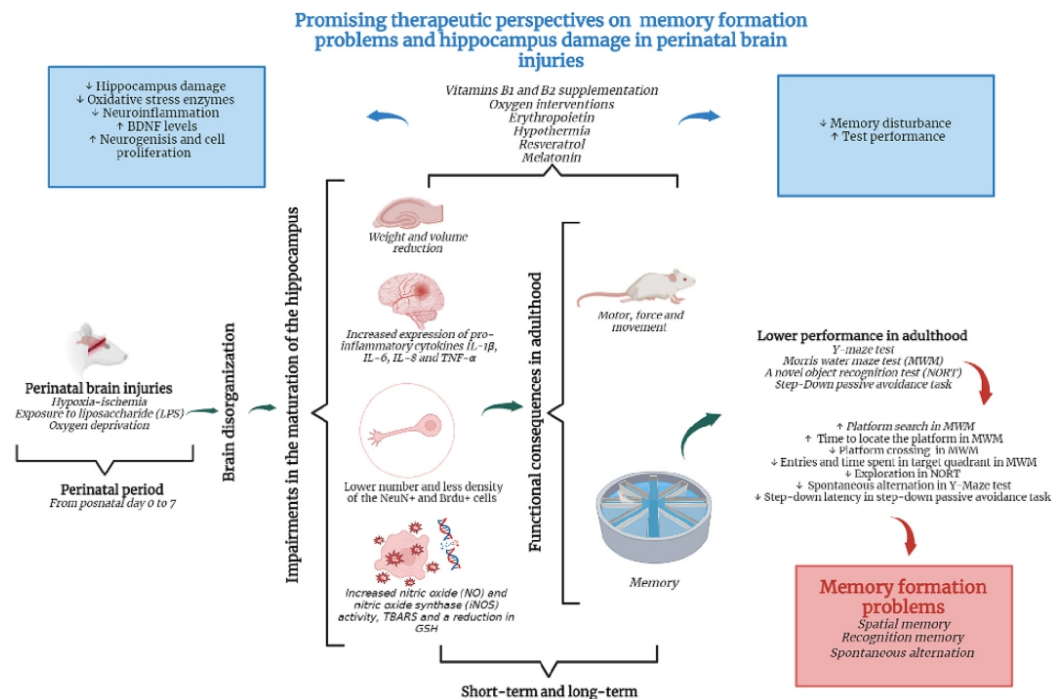


Fig. 10. Graphic abstract with the main findings of the study. Brain injuries in the perinatal period cause long-term and short-term memory, behavioural and motor changes in adulthood. Treatment with vitamin B1 and B2 supplementation, interventions with oxygen, Erythropoietin, Hypothermia, Resveratrol, and Melatonin show promising findings in hippocampal disorganization and memory alterations.

findings are reflections of studies in experimental models with rodents and recent findings point to side effects in the use of hyperbaric oxygen in children with CP (Novak et al., 2020; Novak and Badawi, 2012).

Therapeutic interventions that attenuate the effects of inflammation and oxidative stress in the hippocampus occupied a prominent place in the studies selected for review, together with sensory stimulation, physical exercise, and supplementation with some specific foods that increased brain plasticity and its associated mechanisms, such as expression of BDNF (Sun et al., 2014; Li et al., 2019; Xu et al., 2015). Some organic compounds, such as polyphenols, which are natural compounds present in some plants and foods, have therapeutic properties by increasing BDNF levels in some cases of brain injuries such as ischemia and hypoxia, based on the relationship between metabolic activity, food consumption, and neuronal activity. Some polyphenols, such as resveratrol, act by increasing levels of BDNF in the brain that is related to memory functioning (Ma et al., 1997; Gomez-Pinilla and Nguyen, 2012; Khanna et al., 2020). These results emphasize the importance of multidisciplinary interventions in all impaired skills in CP, starting in childhood. Among the interventions analysed, treatment with erythropoietin was also highlighted, being able to increase neurogenesis and cell differentiation in brain development and to increase the number of cells such as neurons, astrocytes and endothelial cells, exerting a neuroprotective factor in brain lesions (Bernaudin et al., 1999; Siren et al., 2001; Mu et al., 2005), and modulating immune and anti-inflammatory responses (Villa et al., 2003; Agnello et al., 2002; Arvin et al., 1996). Treatment with erythropoietin improved the memory performance of animals with CP by reducing damage to the hippocampus (Gonzalez et al., 2009; Kumral et al., 2004; Ren et al., 2017).

Neither type of intervention presented deleterious effects, opening new perspectives for the treatment of brain injuries and memory formation disturbance present in subjects with CP. Furthermore, in our meta-analysis, regardless of the CP model, studies were included that evaluated the memory performance of animals in the Morris water maze, (Figs. 2 and 4). It is possible to observe promising effects of oxygen interventions, resveratrol and erythropoietin on MWM reducing latency escape. But it was not possible to find significant differences in the interventions in the length of stay on the target platform. Escape latency is a sensitive parameter for elucidation of the memory formation deficits present in neurological diseases, which may explain the results found for this parameter in most studies, rather than time spent in the target quadrant.

Treatment with cell transplantation is presented as an innovative intervention in neurological lesions, where intranasal or intraventricular administration have demonstrated beneficial effects on the analysed parameters, reducing apoptosis, caspase-3 expression and cerebral infarction size (Chen et al., 2015; Farfán et al., 2020; Mori et al., 2015). Thus, cell transplant represents a prominent treatment in this review for its positive effects on the learning of animals. Increase in the exploration time of a new object in the NORT test and reduction of escape latency in the MWM test in CP animals (Chen et al., 2015; Farfán et al., 2020; Mori et al., 2015). This is a very invasive intervention, however, that can have side effects, making it necessary to carefully evaluate its repercussions and whether possible therapeutic effects exist through other less invasive routes of administration before it can be considered a safe intervention in the field of paediatrics.

In this study, it was possible to observe promising results of

environmental stimulation in the improvement of memory and brain damage in preclinical studies, as it occurs in an enriched environment, making it an important tool for use in memory formation disturbance. Preclinical studies (Luvone and Dell'Anna, 1996; Griva et al., 2017; Gonzalez et al., 2009) have demonstrated that enriched environment improved the memory performance of animals with CP in the MWM test, reduced the escape latency and the time to find the platform; clinical trials (Lohaugen et al., 2014; Lieto et al., 2020) have demonstrated the positive repercussions of environmental stimulation on various type memory. Physical exercise and supplementation with specific vitamins were reported to be strong allies in the treatment of CP reinforcing the importance of multidisciplinary interventions in mitigating the

limitations that can arise along with CP in childhood (Cho et al., 2018; Jung and Kim, 2017; Li et al., 2019).

Thus, in preclinical studies, memory formation disturbance similar to those that occur in children with CP can be observed, by behavioural deficits in object discrimination, reduced spatial alternation, shorter time spent in the target quadrant, increased escape latency, and in the time to find the platform in the behavioural tests performed, indicating impairments in recognition memory and spatial memory, in long and short-term assessment protocols. Although the literature is still sparse regarding therapeutic perspectives in the clinic, pre-clinical studies have shown promising effects in the treatment of memory formation deficits present in CP, with improvements in the analysed parameters, such as

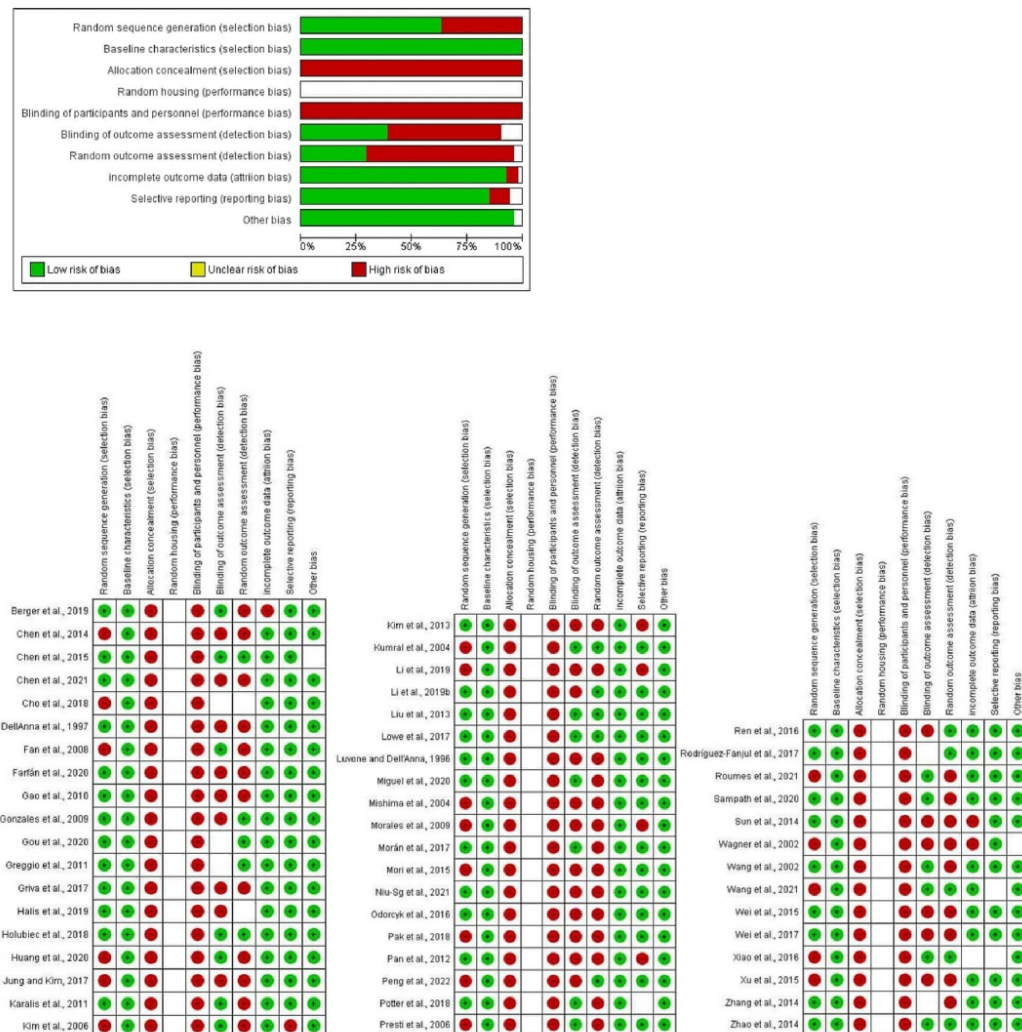


Fig. 11. Risk of bias summary and graph of included studies. Review authors' judgments about each risk of bias item presented as percentages across all included studies. (green) low risk of bias; (red) high risk of bias; (white) unclear risk of bias. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reduced escape latency and time to find the platform in MWM. The experimental perinatal brain lesions models showed biochemical and morphological changes in the hippocampus, in regions such as the dentate gyrus and CA1, an increase in pro-inflammatory cytokines such as IL-1 β , IL-6, IL-8, and a reduction in levels and expression of BDNF. Furthermore, there was a higher expression of nitric oxide (NO) and nitric oxide synthase (iNOS) activity, TBARS, and a reduction in GSH in the hippocampus, indicating the presence of oxidative stress in the hippocampus of animals with CP.

On the other hand, there were reports of therapeutic effects from treatments that attenuated the deleterious effects on the hippocampus caused by neuroinflammation and oxidative stress, which are the main processes that affect the maturation of the hippocampus in CP, bringing improvements in spatial, recognition, short-term and long-term memory. Among the interventions analysed, were the use of oxygen interventions, resveratrol, erythropoietin, stem cells, herbal medicine or natural compounds treatment, and the use of physical exercise, all attenuating damage to the hippocampus and memory formation of animals with CP. These improvements were observed during adulthood even after the end of treatment.

5.1. Strengths and limitations

This is the first systematic review with meta-analysis that investigates therapeutic advances in the treatment of memory formation problems in animals that suffered brain injury in the perinatal period. We systematically evaluated changes in memory formation resulting from environmental insults such as hypoxia-ischemia, maternal exposure to LPS and oxygen deprivation. We observed that the hippocampus is one of the brain structures affected by these insults in the critical period of development in the prenatal and neonatal phases, causing a disturbance in episodic memory formation during adulthood in various behavioural tests. We systematically investigated the literature and summarized the main interventions used in memory formation disturbances present in perinatal brain injuries and their effects on hippocampal damage. For this, we followed strict methodological criteria throughout this review. From the moment of elaborating a search protocol in the databases until the presentation of the results, reducing the risks of possible bias to the maximum. We provide a series of results regarding alterations in the hippocampus maturation present in animals that exposure to brain injuries in the perinatal period, since it is difficult to obtain results regarding morphological, biochemical and functional alterations in children with CP. In the experimental models, it was possible to observe an increase in oxidative stress, neuroinflammation, apoptosis and a reduction in neuroplastic mechanisms such as neurogenesis and a decrease in BDNF levels associated with episodic memory formation disturbance, making it possible to understand. Additionally, we present the outcomes of interventions used after brain injury and their effects on memory formation and hippocampus disturbances, performing a qualitative analysis of these results and a quantitative analysis through a meta-analysis. to verify the effect size of the interventions.

This study has some limitations, that are related to the original studies limitations. First, the studies included in this study only covered brain injuries with implications for CP of environmental origin, being developed from insults such as hypoxia-ischemia, early exposure to LPS or oxygen deprivation (Fragopoulou et al., 2019; Jacobsson and Hagberg, 2004). Due to the complexity of factors involving the CP, there are no reliable models that reproduce all the characteristics of the CP in animals. There are models that use insults during the perinatal period that reproduce some dimensions of the CP phenotype, affecting behaviour and the nervous system in different ways (Coq et al., 2008; Pereira et al., 2021; Visco et al., 2021). This limitation of our study is related to the limitations of the original studies, we were unable to detect genetic models of CP, limiting the study only to CP of environmental origin. That mimics brain damage acquired early in life. Regarding the

methodological quality of the included studies, few studies blinded the animals to reduce possible biases in the presentation of the results due to the observable phenotypic characteristics of the CP that allow the identification of the animals, but the blinding for the pharmacological manipulation and analysis of the results are possible to be carried out, but few studies have described having done this blinding. In addition, it is only possible to evaluate episodic memory, due to the limitations of evaluating other types of memory in animal models. In addition, due to the large scope of this review and the heterogeneity of the methods used to assess memory in animals, it was only possible to carry out the meta-analysis of studies that used the MWM test.

In addition, trying to verify the antecedents of memory formation problems present in CP is complex, due to the number of environmental factors that influence, through epigenetic mechanisms, the adequate development of memory, such as brain injuries, adequate nutrition and sleep (De Souza et al., 2011; Fesser et al., 2021; Potter Pak et al., 2018; Presti et al., 2006). Memory formation alterations are heterogeneous because they manifest themselves in different ways depending on the severity and extent of the brain injury and its sequelae. Different types of CP diagnosis can present a distinct neuropsychological profile, where some cognitive skills are more deficient than others (Stadskleiv et al., 2017). Although we found memory formation disturbances in all studies included in our review, memory formation problems are not present in all subjects diagnosed with CP. Moreover, they are findings from pre-clinical studies, and the results found are not necessarily often observed in humans with CP. Some subjects with CP present deficits on memory acquisition due to early brain injuries, but these are only noticed with the development of the organism and the emergence of functional demands of everyday or school life present in their reality (Pascal et al., 2018; Pak et al., 2018). Although not present in all cases of CP, memory formation impairment is reported in recent studies with children and adults with CP (Fesser et al., 2021; Hoffman et al., 2020), highlighting the importance of investigating interventions that act such as neurological sequelae and memory formation disturbance in adulthood.

5.2. Implications for future investigation

Overall, this study provides evidence of how brain damage in early life affects hippocampal development and promotes problems in the formation of new memories during adulthood in rodents. Furthermore, we provide quantitative and qualitative evidence of the effects of several promising interventions in alleviating the memory formation problems present in animals. These pieces of evidence serve as a guide for future studies to investigate the effects of promising interventions such as the administration of Melatonin or Erythropoietin, or the supplementation of vitamins, such as B1 and B2, or polyphenols, such as resveratrol, as they have strong antioxidant and anti-inflammatory effects, attenuating deficits in hippocampal maturation and consequently reducing memory formation problems in adulthood. Future studies investigating the effects of these interventions on the maturation of other damaged brain structures in CP may promote a better understanding of treatments for the primary problems of CP, such as neuromusculoskeletal sequelae, and the secondary problems such as alterations in memory formation, allowing future investigations of the use these interventions in clinical practice.

This systematic review can also open the way for the investigation of other interventions that act by reducing reactive oxygen species and neuroinflammation and promoting an increase in neuroplastic mechanisms, such as an increase in the levels of BDNF, CREBS and consequently an increase in neurogenesis and cell proliferation in the hippocampus, considering that the interventions that reduced memory formation problems in early brain injury acted through these mechanisms. For this reason, nutritional interventions are present promising due to the relationship between food intake and brain activity.

6. Conclusions

In our study, we observed difficulties in the formation of new episodic memories, a type of declarative memory, from a low performance in tests assessing object recognition and spatial location, in the adult life in animals that were exposed to brain lesions in the perinatal period with implications for the development of cerebral palsy. Our findings indicate that early brain injury impairs the development and maturation of brain structures such as the hippocampus, leading to difficulties in memory formation in adult life, highlighting the importance of the perinatal period in the development of problems in memory formation and acquisition that may be observed during life. These memory formation problems are associated with damage to the hippocampus from brain injury, noting an increase in oxidative stress, neuroinflammation, apoptosis; and a reduction in neuroplastic mechanisms such as neurogenesis and decrease BDNF levels. Interventions that reduced neuroinflammation and the presence of free radicals were highlighted as a therapy for the memory formation problems present in CP. Preclinical studies registered treatments with oxygen interventions, resveratrol, erythropoietin, melatonin and physical exercise, which were able to reduce the damage to the hippocampus and promote improvements in memory and behaviour in experimental models of perinatal brain lesions. These reviewed studies, then, suggest possible avenues of intervention for memory formation disturbance present in perinatal brain lesions, highlighting promising interventions that can mitigate memory problems.

Author contributions

A.E. Toscano was the supervisor and third reviewer. C. M.S. S Calado and S.C. Pereira were the first and second reviewers. C. M.S.S. Calado, S. C. Pereira, V.S. Souza, D.B. Visco, B.S. De Silveira, S.L. Souza, and A.E. Toscano carried out the manuscript preparation process, all of whom participated in determining the eligibility criteria and standardizing the data collection forms and analysing the risk of study bias. R. Manhaes-de-Castro, S.L. Souza, and A.E. Toscano participated in the review and editing for preparation. They contributed their experience regarding intellectual content and guidance. All authors reviewed and agreed with the final manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Reporting no conflict of interest.

Data availability

No data was used for the research described in the article.

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APÊNDICE C – NEONATAL RESVERATROL TREATMENT IN CEREBRAL PALSY MODEL RECOVERS NEURODEVELOPMENT IMPAIRMENTS BY RESTORING THE SKELETAL MUSCLE MORPHOLOGY AND DECREASES MICROGLIAL ACTIVATION IN THE CEREBELLUM

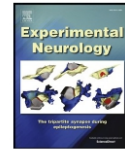
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Research paper

Neonatal resveratrol treatment in cerebral palsy model recovers neurodevelopment impairments by restoring the skeletal muscle morphology and decreases microglial activation in the cerebellum

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ABSTRACT

Cerebral Palsy (CP) is the main motor disorder in childhood resulting from damage to the developing brain. Treatment perspectives are required to reverse the primary damage caused by the early insult and consequently to recover motor skills. Resveratrol has been shown to act as neuroprotection with benefits to skeletal muscle. This study aimed to investigate the effects of neonatal resveratrol treatment on neurodevelopment, skeletal muscle morphology, and cerebellar damage in CP model. Wistar rat pups were allocated to four experimental groups ($n = 15/\text{group}$) according CP model and treatment: Control+Saline (CS), Control+Resveratrol (CR), CP + Saline (CPS), and CP + Resveratrol (CPR). CP model associated anoxia and sensorimotor restriction. CP group showed delay in the disappearance of the palmar grasp reflex ($p < 0.0001$) and delay in the appearance of reflexes of negative geotaxis ($p = 0.01$), and free-fall righting ($p < 0.0001$), reduced locomotor activity and motor coordination ($p < 0.05$) than CS group. These motor skills impairments were associated with a reduction in muscle weight ($p < 0.001$) and area and perimeter of soleus end extensor digitorum longus muscle fibers ($p < 0.0001$), changes in muscle fibers typing pattern ($p < 0.05$), and the cerebellum showed signs of neuroinflammation due to elevated density and percentage of activated microglia in the CPS group compared to CS group ($p < 0.05$). CP animals treated with resveratrol showed anticipation of the appearance of negative geotaxis and free-fall righting reflexes ($p < 0.01$), increased locomotor activity ($p < 0.05$), recovery muscle fiber types pattern ($p < 0.05$), and reversal of the increase in density and the percentage of activated microglia in the cerebellum ($p < 0.01$). Thus, we conclude that neonatal treatment with resveratrol can contribute to the recovery of the delay neurodevelopment resulting from experimental CP due to its action in restoring the skeletal muscle morphology and reducing neuroinflammation from cerebellum.

1. Introduction

Brain injuries during early development can result in a permanent childhood movement and posture disorder known as cerebral palsy (CP). It affects children around the world differently varying depending on

access to obstetric and neonatal care in the sociodemographic region (Graham et al., 2019; Gulati and Sondhi, 2017; Maenner et al., 2016; Oskoui et al., 2013; McIntyre et al., 2022). The Global prevalence of CP is estimated at approximately 1.6 per 1000 live births in high-income countries and its prevalence is markedly higher in low- and middle-

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income countries reaching up to 3.4 per 1000 live births (McIntyre et al., 2022). High-income countries deal with prematurity and morbidities related to low birth weight, while low- and middle-income countries tend to deal with infections in the pre- or post-natal period and perinatal asphyxia (Gulati and Sondhi, 2017; Wimalasundera and Stevenson, 2016).

The early insult that leads to CP occurs during the critical period of development, in other words, the insult occurs at some stage of the window of vulnerability of the central nervous system (CNS) (Marret et al., 2013; Semple et al., 2013). For example, acute or subacute perinatal events such as birth asphyxia are determinants in the white and gray matter lesions generally observed in children with CP (Marret et al., 2013). A window of greatest vulnerability of the white matter occurs between 24 and 34 weeks of gestation and is related to the active growth of brain pathways. It is a phase of high proliferation, migration and maturation of glial cells. At this stage, axonal and dendritic growth, synapse formation and myelination are also occurring, as well as stabilization processes such as synaptic pruning, and specialization of circuits that reach their peak in the postnatal period at around two years of age (Marret et al., 2013; Semple et al., 2013). Thus, isolated white matter damage or combined gray and white matter abnormalities are frequent neuroimaging findings (Korzeniewski et al., 2008) as well as reduced total brain, cerebellum, and gray matter volumes in children with cerebral palsy (Kulak et al., 2016).

Due to early damage to the CNS, children often experience delays in the acquisition of motor skills, including delays in the appearance of primitive reflexes or their inhibition. Primitive reflexes are automatic involuntary movements that occur in response to a stimulus. In humans, satisfactory brain maturity is essential to allow the inhibition of primitive reflexes and the appearance of postural responses for the normal progression of psychomotor functions; this requires a transition from an involuntary brainstem reflex response to one controlled by the cortex. A child with delayed psychomotor development, as happens at CP, may demonstrate difficulties in motor performance such as locomotion and movement coordination, these being related to the greatest risk for abnormal development (Chandradasa and Rathnayake, 2020; Kobesova and Kolar, 2014). The control of these primitive reflexes is carried out at levels of sensorimotor control within the CNS with the cerebellum involved in all levels of integration, acting in muscle tone regulation, postural and balance maintenance (Kobesova and Kolar, 2014). This plays an important role of the cerebellum in the study of CP motor damage. These studies, however, are scarce.

Experimental studies have shown outcomes similar to what occurs in children with CP and its associated mechanisms. Animal models show that after insults during fetal or perinatal development, an inflammatory cascade and cellular apoptosis are triggered, leading to brain tissue damage and consequent deficiency in motor control, delay in appearance or inhibition of primitive reflexes, delay in the development of motor skills, loss of muscle mass (sarcopenia), muscle weakness, change in the distribution of muscle fiber types, dyskinesia, spasticity, hyperreflexia, culminating in functional deterioration (Shi et al., 2019; Zhang et al., 2016; Peterson et al., 2013; Gutman et al., 2011; Costa-de-santana et al., 2023; Lacerda et al., 2017a). Motor disorders are characteristic of children with CP (Peterson et al., 2013; Brandenburg et al., 2019) and they are often accompanied by disturbances of sensation, perception, cognition, communication and behavior (Brandenburg et al., 2019; Rosenbaum et al., 2007). Even the very survival of children is often related to the severity of functional disability (Gulati and Sondhi, 2017) and intellectual disability (Liptak et al., 2004).

In this context, CP models have advanced in the scientific literature to study this disease. Among these models of CP, the model of combined insults anoxia and sensorimotor restriction (SR) stands out for promoting changes similar to CP in children, such as neuromuscular damage (Strata et al., 2004a; da Silva et al., 2016; Calado et al., 2023a; da Silva et al., 2023). The isolated anoxia model determines most of the damage to the central nervous system but it promotes subtle and reversible

damage to motor skills, especially locomotion and SR, contributes strongly to the deterioration maintenance of the motor function (da Conceição et al., 2021). Both, anoxia and SR cause damage to the CNS such as increased pro-inflammatory cytokines (Stigger et al., 2013) and oxidative stress in the cerebral cortex (da Silva et al., 2023), decreased neurogenesis in the hippocampus (Calado et al., 2023a; Takada et al., 2016), increased activated microglia in the hippocampus (Calado et al., 2023a) and cerebellum (Costa-de-santana et al., 2023), confirming the inflammatory cascade and excitotoxicity damage installed resulting from experimental model. Consequently, locomotion, motor coordination (da Conceição et al., 2021; Pereira et al., 2021; Coq et al., 2008; Marcuzzo et al., 2010; Strata et al., 2004b) posture, gait pattern (da Silva et al., 2023), masticatory function (Lacerda et al., 2017b; Lacerda et al., 2021), behavior and memory (Calado et al., 2023a; Takada et al., 2016) are impaired, causing a delay in the ontogenesis of developmental reflexes (Costa-de-santana et al., 2023). Thus, experimental models have proven to be important tools for elucidating the mechanisms underlying CP; from these, it is possible to investigate potential therapeutic interventions through phenotypic plasticity.

Phenotypic plasticity can be broadly defined as the ability of a genotype to produce different phenotypes in response to environmental conditions leading to changes in shape, state, movement or rate of activity (West-eberhard, 1986; West-eberhard, 2005). Various biotic and abiotic factors can induce phenotypic plasticity such as local environmental factors, chemical, social and hormonal changes (West-eberhard, 1986; Kelly et al., 2012; Turcotte and Levine, 2016). Among these factors, nutritional agents are strongly involved in recovery of CP model impairments, as observed in previous studies (da Silva et al., 2023; Lacerda et al., 2021; Calado et al., 2023b; Visco et al., 2022) and can be a resource to be used in CP during the critical period of development such as the neonatal period. However, current pharmacological and non-pharmacological treatments aim to minimize the limitation of the child's functionality and improve the comorbidities associated with CP but they are not able to reversing primary neuromuscular disorders.

Therapeutic perspectives that act both CNS and skeletal muscle damage have been highlighted such as resveratrol, included within the group of functional foods named polyphenols (Adefegha, 2018). Resveratrol, administered during the critical period of development, has shown promising effects in models of early brain injury and models of muscular atrophy. Resveratrol is known for its neuroprotective and antioxidant effects in several neurological disease models including models with implications for CP (Arteaga et al., 2014; Arteaga et al., 2015; Girbovan and Plamondon, 2015; Pan et al., 2016; Cai et al., 2018). Pre-treatment with resveratrol has been shown to prevent neuronal damage in the dentate gyrus and parietal cortex, reducing infarct volume, preserving myelination and minimizing the astroglial reactive response in a hypoxic-ischemia model (Arteaga et al., 2015). In post-treatment, resveratrol has also shown similar effects including reduction of the expression levels of key inflammatory factors (Pan et al., 2016). In a model of intracerebral hemorrhage, treatment with resveratrol was also reported to be beneficial in reducing neuronal damage in the hippocampus and decreasing the activation of microglia in the cortex (Cai et al., 2018). Further, in a model of cerebral ischemia reperfusion, a reduction in microglial activation was reported in the hippocampus (Girbovan and Plamondon, 2015). Regarding the effects of resveratrol on skeletal muscle, studies have shown the attenuation of denervation-induced muscle atrophy (Asami et al., 2018) and obesity-related muscle atrophy (Bai et al., 2020; Huang et al., 2019).

On the other hand, resveratrol has been highlighted in previous studies of *in vitro* models for its antioxidant effects on the cerebellum as well, directly related to muscular control. Resveratrol showed an anti-apoptotic effect in apoptotic models in rat cerebellar granule neurons (CGNs) (an *in vitro* model of Parkinson's disease) (Alvira et al., 2007), as well as a neuroprotective effect on cerebellar granule neurons in models of ammonia (Bobermin et al., 2015) and ethanol neurotoxicity (Kumar et al., 2011). Resveratrol also appears to act in models of neurological

disease that affect motor performance. Post-treatment with resveratrol evidently improved motor performance as well as muscle activity accompanied by a protection of Purkinje cells in ataxic rats (Ghorbani et al., 2018), in addition to benefits CP model in posture and behavior (Calado et al., 2023a; da Silva et al., 2023).

This makes resveratrol a possible treatment in CP models. Considering that studies on the effects of resveratrol have provided a new window of promising therapeutic action in CP, the present work aimed to investigate the effects of resveratrol neonatal treatment on neurodevelopment, skeletal muscle morphology and cerebellar damage in CP model. The hypothesis of this study was that resveratrol recovers cerebellum and musculoskeletal damage caused by CP model, reversing the damage to motor development.

2. Material and methods

2.1. Animals and experimental groups

This was an experimental study with animals carried out by the Studies in Nutrition and Phenotypic Plasticity Unit, Department of Nutrition, Federal University of Pernambuco, Recife, Pernambuco, Brazil. All procedures were carried out in accordance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA) and the international standards of the National Institute of Health Guide for Care and Use of Laboratory Animals (8th ed.). The project was approved by the Animal Use Ethics Committee (CEUA) of Federal University of Pernambuco n°0009/20.

Male Wistar rat pups were used. They were kept in standard vivarium conditions with a temperature of 22 ± 2 °C, inverted light-dark cycle of 12/12 h (Light cycle - 8:00 p.m. to 8:00 a.m.; Dark cycle - 08:00 to 20:00 h), housed in polypropylene cages (46cmx34cmx20cm) lined with sterile wood shavings with free access to water and a standard vivarium diet. The offspring were obtained from 30 nulliparous Wistar rats. On the day of birth, male puppies were randomized, with an ideal body weight (6 to 8 g), distributed into experimental groups, based on the induction of cerebral palsy and the neonatal pharmacological manipulation administered: Control Saline (CS, $n = 15$), consisting of pups that received saline solution; Control Resveratrol (CR, $n = 15$), consisting of pups that received treatment with resveratrol; CP Saline (CPS, $n = 15$), consisting of pups subjected to the experimental CP model and which received saline solution; 4- CP resveratrol (CPR, $n = 15$), consisting of pups subjected to the experimental CP model and which received resveratrol. Each litter consisted of 8 pups. Half of the pups ($n = 4$) were subjected to the CP model and the other half ($n = 4$) were controls. All litters were composed of the same number of pups with similar levels of motor function to avoid disproportionality between litters in terms of competitiveness for breast milk. Male animals present were distributed according to pharmacological intervention with four groups in each litter. They remained with their mothers until the 25th postnatal day, when they were weaned and the males were placed in individual cages until euthanasia on the 29th postnatal day. Some animals were euthanized by decapitation and others by transcardiac perfusion.

2.2. Cerebral Palsy model

The CP model that was performed is based on experiments that associate perinatal anoxia and sensorimotor restriction of the hind paws (da Silva et al., 2016; Coq et al., 2008; Lacerda et al., 2017b). The pups in the CP groups were subjected to two episodes of postnatal anoxia, on the day of birth and on the first day of life (P0 and P1). The pups were placed inside an acrylic chamber partially immersed in water at 37° and exposed to nitrogen (100%) at 9 L/min for 12 min. Then returned to their respective mothers when their color and respiratory rate was completely restored. From the 2nd to the 28th day of life (P2 to P28), sensorimotor restriction of the hind paws was carried out for 16 h a day, with the animal being allowed free movement for the remaining 8 h. For

sensorimotor restriction, an orthosis was fixed in an epoxy mold, keeping the hind legs extended, without the elimination of urine and fecal material, and maternal care being compromised (Coq et al., 2008).

2.3. Resveratrol treatment

After the birth of the pups, the males were randomly allocated according to the experimental groups. During the neonatal period, from the 3rd to the 21st day of postnatal life, saline or resveratrol were administered intraperitoneally (Girbovan and Plamondon, 2015), were distributed in 1 - saline (0.9% NaCl) or 2- treated with resveratrol (daily dose, 10 mg/kg), injection volume was 0.1 ml/100 g rat weight. The rats were weighed daily and the injection volume was adjusted to match the animal's body weight.

2.4. Assessment of neurodevelopment

2.4.1. Ontogenesis of primitive reflexes

Daily, from the 1st to the 21st postnatal day, primitive reflexes related to neuromotor maturation were evaluated from randomized animals ($n = 10$ per group) (Falcão-Tebas et al., 2012; Fox, 1965). Palmar grasp reflex (PG) was verified by a rapid flexion of the fingers after two light percussions on the front paw palm. The first of a series of three consecutive days in which the expected response disappeared completely was considered the day of PG reflex maturation. Similarly, other primitive reflexes were also assessed, however, the first of a series of three consecutive days in which the expected response appeared completely was considered the day of reflex maturation. The following reflexes were: righting reflex (RR) - turning the body from supine to prone in up to 10 s; vibrissa placing (VP) - reflex of placing the front paws on the table, trying to walk when suspended by the tail within 10 s; cliff avoidance (CA) - 45° angular displacement of the animal in up to 10 s, when the animal is placed with its front paws on the edge of a flat, high surface considered a cliff; auditory startle response (ASR) - simultaneous and rapid retraction with the involuntary immobilization of the animal's body after an acute shock; negative geotaxis (NG) - center of a 45° inclined ramp, the animal with its head in a downward direction turns in up to 10 s and positions its head in an upward direction; free-fall righting (FFR) - the animal's turn in free fall from 30 cm in height, when held by all four paws in dorsal decubitus, resting on four paws on a cotton bed (Falcão-Tebas et al., 2012; Fox, 1965).

2.4.2. Locomotor activity

Randomized animals for locomotor activity were evaluated at the ages of 8, 14, 17, 21 and 28 postnatal days through the automatic evaluation of locomotor parameters using AnyMaze® software (San Diego Instruments). The animals executed their trajectory in a circular open field ($n = 10$ per group). The animals were placed in the center of an open field and recorded by video for a period of 5 min each. The test was performed in a dark room attached to the maintenance vivarium, during the dark cycle, in when the animal was naturally in a state of wakefulness, due to the stress caused by having had application of the drug three hours before. In the interval between filming the different animals, the field was cleaned with 3% hypochlorite and the EVA was changed so that the odor of the previous animal would not influence the test of the next animal. The locomotor parameters obtained were: distance traveled (m); average speed (m/s); time stopped (s); total number of stops made by the animal (n); time spent in the central (zone 1), intermediate (zone 2) and peripheral (zone 3) areas of the open field (s).

2.4.3. Motor coordination assessment

The performance test was carried out with a rotarod apparatus (Insight) at 29 days of postnatal life. Each animal was randomly grouped ($n = 10$ per group) and placed in the rotarod on a rotating rod measuring 60 mm in diameter and 75 mm in length. Initially, the animals underwent an adaptation period on the apparatus for 2 min at a speed of 16

rpm. After waiting for a 2 min rest period, the animals were placed individually on the rotarod for 5 attempts, respecting the 2 min rest interval, with a speed of 25 rpm for a maximum of 3 min so that the fall latency could be recorded (adapted from (Stigger et al., 2011a)). The fall latency analysis was summarized as the average of the 5 attempts performed by each animal.

2.5. Analysis of skeletal muscle

2.5.1. Euthanasia, collection and weight of the soleus and EDL muscles

At 29 days of postnatal life, part of the animals were euthanized by decapitation ($n = 6$ per experimental group). Then, soleus and extensor digitorum longus muscles of the right paws were dissected, weighed in a precision scale (model Marte AUW220, 220 g capacity and 0.1 mg sensitivity). The absolute weight of both muscles and their relative weight were obtained, normalizing the weight of each muscle by body weight. Both muscles were frozen in n-hexane (pre-cooled carbon dioxide solidifies -78.5°C) and stored at -80°C for subsequent histochemical procedures and morphologic analysis.

2.5.2. Morphological analysis of the soleus and EDL muscles

Coronal sections ($8\text{ }\mu\text{m}$) were taken in a cryostat at -30°C , of the soleus and extensor digitorum longus muscles, collected from the right paw on the 29th day of life. The sections were fixed on slides and stained using the myofibrillar ATPase technique after reaching room temperature (Lacerda et al., 2017; Brooke and Kaiser, 1970). Muscle fibers were stained for the three main fiber types (I, IIa, IIb), according to differences in the intensity of ATPase staining after acid pre-incubation (pH 4.3 and 4.55). In pre-incubation at pH 4.3 the fibers were stained type I (darker) and type II (lighter) and at pH 4.55 the fibers were stained type I (darker), type IIa (pale) and type IIb (gray). The sections were analyzed with an optical microscope (Olympus BX-41, $40\times$) connected to a computer with Analysis Get It image capture software. All muscle fibers were counted in each histological section, with the values presented for the different types of fibers as a percentage of the total number. The area and perimeter from muscle fibers were also analyzed, defining the area as the measurement of the surface of each fiber and the perimeter as the sum of the measurements of all sides of each fiber. ImageJ® software (version 1.51p) was used to fiber count and analyze the measure area and perimeter of the fibers.

2.6. Analysis of the cerebellum

2.6.1. Euthanasia and collection of cerebellum

The other part of the animals in the experimental groups ($n = 6$ per experimental group) received an intraperitoneal injection of BrdU (200 mg/kg body weight) at P5 e P6 of postnatal life and were sacrificed at 29 days by transcardiac perfusion, anesthetized and perfused with PBS and a 4% paraformaldehyde solution. Then the brain tissue was dissected, post-fixed, and stored in a cryoprotectant solution. Sections $40\text{ }\mu\text{m}$ thick were collected from cerebellum and subjected to immunohistochemistry (Visco et al., 2022).

2.6.2. Analysis of cell proliferation in the cerebellum

The cerebellum slices were washed in a phosphate-buffered substance (PB) and incubated in Triton (X-100 0.3%, St. Louis, MO, USA) containing 10% hydrogen peroxide. The sections were then incubated in absolute methanol and washed in PB and incubated in formamide (50% in sodium citrate salt solution, Sigma-Aldrich) at 65°C for 2 h. After washing, denaturation of DNA was performed in HCl (1 N) at 37°C . The sections were then incubated in borate buffer solution (pH 8.4) (Ye et al., 2012). The sections were subsequently incubated overnight (4°C) in a solution of primary anti-BrdU antibody (mouse anti-BrdU, 1: 30,000, Roche Molecular). The following day, the sections were washed for 4 to 5 min in PBS and incubated in the dark for 2 h with biotinylated secondary antibody (1: 750, Vector Laboratories) and developed in an

avidin-biotin complex (Elite ABC kit, Vector Laboratories) and diaminobenzidine (DAB staining kit, Vector Laboratories). The cerebellum sections were subsequently washed, dried, and mounted on slides with Cytoseal, and covered with coverslips. Finally, images were captured using a $20\times$ objective optical microscope of 4–5 sections per animal. The number of BrdU + cells in the cerebellum was counted by a blinded researcher for each experimental group (Visco et al., 2022). The boundaries of the Crus 1 and Crus 2 regions of the cerebellum were digitally delineated.

2.6.3. Analysis of microglia density and activated microglia in the cerebellum

The microglia profile was conducted using brain sections incubated first in 10% H₂O₂ in methanol and subsequently in 10% H₂O₂ in phosphate buffer (0.1 M, pH 7.4) containing 3% Triton X-100 (PBT). The sections were then incubated at 4°C for 48 h in primary ionized calcium-binding adapter molecule 1 antibody (Iba1) (rabbit anti-Iba1/ IAF1, 1: 30,000, Wako), diluted with 5% horse serum in PBT (Saavedra et al., 2021). The sections were subsequently incubated in a secondary antibody (biotinylated anti-rabbit; 1:750, Sigma-Aldrich) for a period of 2 h at 4°C . The brain sections were then incubated in solutions of avidin-biotin-peroxidase complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) and a solution of the diaminobenzidine staining kit (DAB Kit; Vector Laboratories) to stain the microglia (Saavedra et al., 2021). Sections from each group were processed in parallel to avoid non-specific staining effects (Visco et al., 2022). The brain sections were then mounted on gelatinized slides and covered with Cytoseal coverslips (Thermo Scientific, USA). Evaluation of Iba1 + microglial cells in the cerebellum ($n = 6$ per experimental group) was conducted by selecting two fields per section from a total of four sections randomly selected by brain section from the Crus 1 and Crus 2 regions, giving a total of eight photographs per area of the cerebellum for each animal. The selected fields were captured using a light microscope (Zeiss, Germany) $20\times$ objective. A blinded researcher used ImageJ software to count the number of cells Iba1-immunoreactive by area and classify the profile of the microglia according to the previous descriptions. Microglial cells with a small soma and few to numerous processes were considered to be branched microglia (types I–III), while those with a large soma or amoeboid body and thicker, shorter processes were considered to be activated microglia (types IV–V) (Saavedra et al., 2021; Roque et al., 2016). The data presented were microglia density (n/mm³—total cells divided by the area assessed, 1000,000. 0.001. 40) and the percentage of microglia activation relative to the total (%) (Visco et al., 2022).

2.7. Statistical analysis

The data obtained were initially analyzed for normal distribution with the Shapiro-Wilk test. If a normal distribution was found, appropriate parametric tests were carried out, such as the Two-Way Anova, to compare groups in analyses, carried out for animals of the same age, or the Two-Way Anova Repeated Measures to compare groups in analyses carried out with animals of different ages. If a normal distribution was not observed, non-parametric statistical tests such as the Kruskal-Wallis and Friedman tests were adopted. The results were expressed as mean $\pm$ standard error of mean or as median and maximum and minimum values, with a significance level of 5%. The GraphPadPrism® version 9 software was used to analyze the data and construct the graphs.

3. Results

3.1. Neonatal treatment with resveratrol reverses the delay in reflex ontogenesis caused by experimental CP

The cerebral palsy model caused changes in developmental reflexes. There were delay in the disappearance of the palmar grasp reflex (CPS: 14.7 ± 0.67 days / CS: 10.4 ± 0.79 days, $p < 0.0001$) and a delay in the

appearance of reflexes of negative geotaxis (CPS: 15.4 ± 0.69 days / CS: 12.7 ± 0.3 days, $p = 0.01$) and free-fall righting (CPS: 15.2 ± 0.53 days / CS: 9.0 ± 0.89 days, $p < 0.0001$) compared to the control group. Treatment with resveratrol was able to reverse this delay in the appearance of negative geotaxis reflexes (CPR: 12.2 ± 0.53 days / CPS: 15.4 ± 0.69 days, $p < 0.01$) and free-fall righting (CPR: 12.2 ± 0.55 days / CPS: 15.2 ± 0.53 days, $p < 0.01$) (Fig. 1). No differences were observed between the groups regarding righting, vibrissa placing, cliff avoidance reflex and auditory startle response ($p > 0.05$) (Fig. 1).

3.2. Neonatal treatment with resveratrol reverses impairments in locomotor development

The experimental CP reduced the locomotor activity of the animals. At 21 and 28 days of postnatal life, the CP group performed shorter distance traveled (P21: CPS: 15.30 ± 2.02 m / CS: 26.76 ± 7.42 m, $p = 0.02$ / P28: CPS: 15.45 ± 2.91 m / CS: 40.57 ± 4.03 m, $p < 0.0001$) and average speed (P21: CPS: 0.51 ± 0.06 m/s / CS: 0.89 ± 0.08 m/s, $p = 0.02$ / P28: CPS: 0.52 ± 0.09 m/s / CS: 1.35 ± 0.13 m/s, $p < 0.0001$) compared to control group (Figs. 2A and B). The time stopped (CPS: 148.46 ± 14.82 s / CS: 52.03 ± 3.44 s, $p < 0.0001$) and the number of stops (CPS: 24.50 ± 1.78 / CS: 12.88 ± 1.64 , $p < 0.001$) was increased in the CP group compared to the control group at 28 days of life (Figs. 2C and D).

The animals' exploratory capacity was also affected by the CP model. Animals in the CPS group had more time spent in zones 1 and 2 (central and intermediate area of the open field) at 17 days of life (Zone 1: CPS: 91.05 ± 29.35 s / CS: 25.94 ± 11.79 s, $p < 0.0001$ / Zone 2: CPS: 86.54 ± 20.70 s / CS: 28.87 ± 10.15 s, $p < 0.0001$), and less time spent in zone 3 (peripheral region of the field) at 14 (CPS: 87.75 ± 25.79 s / CS: 187.78 ± 18.83 s, $p < 0.0001$) and 17 (CPS: 129.36 ± 29.65 s / CS: 243.94 ± 20.63 s, $p = 0.0007$) days of postnatal life compared to control group (Figs. 2E, F and G).

Treatment with Resveratrol was able to reduce damage in locomotion. CPR group showed an increase in the distance traveled (P21: CPR: 27.37 ± 3.50 m / CPS: 15.30 ± 2.02 m, $p < 0.05$ / P28: CPR: 30.34 ± 4.48 m / CPS: 15.45 ± 2.91 m, $p < 0.01$) and average speed (P21: CPR: 0.92 ± 0.12 / CPS: 0.51 ± 0.07 m/s, $p < 0.05$ / P28: CPR: 1.01 ± 0.15 / CPS: 0.52 ± 0.09 m/s, $p < 0.01$) at 21 and 28 compared to CPS (Fig. 2A and B). Consistently, resveratrol in experimental CP led to a decrease in

time stopped (CPR: 89.42 ± 16.76 s / CPS: 148.46 ± 14.82 s, $p < 0.0001$) and the number of stops (CPR: 16.56 ± 2.93 / CPS: 24.50 ± 1.78 , $p < 0.001$) made in the open field at 28 days of life compared to the CPS group (Fig. 2C and D).

Similarly, resveratrol also had positive effects on open field exploratory ability. Animals in the CPR group had less time spent in open field zones 1 and 2 at 17 days of life (Zone 1: CPR: 27.45 ± 6.03 s / CPS: 91.05 ± 29.35 s, $p < 0.05$ / Zone 2: CPR: 32.62 ± 5.25 s / CPS: 86.54 ± 20.70 s, $p < 0.05$), and more time spent in zone 3 at 14 (CPR: 176.87 ± 80.91 s / CPS: 87.75 ± 25.79 s, $p < 0.001$) and 17 (CPR: 256.57 ± 8.51 s / CPS: 129.36 ± 29.65 s, $p < 0.0001$) days of postnatal life compared to CPS group (Figs. 2E, F and G).

Exposure to neonatal resveratrol in control animals also stimulated locomotion in the initial ages of locomotor development but this was not maintained at the other ages evaluated. CR group showed a decrease in the number of stops at 8 days (CR: 17.5 ± 1.79 / CS: 32.70 ± 2.45 , $p < 0.0001$) and greater average speed at 14 days (CR: 0.77 ± 0.07 / CS: 0.39 ± 0.06 m/s, $p < 0.05$) compared to CS group (Figs. 2B and D).

3.3. Motor coordination in a CP model is not influenced after neonatal treatment with resveratrol

Motor coordination was assessed using the rotarod performance test at 29 days of age. We observed a reduction in the animals' ability to stay on the moving rod, thus they reduced their fall latency in the rotarod (CPS: 2.66 ± 0.24 s / CS: 21.37 ± 7.06 s, $p = 0.0114$) (Fig. 3).

3.4. Muscle weight is not influenced after neonatal treatment with resveratrol in a CP model

The animals subjected to CP had absolute weight of the soleus muscles (CPS: 0.0091 ± 0.002 g / CS: 0.0313 ± 0.001 g, $p < 0.0001$) and EDL muscles reduced (CPS: 0.0155 ± 0.002 g / CS: 0.0277 ± 0.001 g, $p < 0.0003$) as well as the relative weight of the soleus muscles (CPS: 0.0002 ± 0.000 g / CS: 0.0004 ± 0.000 g, $p < 0.0001$) at 29 days compared to the control group (Fig. 2A, B and C). This demonstrates that the CP model used in this study caused atrophy of the muscles in the animals' hind limbs. However, there was no significant difference between animals in the CPS and CPR groups, which demonstrated that treatment with resveratrol had not affected muscle mass in this CP model (Fig. 4).

3.5. Neonatal resveratrol treatment reverses damage to distribution pattern of muscle fiber types

Corroborating the reduction in muscle weight, the area and perimeters of the soleus and EDL muscle fibers were reduced in animals subjected to the CP model compared to the control (Soleus area: CPS: $1241 \pm 108.29 \mu\text{m}^2$ / CS: $4058 \pm 382.53 \mu\text{m}^2$, $p < 0.0001$; Solo Perimeter: CPS: $148 \pm 6.08 \mu\text{m}$ / CS: $263 \pm 9.71 \mu\text{m}$, $p < 0.0001$; EDL Area: CPS: $885 \pm 68.62 \mu\text{m}^2$ / CS: $3326 \pm 228.52 \mu\text{m}^2$, $p < 0.0001$; EDL Perimeter: CPS: $122 \pm 3.79 \mu\text{m}$ / CS: $238 \pm 5.10 \mu\text{m}$, $p < 0.0001$). Resveratrol treatment in CP led to an increase in the perimeter of muscle fibers in the EDL muscle compared to the CPS group (CPR: $172 \pm 10.50 \mu\text{m}$ / CPS: $122 \pm 3.79 \mu\text{m}$, $p < 0.0232$) (Fig. 5A, B and G).

The induction of experimental CP also resulted in a distortion of the distribution pattern of muscle fiber types. The CPS group had a lower percentage of type I fibers in the soleus muscle (pH 4.3 CPS: $30.1 \pm 5.40\%$ / CS: $61.6 \pm 2.74\%$, $p = 0.0001$ / pH 4.55 CPS: $26.6 \pm 5.40\%$ / CS: $67.6 \pm 4.23\%$, $p < 0.001$) and a higher percentage of type II fibers (pH 4.3 CPS: $69.9 \pm 5.40\%$ / CS: $38.4 \pm 2.74\%$, $p = 0.0001$ / pH 4.55 fibers IIa CPS: $36.5 \pm 9.05\%$ / CS: $16.4 \pm 3.23\%$, $p = 0.0379$; fibers IIb CPS: $42.1 \pm 2.01\%$ / CS: $16.0 \pm 3.10\%$, $p < 0.0004$) compared to the CS group (Figs. 5C and D). In the EDL muscle, the CPS group showed an increase in type I fibers (pH 4.3 CPS: $29.3 \pm 5.40\%$ / CS: $12.1 \pm 1.37\%$, $p = 0.0006$ / pH 4.55 CPS: $33.8 \pm 4.79\%$ / CS: $12.5 \pm 2.35\%$, $p < 0.001$)

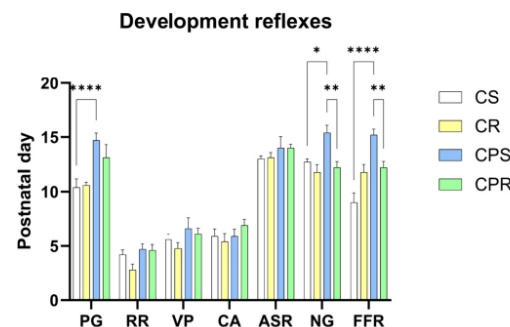


Fig. 1. Effects of neonatal resveratrol treatment on reflex ontogenesis in a CP model. Control Saline (CS, $n = 10$), Control Resveratrol (CR, $n = 10$), Cerebral Palsy + Saline (CPS, $n = 10$) and Cerebral Palsy + Resveratrol (CPR, $n = 10$). Primitive reflex maturation was considered the disappearance of the palmar grasp reflex (PG) and the appearance of righting reflex (RR), vibrissa placing reflex (VP), cliff avoidance (CA), auditory startle response (ASR), negative geotaxis (NG) and free-fall righting (FFR) reflexes. Data were expressed as mean $\pm$ SEM. Two-Way ANOVA and Tukey's post hoc test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

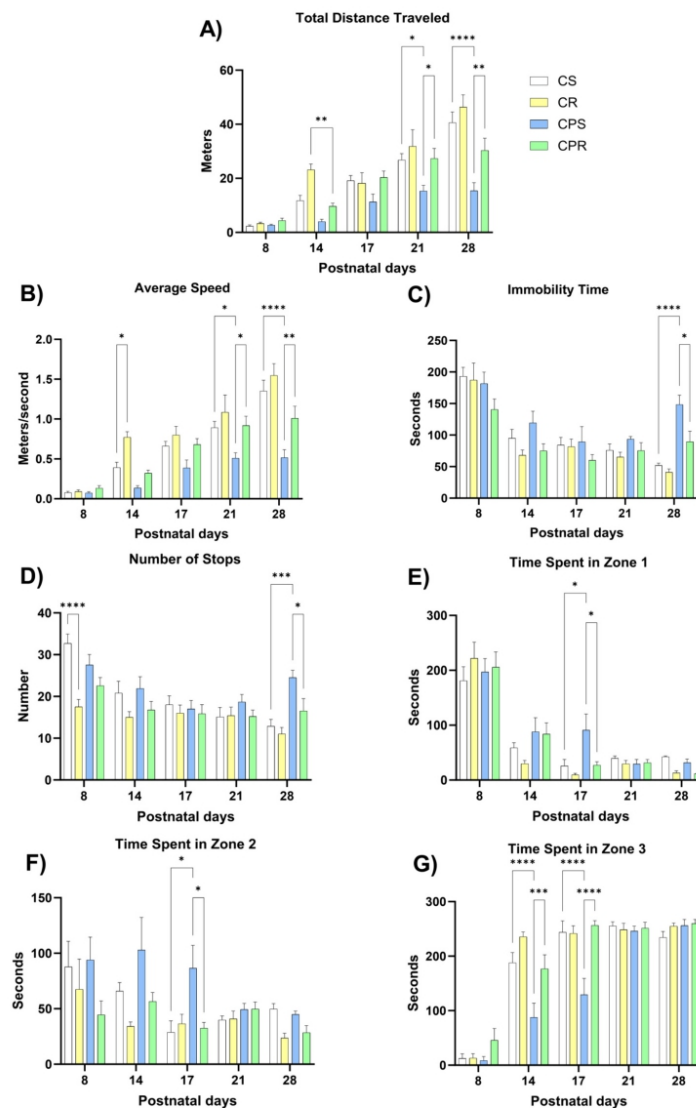


Fig. 2. Effects of neonatal resveratrol treatment on development of locomotor activity in CP model and control animals. Control Saline (CS, $n = 10$), Control Resveratrol (CR, $n = 10$), Cerebral Palsy + Saline (CPS, $n = 10$) and Cerebral Palsy + Resveratrol (CPR, $n = 10$). A) Distance traveled (m); B) Average speed (m/s); C) Immobility Time (s); D) Total number of stops made by the animal (n); E) Time spent in the central area (zone 1), F) Time spent in the intermediate area (zone 2) and G) Time spent in the peripheral area (zone 3) of the open field. Data were expressed as mean $\pm$ SEM. Two-Way ANOVA and Tukey's post hoc test $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

and decrease in type II fibers (pH 4.3 CPS: $70.7 \pm 5.40\%$ / CS: $87.1 \pm 1.65\%$, $p < 0.01$ / pH 4.55 fibers IIa CPS: $29.0 \pm 8.36\%$ / CS: $65.8 \pm 7.34\%$, $p = 0.0088$; fibers IIb CPS: $41.2 \pm 6.03\%$ / CS: $16.3 \pm 3.58\%$, $p = 0.0083$) compared to the CS group (Figs. 5E, F and G).

Treatment with resveratrol led to a reduction in the damage caused by the CP model in the typing of muscle fibers in both muscles. In the soleus muscle, animals in the CPR group compared to the CPS group

showed an increase in type I fibers (pH 4.3 CPR: $56.4 \pm 4.75\%$ / CPS: $30.1 \pm 5.40\%$, $p = 0.0004$ / pH 4.55 CPR: $62.2 \pm 5.13\%$ / CPS: $26.6 \pm 5.40\%$, $p < 0.001$), decrease in type II fibers, specifically type IIb fibers (pH 4.3 CPR: $43.6 \pm 4.75\%$ / CPS: $69.9 \pm 5.40\%$, $p < 0.01$ / pH 4.55 fibers IIb CPR: $14.1 \pm 2.75\%$ / CPS: $42.1 \pm 2.01\%$, $p = 0.0001$) (Figs. 5C and D). In the EDL muscle there was a decrease in type I fibers (pH 4.3 CPR: $17.3 \pm 1.61\%$ / CPS: $29.3 \pm 5.40\%$, $p < 0.05$ / pH 4.55

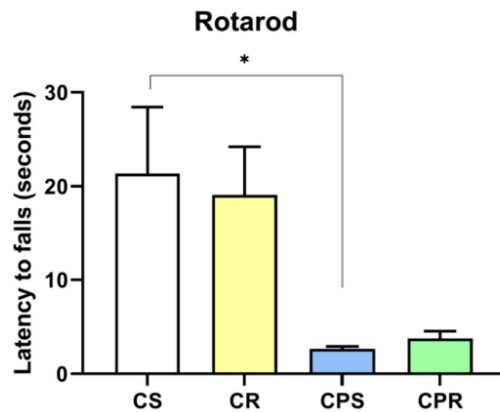


Fig. 3. Effects of experimental CP on motor coordination in rats. Control Saline (CS, $n = 10$), Control Resveratrol (CR, $n = 10$), Cerebral Palsy + Saline (CPS, $n = 10$) and Cerebral Palsy + Resveratrol (CPR, $n = 10$). Data were expressed as mean $\pm$ SEM. Two-Way ANOVA and Tukey's post hoc test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

CPR: $17 \pm 1.72\%$ / CPS: $33.8 \pm 4.79\%$, $p = 0.0032$) and increase in type II fibers (pH 4.3 CPR: $82.7 \pm 1.61\%$ / CPS: $70.7 \pm 5.40\%$, $p < 0.05$ / pH 4.55 fibers IIa CPR: $73.7 \pm 2.50\%$ / CPS: $29.0 \pm 8.36\%$, $p = 0.0012$ / fibers IIb CPR: $9.3 \pm 2.95\%$ / CPS: $41.2 \pm 6.34\%$, $p = 0.00122$) (Figs. 5E, F and G).

3.6. Cell proliferation in the cerebellum is not influenced by the CP model or neonatal resveratrol treatment

Neither the CP model used in the present study nor the treatment with resveratrol influenced cell proliferation in Crus 1 areas (CS: 1264.3

± 100.37 / CR: 1164.84 ± 200.89 / CPS: 960.94 ± 105.05 / CPR: 1291.55 ± 185.54 cells/mm², $p > 0.05$) and Crus 2 (CS: 1009.81 ± 78.64 g / CR: 812 ± 152.51 / CPS: 601.74 ± 127.12 / CPR: 845.69 ± 104.53 cells/mm², $p > 0.05$) of the rat cerebellum as observed by immunohistochemical analysis of BrdU+ cells (Figs. 6A, B and C).

3.7. Neonatal treatment with resveratrol reverses damage in the density of microglia and percentage of activated microglia in the cerebellum

Immunohistochemical analysis of the cerebellum Crus 1 and Crus 2 areas revealed changes in the density of microglia and percentage of activated microglia from experimental groups through the presence of Iba + cells. In the Crus 1 and Crus 2 cerebellum, the density of microglia was increased in rats subjected to CP (Crus 1 CPS: 1157.12 ± 66.68 cells/mm² / CS: 601.27 ± 17.21 cells/mm², $p < 0.0001$ / Raw 2 CPS: 1006.85 ± 50.29 cells/mm² / CS: 560.64 ± 53.80 cells/mm², $p < 0.0001$) (Figs. 7A and B), as well as an increase in the percentage of activated microglia in the CPS group compared to the control (Crus 1 CPS: $57.46 \pm 1.41\%$ / CS: $42.37 \pm 1.66\%$, $p < 0.01$ / Crus 2 CPS: $63.11 \pm 1.58\%$ / CS: $51.24 \pm 3.04\%$, $p < 0.05$) (Figs. 7C, D and E). Thus, the CPS group cerebellum showed signs of neuroinflammation due to elevated density of microglia and the percentage of activated microglia compared to CS group.

Treatment with neonatal resveratrol in CP compared to the CP group treated with saline reduced the density of microglia (Crus 1 CPR: 762.94 ± 51.12 cells/mm² / CPS: 1157.12 ± 66.68 cells/mm², $p < 0.001$ / Crus 2 CPR: 544.08 ± 46.46 cells/mm² / CPS: 1006.85 ± 50.29 cells/mm², $p < 0.0001$) and percentage of activated microglia (Crus 1 CPR: $43.79 \pm 2.41\%$ / CPS: $57.46 \pm 1.41\%$, $p < 0.01$ / Crus 2 CPR: $32.47 \pm 1.71\%$ / CPS: $63.11 \pm 1.58\%$, $p < 0.0001$), demonstrating a reverse of the damage to the Crus 1 and Crus 2 cerebellum caused by the experimental CP (Fig. 7A, B, C, D and E).

Resveratrol also led to changes in the pattern of cerebellar microglia in control animals. The CR group also had increased microglia density in the cerebellum area Crus 1 (Crus 1 CR: 855.58 ± 40.46 cells/mm² / CS: 601.27 ± 17.21 cells/mm², $p < 0.0001$) (Fig. 7A); the percentage of

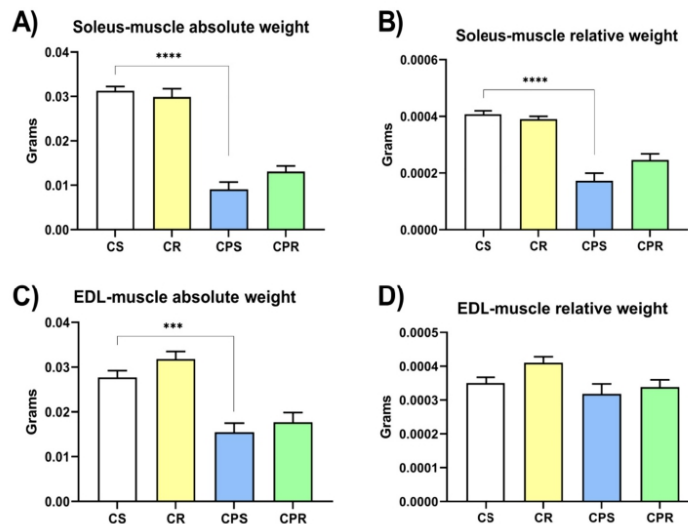


Fig. 4. Effects of experimental CP on soleus and EDL muscle weight in rats. Muscle absolute weight (g) and Muscle relative weight (g) of soleus muscle (A e B) and EDL muscle (C e D). Control Saline (CS, $n = 8$), Control Resveratrol (CR, $n = 8$), Cerebral Palsy + Saline (CPS, $n = 8$) and Cerebral Palsy + Resveratrol (CPR, $n = 8$). Data were expressed as mean $\pm$ SEM. Two-Way ANOVA and Tukey's post hoc test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

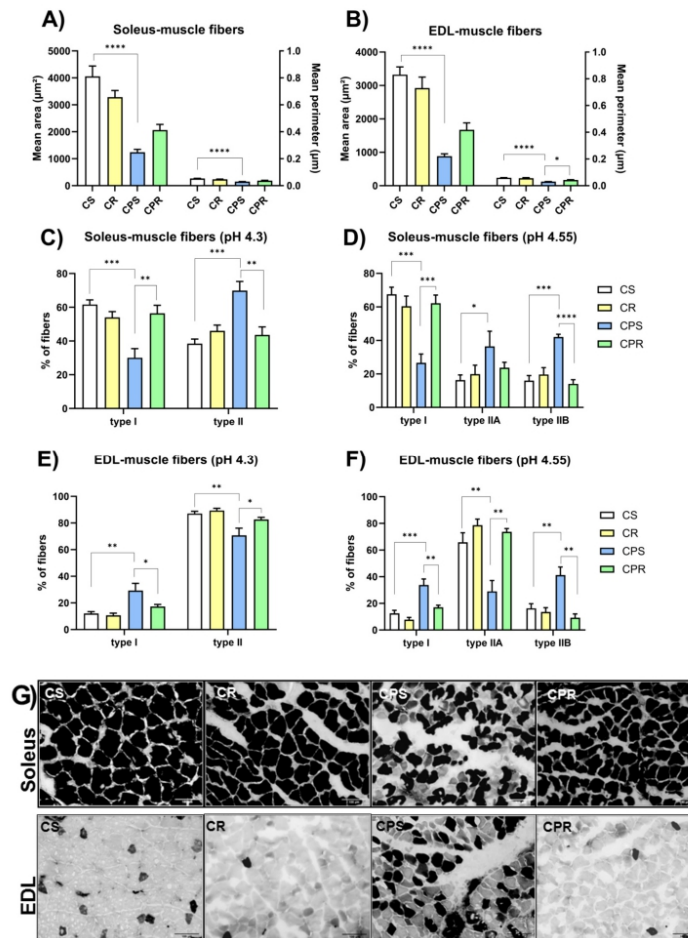


Fig. 5. Effects of neonatal resveratrol treatment on the distribution pattern of muscle fiber types in a CP model. Mean area (μm^2) and perimeter (μm) of soleus muscle (A) and EDL muscle (B); Distribution percentage of fiber types after pre-incubation in a buffer at pH 4.3 (%) in soleus muscle (C) and EDL muscle (D); Distribution percentage of fiber types after pre-incubation in a buffer at pH 4.55 (%) in soleus muscle (E) and EDL muscle (F); (G) Soleus (first row) and EDL (second row) muscle sections stained for myofibrillar ATPase activity after pre-incubation in a buffer at pH 4.55 showing the variability in fiber type content between each experimental group: type I (dark); type IIA (light) and type IIB (gray). Control Saline (CS, $n = 6$), Control Resveratrol (R, $n = 6$), Cerebral Palsy + Saline (CPS, $n = 6$) and Cerebral Palsy + Resveratrol (CPR, $n = 6$). Data were expressed as mean $\pm$ SEM. Two-Way ANOVA and Tukey's post hoc test $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

activated microglia was decreased in Crus area 2 (CR: $35.94 \pm 1.15\%$ / CS: $51.24 \pm 3.04\%$ $p < 0.05$) compared to the saline control group (Fig. 7D).

4. Discussion

The present study investigated the effects of neonatal resveratrol on neurodevelopment, skeletal muscle morphology and cerebellar damage in experimental CP. The CP model caused impairments in motor skills as seen by the delay in the maturation of neuromotor development reflexes, decreased locomotion and motor coordination. The experimental CP led to a reduction in weight, area and perimeter of muscle fibers in the soleus and EDL muscles, a change in the pattern of fiber types also in

both muscles. In the cerebellum, CP increased the density of microglia and the percentage of activated microglia in the areas Crus 1 and Crus 2. Application of neonatal resveratrol showed potential therapeutic effect on the damage caused by the CP model. Resveratrol avoided the delay in the acquisition of neurodevelopment skills with benefits in the ontogenesis of reflexes and locomotion. It restored the phenotype of muscle fibers in the soleus and EDL, and decreased density and activation of cerebellar microglia in rats affect by experimental CP.

As is well established in the literature, CP models lead to impairments in motor development, especially locomotion, corroborating the present study in which we applied a model of combined insults in the perinatal period (da Conceição et al., 2021; Pereira et al., 2021; Strata et al., 2004c; Silva et al., 2016). Neonates exposed to oxygen

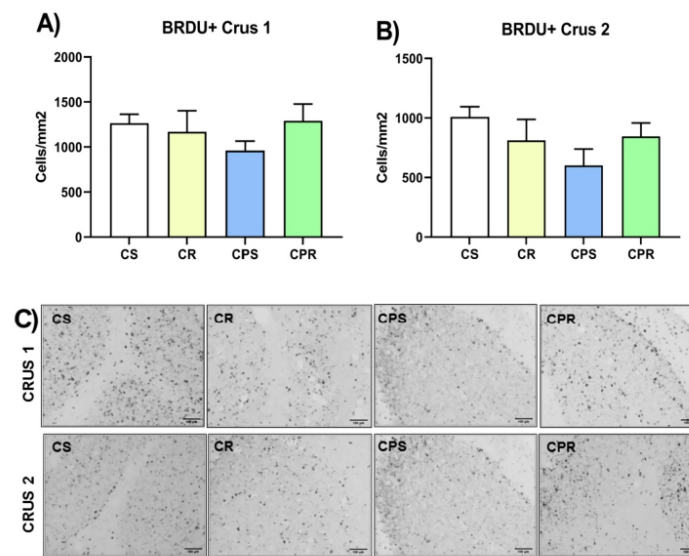


Fig. 6. Effects of the CP model on immunohistochemistry for BrdU+ cells in the cerebellum on the 29th day of postnatal life. BrdU+ cells in Crus 1 (A) and Crus 2 (B) area; (C) Immunohistochemistry for BrdU+ cells in the cerebellum Crus 1 (first row) and Crus 2 (second row) each experimental group. Control Saline (CS, n = 6), Control Resveratrol (CR, n = 6), Cerebral Palsy + Saline (CPS, n = 6) and Cerebral Palsy + Resveratrol (CPR, n = 6). Data were expressed as mean ± SEM. Two-Way ANOVA and Tukey's post hoc test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

deprivation, as occurs in anoxia, developed early brain damage triggered by a diffuse inflammatory response resulting in an increase in pro-inflammatory cytokines such as IL1 in the cerebral cortex and an increase in glial cells but with reversible changes in gait; thus, it is not used in isolation in CP models (Costa-de-santana et al., 2023; Stigger et al., 2013; Marcuzzo et al., 2010). Complementary sensorimotor restriction leads to an increase in the tone of the muscles of the hind limbs of rats associated with restriction in the range of joint movement and distortion in the representation of the hind limbs in the primary motor cortex and in the S1 somatosensory cortex (Strata et al., 2004a; Coq et al., 2008). Thus, the association of these perinatal insults is fundamental in maintaining the rat's motor phenotype similar to diplegic CP in humans where there is predominant involvement of the lower limbs (Strata et al., 2004a; da Conceição et al., 2021; Coq et al., 2008; Stigger et al., 2011b).

Furthermore, the ontogenesis of primitive reflexes may be impaired in experimental CP, contributing to worse locomotor performance and movement coordination (Costa-de-santana et al., 2023). This is what we demonstrated through the adopted model of experimental CP, which caused an average delay of between three and six days in the acquisition of reflexes for the development of negative geotaxis and free fall, respectively, corroborating previous studies with the same CP model (Costa-de-santana et al., 2023; Marcuzzo et al., 2010). This delay in the ontogenesis of primitive reflexes was directly related to the maturation of ideal locomotor milestones for the animal's age. Thus, locomotion was impaired in animals subjected to experimental CP, which showed less ability to explore the peripheral areas of the open field at 14 and 17 days of postnatal life, ages, coinciding with the delay in the appearance of the negative geotaxis reflex and free fall. Consequently, at more advanced ages, CP animals also exhibited reduced ability to generate movement. The animals in the CPS group showed shorter distance covered, lower average speed, increased time stopped and increased number of stops at 21 and 28 days of life, the period of locomotor development when the animal should be reaching the adult gait pattern (da Conceição et al., 2021; Westerga and Gramsbergen, 1990),

reaffirming this relationship between reflex ontogenesis and locomotor activity.

Resveratrol administered during the neonatal period in animals subjected to CP was able to reverse the delay in the appearance of negative geotaxis and free fall reflexes and, consequently, recovered the losses in locomotion, improving exploratory capacity in open field areas and increasing generation of movement. Therefore, resveratrol administered during the neonatal period was deemed to help restore neurodevelopmental impairments, subsequently influencing locomotion and functionality in a CP model. The neonatal period is considered a critical period of brain development in which synaptogenesis and activity-dependent plasticity are at their peak (Jiang and Nardelli, 2016) and the organism has greater susceptibility to environmental agents responding to stimuli imposed by the environment (Jiang and Nardelli, 2016; Chakraborty et al., 2021; Del, 2015). Other authors have already proposed that this critical period may represent a window of opportunity for neuromodulator interventions that are not commonly seen in the adult brain but that can enhance the therapeutic response in the developing brain (Ismail et al., 2017). Therefore, it is suggested that the neonatal period may be the therapeutic window for resveratrol to act, attenuating the damage to neurodevelopment caused by experimental CP.

As we expected, motor coordination was also impaired by the CP model, corroborating previous studies that have used the same model that associates anoxia and sensorimotor restriction as performed on the rotarod test (Pereira et al., 2021; Stigger et al., 2011b) as well in other tests of motor coordination such as Horizontal ladder walking (Marcuzzo et al., 2010), which is similar to what happens with children with CP who present impairments in executive function outcomes (Babik et al., 2023). Motor coordination is a skill that allows broad or refined movements to be carried out based on muscular synergy. Muscular synergy is related to motor control and learning, that is, it depends on the activation of the motor unit resulting in the contraction of specific muscle groups in order to adequately execute the movements required in

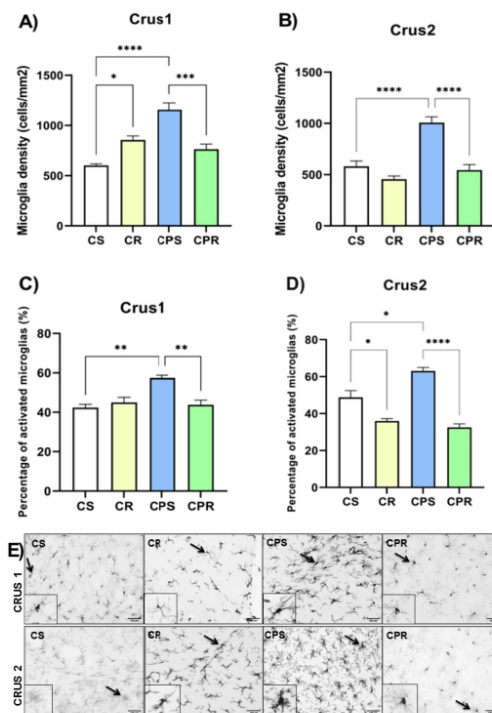


Fig. 7. Effects of neonatal resveratrol treatment on immunohistochemistry for Iba+ cells in the cerebellum in the Crus 1 and Crus 2 areas on the 29th day of postnatal life. Microglia density (cells/mm²) in Crus 1 (A) and Crus 2 (B) area; Percentage of activated microglia (%) in Crus 1 (C) and Crus 2 (D) areas; (E) Immunohistochemistry for Iba+ cells in the cerebellum - black arrows indicate the activated microglia represented in the inset from Crus 1 (first row) and Crus 2 (second row) each experimental group. Control Saline (CS, n = 6), Control Resveratrol (R, n = 6), Cerebral Palsy + Saline (CPS, n = 6) and Cerebral Palsy + Resveratrol (CPR, n = 6). Data were expressed as mean ± SEM. Two-Way ANOVA and Tukey's post hoc test *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

an activity (Singh et al., 2018). Furthermore, sensory information also influences motor control. A previous study has suggested that for the swing-to-stance phase transition, sensory regulation based on foot contact information increases the robustness of locomotion (Aoi and Funato, 2016). Thus, this impairment in motor coordination in animals subjected to CP is justified since the insults carried out in the model lead to changes in neuromotor development. Sensorimotor restriction itself leads to sensory deprivation of the hind limbs, associated with anoxia, leading to topographical disorganization of the S1 cortex foot maps (Coq et al., 2008). Therefore, this understanding of the complexity of recovering motor coordination in CP suggests that multidimensional treatment is often necessary, including, in addition to pharmacological interventions, environmental enrichment and treadmill training (Novak et al., 2020). This may explain why we did not observe significant differences with exclusive pharmacological treatment with resveratrol in CP.

The impairments in motor skills highlighted in this study due to experimental CP are the result of primary damage, such as musculoskeletal and neurological damage. Our research showed that the CP model caused a reduction in muscle weight and in the area and

perimeter of the soleus and EDL muscle fibers, confirming the muscular atrophy that occurs in the animals' hind legs. Muscle atrophy is linked to the fact that sensorimotor restriction is one of the insults of the CP model, similar to that observed in previous studies (Pereira et al., 2021; Marcuzzo et al., 2010; Stigger et al., 2011b). It is already known that muscular atrophy in an experimental model is not restricted only to the peripheral muscles of the hind limbs and can also affect masticatory muscles, influencing their feeding process and consequently causing gain in muscle mass in different regions of the body (da Silva et al., 2016; Pereira et al., 2021; Lacerda et al., 2021; Lacerda et al., 2017c; Lacerda et al., 2017d). Atrophy is a key outcome, quite evident and known in CP, and is important to be evaluated as confirmation of the effectiveness of the model. Furthermore, we also observed in the analysis of microscopic anatomy polymorphic fibers, disorganization of the muscle tissue with spaced fascicles suggesting loss of continuity of the perimysium in CP animals compared to the control. Therefore, in this study we confirmed the similarity between the model used in this study and the muscle fiber phenotype observed in humans when evaluated in three dimensions by Synchrotron X-ray computed tomography (Borg et al., 2019).

Polyphenols have been demonstrated to have a potential preventive effect on musculoskeletal atrophy (Salucci and Falcieri, 2020). Among them, resveratrol has shown benefits in preventing muscle atrophy induced by denervation (Asami et al., 2018) and obesity (Bai et al., 2020; Huang et al., 2019). Studies in the literature, however, are scarce regarding its effects on muscle atrophy induced by early brain injury, as was researched in the present study. We observed that resveratrol did not appear to act directly on muscle mass gain in our CP model, as it did not recover muscle weight or the fiber area of the soleus and EDL muscles, but it was able to reverse the changes in the distribution pattern of the types of muscle fibers resulting from the CP model.

In this context of musculoskeletal changes, the distribution pattern of fiber types was also impaired by experimental CP. The soleus muscle is known for supporting postural maintenance, with a fatigue-resistant characteristic consisting predominantly of type I (slow-twitch) fibers, unlike the EDL muscle, which is made up of type II fibers (fast-twitch) and is less resistant to fatigue during their recruitment (Komiya et al., 2017; Schiaffino and Reggiani, 2011). CP animals showed distortion of this muscle fiber distribution pattern. Type I muscle fibers in the CPS group were decreased in the soleus and increased in the EDL, and type II were increased in the soleus and decreased in the EDL. Neuromuscular changes in the CP model associated with the decrease in muscle activity due to immobilization may justify the changes in the types of soleus and EDL muscle fibers. Motor nerves have a high potential to influence the composition and properties of the functional elements of the muscle fiber, with the reinnervation mechanism being a key outcome for muscle plasticity (Pette, 2001; Bassel-duby and Olson, 2006). When the slow-twitch soleus muscle became reinnervated by nerve fibers normally supplying the fast-twitch flexor digitorum longus muscle contractile speed increased (Pette, 2001). When the fast-twitch muscle was reinnervated with the soleus nerve it became slower contracting (Pette, 2001). Therefore, distortion in the pattern of muscle fiber typing may be a form of muscular adaptation to the current functional demand. It may be related to the limited resistance to fatigue seen by the lower capacity for locomotion and motor coordination in the present study. Our result corroborates other studies that have used the same CP model; chewing muscles were evaluated, which showed distortion in the pattern of muscle fiber typing (Lacerda et al., 2021; Lacerda et al., 2017c) and reinforcement of the global impairment of the musculoskeletal system to the detriment of the perinatal insult.

In the soleus muscle, treatment with resveratrol restored the percentage of type I fibers, also called slow-twitch, in CP animals, corroborating a study that used an intrauterine growth delayed pig model (Cheng et al., 2020). This may be related to the role of resveratrol in the AMPK/SIRT1/PGC-1α signaling pathway. In bovine myotubes, protein and gene expression of AMPK, SIRT1 and PGC-1α were upregulated by resveratrol; these are proteins involved in metabolic homeostasis,

including protein synthesis that might promote muscle fiber type transition from fast-twitch to slow-twitch as observed in a previous study with bovine myotubes (Zhang et al., 2023). Similarly, in porcine myotubes and mouse C2C12 AMPK, SIRT1 and PGC-1 α were also increased with resveratrol treatment and inhibited the expression of miR-22-3p (Wen et al., 2022), suggesting that resveratrol regulates muscle fiber type gene expression through the AMPK signaling pathway and miR-22-3p. However more studies are needed to clarify the mechanisms involved in the differentiation of muscle fibers in experimental CP.

In the EDL muscle, resveratrol restored the fiber typing pattern expected for this muscle; that is, it increased the percentage of type II fibers, also called fast-twitch. Our results raise the hypothesis that the action of resveratrol goes beyond the AMPK signaling pathway and possibly its antioxidant effects may be the mechanism of action of resveratrol on EDL. In diabetic rats, fast-twitch muscle incurs more oxidative stress (Chang et al., 2014), suggesting that fast-twitch muscle fibers may be more susceptible to oxidative stress than slow-twitch muscle fibers (Nonaka et al., 2014). However, further investigation is needed regarding the mechanisms of action of resveratrol on fast-twitch muscle fibers in a CP model.

Damage to the musculoskeletal system was not solely responsible for the impairments in motor and behavioral skills resulting from the CP model. It is known that the central nervous system is damaged by insults in early life (Cioni et al., 2011; Bersani et al., 2020). The brain that undergoes a prenatal CP model is impacted by losses in neurogenesis and curbs generation of neural stem cells, whereas postnatal models show increased proliferation of neural precursor cells, improper migration, and reduced survival of new neurons (Visco et al., 2021). Thus, we observed in our study that cell proliferation in the cerebellum was not influenced by our postnatal CP model since BrdU+ cells in the Crus I and Crus 2 areas of the cerebellum did not change. However, studies with a CP model using anoxia associated or not with sensorimotor restriction showed that other areas may present damage to neurogenesis such as the hippocampus (Calado et al., 2023a; Takada et al., 2016) and may even benefit from treatment with resveratrol (Calado et al., 2023a). Thus, the literature reports heterogeneous outcomes, but also there is a scarcity of studies showing the effects of resveratrol on the cerebellum. With human subjects, an analysis through neuroimaging examinations of cerebellar lesions in neonates after early brain injury is unclear and often underestimated, requiring further studies in the cerebellar histopathological area (Chalak, 2021; Annink et al., 2021). This makes us hypothesize that the cerebellum may present a different pattern of response to perinatal injury.

Although the cerebellum did not change its neurogenesis due to the experimental CP, it was sensitive to changes in microglia. In a previous study, motor deficits in experimental CP were seen to be associated with the activation of glial cells in the cerebellum, corroborating our study (Costa-de-santana et al., 2023). We showed that there was an increase in the density of microglia and an increase in the percentage of activated microglia in animals subjected to the CP model, corroborating studies that showed that insults in the developing brain (prenatal or early life) provoke the microglial defense response, increasing its density in the hippocampus (Costa-de-santana et al., 2023; Orso et al., 2023) and in the cerebellum (Costa-de-santana et al., 2023). Microglia are primary innate immune cells of the brain that trigger the release of cytokines and chemokines and have phagocytic action on dead neurons. Their sustained activation can be harmful to the developing nervous system (Orso et al., 2023; De Pablos et al., 2014; Lenz and Nelson, 2018). Thus, after oxygen deprivation, as occurred in perinatal anoxia in our CP model, there was intense activation of microglia leading to the production of mitochondrial free radicals and brain injuries due to excitotoxicity (Costa-de-santana et al., 2023), confirming our result.

Resveratrol is known to act as a neuroprotector with effects on neuroinflammation and antioxidants in models of insults to the nervous system (Miguel et al., 2021; Zhou et al., 2014). A similar action was observed in the present study, as the resveratrol reversed the changes in

microglia density and percentage of activated microglia in those animals subjected to the CP model. Considering that brain neuroplasticity is modulated during postnatal development (Kourosh-Arabi et al., 2021), we have presented an original therapeutic potential of neonatal resveratrol in experimental CP. We show that resveratrol can contribute to the control of neuroinflammation with a consequent reduction in neurodevelopmental damage.

The proposed neonatal treatment with resveratrol at a dose of 10 mg/kg proved to be sufficient to promote benefits in the acquisition of important skills for the animals' motor development and to promote changes in the muscle phenotype and microglial activation. It did not demonstrate adverse effects beyond what was already expected with this route of administration, such as discomfort during intraperitoneal application. However, in the present study we can highlight as limitations the exclusive period of treatment that was carried out, only during the neonatal period, while other studies have demonstrated preventive benefits of pre-insult resveratrol (Arteaga et al., 2015; Zhou et al., 2014). Therefore, new studies are suggested in this therapeutic approach with resveratrol comparing prenatal treatment in pregnant rodent and postnatal care, and even combining resveratrol with other interventions such as environmental enrichment to gain motor coordination. Furthermore, we showed the effects of resveratrol in the short and medium term, suggesting that future research into the long-term effects of resveratrol in adult or elderly animals is also required.

5. Conclusion

A CP model in neonatal rats caused neurodevelopment impairments observed by deficiency in locomotor activity and motor coordination similar to CP in humans, associated with delay in the acquisition of developmental reflexes, muscle atrophy, change in muscle fiber typing pattern and microglia density in the cerebellum. Treatment with resveratrol was shown to act on neurodevelopmental impairments caused by this experimental CP, benefiting the acquisition of motor skills such as locomotion, by recovering the muscle fibers morphology and reversing neuroinflammation in the cerebellum.

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CRediT authorship contribution statement

Sabrina da Conceição Pereira: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Raul Manhães-de-Castro:** Writing – review & editing, Software, Resources, Project administration, Funding acquisition, Conceptualization. **Vanessa da Silva Souza:** Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Caio Matheus Santos da Silva Calado:** Writing – original draft, Supervision, Methodology, Investigation, Formal analysis. **Beatriz Souza de Silveira:** Writing – original draft, Supervision, Project administration, Methodology, Formal analysis. **Leticia Nicoly Ferreira Barbosa:** Writing – original draft, Visualization, Supervision, Methodology, Formal analysis. **Luz Törner:** Writing – review & editing, Validation,

Supervision, Conceptualization. **Omar Guzmán-Quevedo:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition. **Ana Elisa Toscano:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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APÊNDICE D – NEONATAL TREATMENT WITH RESVERATROL DECREASES POSTURAL AND STRENGTH IMPAIRMENTS AND IMPROVES MITOCHONDRIAL FUNCTION IN THE SOMATOSENSORY CORTEX RATS SUBMITTED TO CEREBRAL PALSY

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Neonatal treatment with resveratrol decreases postural and strength impairments and improves mitochondrial function in the somatosensory cortex rats submitted to cerebral palsy

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ABSTRACT

Cerebral palsy is a neurodevelopmental disease characterized by postural, motor, and cognitive disorders, being one of the main causes of physical and intellectual disability in childhood. To minimize functional impairments, the use of resveratrol as a therapeutic strategy is highlighted due to its neuroprotective and antioxidant effects in different regions of the brain. Thus, this study aimed to investigate the effects of neonatal treatment with resveratrol on postural development, motor function, oxidative balance, and mitochondrial biogenesis in the brain of rats submitted to a cerebral palsy model. Neonatal treatment with resveratrol attenuated deficits in somatic growth, postural development, and muscle strength in rats submitted to cerebral palsy. Related to oxidative balance, resveratrol in cerebral palsy decreased the levels of MDA and carbonyls. Related to mitochondrial biogenesis, was observed in animals with cerebral palsy treated with resveratrol, an increase in mRNA levels of TFAM, in association with the increase of citrate synthase activity. The data demonstrated a promising effect of neonatal resveratrol treatment, improving postural and muscle deficits induced by cerebral palsy. These findings were associated with improvements in oxidative balance and mitochondrial biogenesis in the brain of rats submitted to cerebral palsy.

1. Introduction

Cerebral palsy (CP) is a heterogeneous group of diseases that result from non-progressive damage to the brain at an early stage of development. CP symptoms include irregular movement and irregularities in posture (Hadders-algra, 2014). According to the definition, the damage is not progressive, but the general condition can change over the years due to treatment and the plasticity of the maturing nervous system

(Młodawski et al., 2019). People with CP often perform poorly in activities of daily living due to limited limb, trunk, and head control (Velasco et al., 2017), have abnormal muscle tone and motor control, which contributes to the loss of postural control and motor coordination, compromising functionality and, consequently, quality of life (McNish et al., 2019).

CP is the most common motor development disorder in children (McIntyre et al., 2012), with an incidence of 3,4 per 1000 live births in regions from low- and middle-income countries and 1,5 per 1000 live

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Abbreviations

CP	Cerebral Palsy
ROS	Reactive Oxygen Species
RES	Resveratrol
TBA	Thiobarbituric Acid
MDA	Malondialdehyde
TCA	Trichloroacetic Acid
DNPH	Dinitrophenylhydrazine
SOD	Superoxide dismutase
CAT	Catalase
GST	Glutathione-S-Transferase
GSH/GSSG	Glutathione/Oxidized Glutathione ratio
OPT	o-phthalaldehyde
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
β 2M	β -2 microglobulin
RT-PCR	Real-time polymerase chain reaction
LPS	liposaccharide
OS	oxidative stress
PGC-1 α	Peroxisome proliferator-activated receptor-gamma coactivator-1 α
TFAM	transcription factor A

births in regions from high-income countries (McIntyre et al., 2022). The etiology of CP is diverse and multifactorial, and the risk factors are found in the periods before and around the time of conception, and up to 2 years of age (McIntyre et al., 2022; Pakula et al., 2009). The following risk factors are described as associated with CP: genetic variants, congenital anomalies, neonatal respiratory distress syndrome, meconium aspiration, birth asphyxia, kernicterus, hypoxic-ischemia, and cerebrovascular insults during pregnancy and in infancy, and accidental and non-accidental brain injury (McIntyre et al., 2012, 2022). Among these factors, the mechanisms that cause oxygen and nutrient deprivation to the central nervous system stand out, such as perinatal asphyxia (Hakobyan et al., 2019; Perlman and Shah, 2011), which are associated with the severe neurofunctional consequences of CP (Low and Roland, 2004; Rainaldi and Perlman, 2016; Vannucci et al., 2004; Volpe, 2001).

Oxidative stress (OS) is considered a major contributor to ischemic brain injury (Warner et al., 2004) because it is an important consequence of neurotransmitter-mediated toxicity in combination with hyperoxygenation after hypoxia-ischemia (Ferriero, 2001). The generation of ROS and consequent oxidative stress and mitochondrial dysfunction is related to the onset of neuronal injury, leading to neurodegeneration (Manzanero et al., 2013; Sun et al., 2022). The effects of OS are observed in the pathogenesis of different diseases that affect the brain, such as mitochondrial disorders, cerebral ischemia, epilepsy, and cerebral palsy (Ayiccek and Iscan, 2006). Understanding the role of OS in cerebral palsy is important as therapy with agents that increase the body's antioxidant capacity may be a treatment option for preventing the neurological insult (Ayiccek and Iscan, 2006).

In this context, experimental models emerge as tools that mimic the pathophysiological mechanisms of CP and its motor disorders similar to those that occur in humans (Lacerda et al., 2017; Pereira et al., 2021a; Pereira et al., 2021b). In this sense, the model that associates perinatal anoxia and sensory-motor restriction of the lower limb reproduces the motor deficits found in children with cerebral palsy, such as reduced locomotor activity (Marcuzzo et al., 2010; Pereira et al., 2021a), reduced sarcomere density (Stigger et al., 2011), changes in oral functions (Lacerda et al., 2017), in addition to impairments in motor performance (Marcuzzo et al., 2010).

Through experimental models, early therapeutic perspectives may emerge for the treatment of CP based on phenotypic plasticity. Phenotypic plasticity is defined as 'the ability of individual genotypes to

produce different phenotypes when exposed to different environmental conditions (Pigliucci et al., 2006). This includes the ability of neural synapses and brain pathways to be modified by altered thoughts and emotions, as well as environmental, behavioral, and neural stimuli, thus enabling neural plasticity (Kourosh-Armani et al., 2021). Studies with polyphenols have shown that the consumption of these natural compounds can improve neural plasticity (Bensalem et al., 2018) and resveratrol (RES), a polyphenolic compound present in many plant species (Peng et al., 2022; Song et al., 2014), improves neuroplasticity through a variety of mechanisms (Peng et al., 2022). It is important to highlight the importance of interventions due to their ability to modulate neuronal activity, generating adaptive responses that can cause normal differentiation of motor neurons and consequent ideal neuromuscular performance in adulthood (Stigger et al., 2011).

Several researches have indicated that the use of neuroprotective drugs can improve the prognostic perspectives for patients with cerebral palsy (Sun et al., 2021) and interventions on the central nervous system, mainly in early life, have been suggested as therapies aimed at reducing oxidative stress by interrupting the lesion cascade (Juil and Ferriero, 2014; Visco et al., 2022). Resveratrol has received considerable attention recently for its strong brain protection (Peng et al., 2022). RES is a substance that has shown effective treatment of a wide range of pathologies, including neurodevelopmental disorders and neurocognitive disorders (Baur and Sinclair, 2006). Resveratrol is among the polyphenols that are being used in acute central nervous system injury (Peng et al., 2022) and hypoxia-ischemia models as a neuroprotective strategy and (Juil and Ferriero, 2014; Ortega et al., 2014; Pan et al., 2016). Several lines of evidence have also demonstrated its antioxidant, anti-inflammatory and anti-aggregating properties (Jing et al., 2013; Marques et al., 2009; Pandey and Rizvi, 2009). In addition, a study on early intervention with RES in hypoxia-ischemia brain damage was reported (Karalis et al., 2011), however, the outcomes evaluated in this study have not yet been explored in the literature. Thus, this study aims to investigate the effects of neonatal treatment with resveratrol on postnatal development, motor function, and oxidative balance in the brain of rats submitted to a cerebral palsy model through neonatal anoxia and sensorimotor restriction.

2. Methodology

2.1. Animals

This study was performed by the guidelines of the National Council for the Control of Animal Experiments (CONCEA) and with the international standards of the National Institute of Health Guide for Care and Use of Laboratory Animals (8th ed) and has been approved by the Ethics Committee for Animal Use (CEUA) of the Federal University of Pernambuco (process number CEUA: 0032/2021). Twenty-one female *Wistar* rats were mated with breeding males in the proportion of two females to one male in a 12h dark-light-cycle (dark 8 a.m.-8 p.m.) at 22 $\pm$ 2 °C with free access to water and diet. On the day of birth, male pups were randomly assigned with an ideal body weight (6–8g) and they were divided into four groups, based on resveratrol manipulation and cerebral palsy induction: 1- saline control (CS, n = 12), consisting of pups that received a saline solution from the 3rd to the 21st day of life; 2- resveratrol control (CR, n = 11), consisting of pups that received resveratrol from the 3rd to the 21st day of life; 3- CP salina (CPS, n = 11), constituted by pups submitted to the experimental model of CP and that received a saline solution from the 3rd to the 21st day of life; 4- CP resveratrol (CPR, n = 12), constituted by pups submitted to the experimental model of CP and that received resveratrol from the 3rd to the 21st day of life. Each litter consisted of 8 pups that remained with their mothers until the 25th postnatal day (P25) when they were weaned, and the males were placed in individual cages until euthanasia by decapitation at P29.

2.2. Experimental model of cerebral palsy

The experimental CP model used was the same as described by [Strata et al. \(2004\)](#), [Coq et al. \(2008\)](#), [Lacerda et al. \(2017\)](#), and [Pereira \(2021a\)](#) which associates perinatal anoxia with sensorimotor restriction of the hind paws. The pups in the CP groups underwent two episodes of postnatal anoxia, on the day of birth and the first day of life (P0 and P1). Pups were placed inside an acrylic chamber partially immersed in water at 37 °C and exposed to nitrogen (100%) at 9L/min for 12 min, then recovered in air and at room temperature and returned to their respective mothers. From P2 to P28 sensorimotor restriction of the hind paws was performed for 16 h a day, with the animal being allowed free movement for the remaining 8 h. For the sensorimotor restriction, an orthosis made with an epoxy mold was used, leaving the hind legs extended, without affecting the elimination of urine and feces and maternal care ([Strata et al., 2004](#)).

2.3. Administration of resveratrol

After the birth of the animals, the male offspring were randomly allocated according to the experimental groups in the neonatal period, and the drug was administered intraperitoneally from P3 to P21 ([Giribovan and Plamondon, 2015](#)). The animals were divided into 1- treated with resveratrol (daily dose, 10 mg/kg) and 2-saline (0.9% NaCl), injection volume was 0.1 ml/100g rat weight). Rats were weighed daily, and the injection volume was adjusted to match the animal's body weight.

2.4. Weight evolution, somatic growth, and maturation of physical features

The weight of the animals was recorded daily from the day of birth to P29. For this, a Marte scale, models S-100, capacity of 1 kg and sensitivity of 0.01 g was used.

The somatic growth and physical features were evaluated daily between 7:00 and 9:00h until maturation. For the somatic growth, the animals were evaluated with a digital caliper (JOMARCA®), for the following measurements, in centimeters ([H. J. da Silva et al., 2005](#)): lateral skull axis (distance between the ear holes), anteroposterior skull axis (distance between snout and head-neck articulation), tail length (distance from tail tip to tail base) and longitudinal axis (distance between snout and tail base).

The age at the maturation of a particular physical feature was defined as the day when it was first observed ([Smart and Dobbing, 1971](#)). The following features were evaluated: auricle opening, auditory conduit opening, eyes opening, upper incisors eruption, and lower incisors eruption.

2.5. Gait analysis on catwalk

Gait analysis was performed using the Catwalk (Noldus), as described by [Herold et al. \(2016\)](#), at P28. Changes in the components of the gait cycle are determined and deficits in locomotion are quantified. The CatWalk System consists of an enclosed walkway (glass plate) that is illuminated by fluorescent light. The system is equipped with a high-speed color camera connected to a computer with the appropriate detection software (CatwalkXT9.1), which can detect various static and dynamic parameters during the rat's spontaneous locomotion ([Fig. 1](#)). In this way, the animal is positioned in a 1-m-diameter corridor and individually filmed as it crosses the Catwalk to measure static and dynamic parameters of spontaneous locomotion.

2.6. Forelimb grip strength and postural analysis

Muscle strength analysis was performed using the forelimb grip strength test at P22 and P28. Each animal grabbed the support bar,

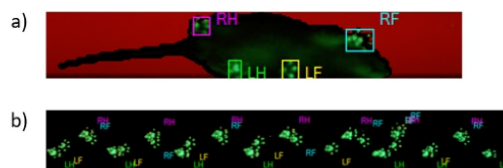


Fig. 1. Evaluation of static and dynamic gait parameters during animal's locomotion by the Catwalk's system. A – Rat walking through the platform. B - Representative images of paw prints during active locomotion of the animals on the platform. LF, left front paw; LH, left hind paw; RF, right front paw; RH, right hind paw.

exerting a traction force on it in a vertical position while suspended by the tail. The force sensor quantifies the peak force of each animal (g). This test was performed on the Animal Grip Strength System (SD Instruments) equipment with a capacity of 200Kgf, resolution 0.1Kgf, and accuracy of $\pm 0.2\%$ ([Takeshita et al., 2017](#)).

For the postural analysis, the animals were submitted to the adapted forelimb grip test and evaluated in a horizontal position, at P14, P21 and P28 for the analysis of postural alignment. The performance of the animals during the test was recorded using video. After that, two images of the corresponding video were captured, an image after the initial 5 s of filming, when the animal was still, and another image before the final 5 s, in the same way. We thus determined two moments of analysis to obtain an average of each animal at the ages studied. The parameters evaluated were head and trunk alignment in the horizontal posture. To evaluate the head alignment, an angle will be delimited using the tragus, the occipito-cervical transition, and the midpoint in the transition between the head and the belly of the animal as reference points. To assess trunk alignment, another angle will be defined, the reference points being the transition between the back and tail, the midpoint on the animal's back, and the occipito-cervical transition (adapted from [Lelard et al., 2006](#)).

2.7. Extraction, tissue preparation, and protein quantification

At P29, the animals were euthanized by decapitation. The somatosensory cortex (here from now just mention as sample) was removed and kept at $-80\text{ }^{\circ}\text{C}$ and prepared for RT-PCR assays and biochemical analysis. The sample was homogenized in cold extraction buffer (100 mM Tris base, pH 7.5; 10 mM EDTA; containing a cocktail of protease inhibitors). After homogenization, samples were centrifuged at $4\text{ }^{\circ}\text{C}$ at $1180\times g$ for 5 min, and supernatants were used for protein quantification according to the Bradford method (BRADFORD, 1976).

2.8. Measurement of citrate synthase activity

Citrate synthase is the first enzyme in the Krebs cycle, its activity capacity is widely used as a marker of cellular and mitochondrial function. Citrate synthase activity was evaluated as previously described ([Patel, 1976](#)). Briefly, the reaction was carried out in a mixture containing Tris-HCl (pH = 8.2), magnesium chloride (MgCl), ethylenediamine-tetra-acetic acid (EDTA), 0.2 of 5.5 dithiobis (2-nitrobenzoic acid) ($E = 13.6\text{ }\mu\text{mol}/(\text{mL}\cdot\text{cm})$), 3 acetyl CoA, 5 oxaloacetate and 0.1 mg/mL of sample. The activity was evaluated by measuring the change in the absorbance rate at 412 nm for 3 min at a temperature of $25\text{ }^{\circ}\text{C}$, using the EVEN UV-VIS spectrophotometer. The levels are expressed as U/mg of protein.

2.9. Oxidative stress biomarkers

- Evaluation of malondialdehyde production

0.2 mg/ml of protein sample was used to measure the thiobarbituric acid (TBA). In this assay, malondialdehyde (MDA) or MDA-like substances produce a pink pigment with maximum absorption at 535 nm. The reaction was carried out using 30% trichloroacetic acid (TCA) and Tris-HCl (3 mM), followed by centrifugation at $2500 \times g$ for 10 min. Then, the supernatant was transferred to a tube, mixed with the same volume of 0.8% TBA (v/v), and boiled for 30 min. The absorbance of the organic phase was read at 535 nm in a spectrophotometer, and the results were expressed as mmol per mg of protein (Buege and Aust, 1978).

3. Evaluation of protein oxidation

The carbonyl content is the primary marker for oxidative damage to protein, measured as previously published (Reznick and Packer, 1994). Briefly, 30% TCA was added to the sample (0.2 mg/ml of protein) on ice, mixed, and centrifuged for 15 min at $1180 \times g$. The pellet was suspended in 10 mM 2,4-dinitrophenylhydrazine (DNPH) and immediately incubated in a dark room for 1 h with shaking every 15 min. Then, samples were centrifuged, washed three times with ethyl/acetate buffer, and the pellet suspended in 6 M guanidine hydrochloride, followed by incubation for 5 min in a water bath at 30 °C. Absorbance was read up to 370 nm, and the results were expressed as mmol per mg of protein.

3.1. Enzymatic antioxidant defense

3.1.1. - Measurement of superoxide dismutase (SOD) activity

The total superoxide dismutase enzyme activity (t-SOD) was determined according to the previously described method (Misra and Fridovich, 1972). Sample (0.2 mg/ml) were incubated with 880 μ l sodium carbonate (0.05%, pH 10.2, 0.1 mM EDTA) at 25 °C, and the reaction started by 30 mM epinephrine (in 0.05% acetic acid). The kinetics of the inhibition of adrenalin auto-oxidation was monitored for 180 s at 480 nm, and the result was expressed as U/mg protein.

3.1.2. Measurement of catalase (CAT) activity

CAT activity was measured according to the method described by Aebi (Aebi, 1984). The assay consisted of 50 mM-phosphate buffers (pH 7.0), 0.300 mM H₂O₂ and 0.3 mg/mL of sample. The constant rate of the enzyme was determined by measuring the absorbance change at 240 nm for 4 min at 25 °C. CAT activity was expressed as U/mg protein.

- Measurement of Glutathione-S-Transferase (GST) activity

GST activity was measured as described previously (Habig et al., 1974). Two hundred micrograms of the sample were added to 0.1 M-phosphate buffer (pH 6.5) containing one mM-EDTA at 25 °C. The assay was initiated with 1 mM of 1-chloro-2,4-dinitrobenzene plus 1 mM-GSH. The formation of 2,4-dinitrophenyl-S-glutathione was monitored at 340 nm of absorbance, and the enzymatic activity was defined as the amount of protein required to catalyze the formation of 1 μ mol 2,4-dinitrophenyl S-glutathione. The results were expressed as U/mg protein.

3.2. Non-enzymatic defense

- REDOX State

Reduced Glutathione/Oxidized Glutathione ratio (GSH/GSSG) was evaluated as previously described by (Hissin and Hilf, 1976). The samples were incubated in a 0.1M phosphate buffer containing 5 mM-EDTA (pH 8.0) and with 1 μ g/ml o-phthalaldehyde (OPT) at room temperature (RT) for 15 min and evaluated by fluorescence with wavelengths of 350 nm and 420 nm. GSSG levels were assessed by incubating the same samples with 40 mM N-ethylmaleimide for 30 min in RT with the addition of a 100 mM NaOH buffer. The same steps of the GSH assay were followed to determine the GSSG levels. The ratio of GSH/GSSG

determined the REDOX state.

- Quantification of total thiol groups

The quantification of sulfhydryls was based on the reduction of 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB). The sample was mixed in a solution containing Tris-EDTA buffer (pH 7.4), with 10 mM of DTNB and incubated at room temperature for 30 min. The absorbance was measured at 412 nm and results are expressed as nmol/mg protein.

3.3. mRNA evaluation

Total RNA was extracted from cortex tissue using TRIzol reagent and the guanidine isothiocyanate method according to the manufacturer's instructions (Invitrogen, USA). RNA quantification was performed in a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and purity was assessed using the ratio of 260/280 nm. Real-time polymerase chain reaction (RT-PCR) experiments were performed using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, USA). All genes used in the experiment were processed in duplicate, and the cycle threshold (Ct) value of each targeted gene was normalized to the β -2 microglobulin (β 2M), and data expressed as $2^{-\Delta\Delta Ct}$ (Silva et al., 2023) (see Table 1).

3.4. Statistical analysis

For data analysis and graph construction we used the statistical software GraphPadPrism® version 9 and the results were expressed as mean $\pm$ standard error. A significance level of 95% was assigned. The Kolmogorov-Smirnov normality test was performed. As the normal distribution, for intergroup comparison, the ANOVA two-way parametric test was used with experimental cerebral palsy and pharmacological manipulation, followed by the Tukey post-test. For the variables in which the analysis was performed at several ages, the ANOVA two-way repeated measures followed by the Tukey post-test was used.

4. Results

4.1. Weight evolution, somatic growth and maturation of physical features

Fig. 2 presents the weight evolution of pups. The animals in the CPS group had lower body weight compared to the control saline group, from P8 to the last day of the experiment (P8: CPS: $12.45g \pm 2.57g/CS$: $18.79g \pm 1.52g$, $p < 0.01$; P14: CPS: $19.72g \pm 4.27g/CS$: $31.93g \pm 2.65g$, $p < 0.001$; P17: CPS: $22.45g \pm 4.4g/CS$: $37.42g \pm 3.67g$, $p < 0.001$; P21: CPS: $26.62g \pm 4.93g/CS$: $47.34g \pm 4.68g$, $p < 0.001$; P29: CPS: $47.87g \pm 12.35g/CS$: $78.35g \pm 4.78g$; $p < 0.001$) (Fig. 1). The treatment with resveratrol in CP attenuated the damage on weight gain only at P21 (P21: CPR: $32.69g \pm 4.2g/CS$: $26.62g \pm 4.93g$, $p < 0.05$).

Somatic growth was also affected by experimental CP, evidenced by

Table 1
Primers sequence used in this study.

Gene Name	Forward Primer Sequence	Reverse Primer Sequence
β 2M	TGACCGTGATCTTTCTGGTG	ACTTGAATTTGGGGAGTTTCTG
PGC-1 α	AAC AGC AAA AGC CAC AAA GA	AAG TTG TTG GTT TGG CTT GA
TFAM	TCT CAT GAT GAA AAG CAG GCA	GAG ATC ACT TCG CCC AAC TT
Complex II Subunit (Sdhb)	TTT ACC GAT GGG ACC CGG AC	CGT GTT GCC TCC GTT GAT GT
Complex V Subunit (Atp5f1a)	TCC CTG AAC TTG GAA CCC GA	GGC ATT TCC CAG GGC ATC AA

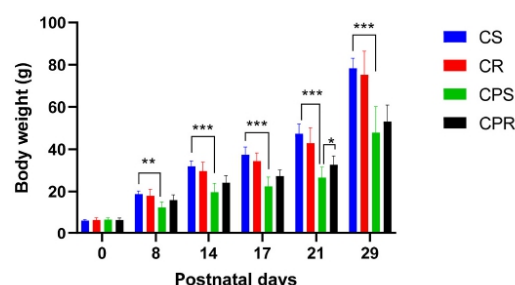


Fig. 2. Weight evolution at P0, P8, P14, P17, P21, and P29 according to the experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 11); CPS: CP + Saline (n = 11); CPR: CP + Resveratrol (n = 12). Data were expressed as mean $\pm$ standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

smaller murinometric measurements in the CPS group compared to the CS group. Thus, in the measurements of the skull axes, the smallest lateral skull axis was observed in the CPS group at P12 (CPS: $1.55 \text{ cm} \pm 0.12 \text{ cm/CS}$: $1.79 \text{ cm} \pm 0.15 \text{ cm}$; $p < 0.001$), P15 (CPS: $1.67 \text{ cm} \pm 0.16 \text{ cm/CS}$: $1.82 \text{ cm} \pm 0.09 \text{ cm}$; $p < 0.05$), P21 (CPS: $1.69 \text{ cm} \pm 0.16 \text{ cm/CS}$: $1.90 \text{ cm} \pm 0.05 \text{ cm}$; $p < 0.01$) and P29 (CPS: $1.83 \text{ cm} \pm 0.11 \text{ cm/CS}$: $2.15 \text{ cm} \pm 0.15 \text{ cm}$; $p < 0.001$), as was observed smaller anteroposterior skull axis of the animals submitted to the CP model compared to the control animals at P3 (CPS: $2.23 \text{ cm} \pm 0.16 \text{ cm/CS}$: $1.93 \text{ cm} \pm 0.26 \text{ cm}$; $p < 0.05$), P12 (CPS: $2.85 \text{ cm} \pm 0.27 \text{ cm/CS}$: $3.20 \text{ cm} \pm 0.17 \text{ cm}$; $p < 0.05$), P15 (CPS: $3.09 \text{ cm} \pm 0.24 \text{ cm/CS}$: $3.39 \text{ cm} \pm 0.13 \text{ cm}$; $p < 0.05$), P18 (CPS: $3.24 \text{ cm} \pm 0.3 \text{ cm/CS}$: $3.59 \text{ cm} \pm 0.17 \text{ cm}$; $p < 0.05$), P21 (CPS: $3.32 \text{ cm} \pm 0.23 \text{ cm/CS}$: $3.81 \text{ cm} \pm 0.15 \text{ cm}$; $p < 0.05$) and P29 (CPS: $4.1 \text{ cm} \pm 0.32 \text{ cm}$; $p < 0.05$) (Fig. 3A and B).

Similarly, tail length was also impaired by experimental CP from the 9th postnatal day onwards (P9: CPS: $2.84 \text{ cm} \pm 0.51 \text{ cm/CS}$: $3.5 \text{ cm} \pm 0.36 \text{ cm}$; $p < 0.05$; P12: CPS: $3.31 \text{ cm} \pm 0.47 \text{ cm/CS}$: $4 \text{ cm} \pm 0.28 \text{ cm}$; $p < 0.05$; P15: CPS: $3.66 \text{ cm} \pm 0.64 \text{ cm/CS}$: $4.41 \text{ cm} \pm 0.35 \text{ cm}$; $p < 0.05$; P18: CPS: $4.19 \text{ cm} \pm 0.96 \text{ cm/CS}$: $4.99 \text{ cm} \pm 0.36 \text{ cm}$; $p < 0.05$, P21:

CPS: $4.71 \text{ cm} \pm 0.7 \text{ cm/CS}$: $6.07 \text{ cm} \pm 0.47 \text{ cm}$, $p < 0.05$; and P29: CPS: $5.46 \text{ cm} \pm 1.23 \text{ cm/CS}$: $8.21 \text{ cm} \pm 0.91 \text{ cm}$, $p < 0.05$), as well as the longitudinal axis from the 12th postnatal day (P12 CPS: $7.92 \text{ cm} \pm 0.95 \text{ cm/CS}$: $9.01 \text{ cm} \pm 0.48 \text{ cm}$, $p < 0.05$; P15: CPS: $8.73 \text{ cm} \pm 0.96 \text{ cm/CS}$: $10.51 \text{ cm} \pm 0.98 \text{ cm}$, $p < 0.001$; P18: CPS: $9.49 \text{ cm} \pm 1.12 \text{ cm/CS}$: $11.63 \text{ cm} \pm 0.59 \text{ cm}$, $p < 0.001$; P21: CPS: $10.02 \text{ cm} \pm 1.22 \text{ cm/CS}$: $12.45 \text{ cm} \pm 0.71 \text{ cm}$, $p < 0.001$; and P29: CPS: $12.32 \text{ cm} \pm 1.66 \text{ cm/CS}$: $13.55 \text{ cm} \pm 1.26 \text{ cm}$, $p < 0.01$) when compared to the control group (Fig. 3C and D).

Treatment with resveratrol in animals with CP attenuated the damage in somatic growth. Therefore, the animals in the CP group treated with resveratrol had greater measurements regarding tail length at P29 (CPR: $6.37 \text{ cm} \pm 1.15 \text{ cm/CPS}$: $5.46 \text{ cm} \pm 1.23 \text{ cm}$, $p < 0.05$) and the longitudinal axis at P6 (CPR: $7.49 \text{ cm} \pm 1.05 \text{ cm/CPS}$: $6.27 \text{ cm} \pm 0.42 \text{ cm}$, $p < 0.01$), P9 (CPR: $8.54 \text{ cm} \pm 1.39 \text{ cm/CPS}$: $7.19 \text{ cm} \pm 0.66 \text{ cm}$, $p < 0.01$) and P12 (CPR: $9.16 \text{ cm} \pm 1.32 \text{ cm/CPS}$: $7.93 \text{ cm} \pm 0.95 \text{ cm}$, $p < 0.01$) compared to the CPS group. Additionally, in control animals submitted to resveratrol treatment, it was also possible to observe an increase in the longitudinal axis of the body at P6 (CR: $7.75 \text{ cm} \pm 1.1 \text{ cm/CS}$: $6.38 \text{ cm} \pm 0.38 \text{ cm}$, $p < 0.01$) and P9 (CR: $8.95 \text{ cm} \pm 1.08 \text{ cm/CS}$: $7.66 \text{ cm} \pm 0.46 \text{ cm}$, $p < 0.01$) compared to the CS group (Fig. 3C and D).

Not only the somatic growth was affected by experimental CP, but the maturation of physical features was also impaired. There were a delay in the post-natal day of auricle opening (CPS: $3.41 \pm 1.08/\text{CS}$: 2.0 ± 0.0 , $p = 0.0124$), auditory conduit opening (CPS: $14.33 \pm 1.87/\text{CS}$: 12.41 ± 0.51 , $p = 0.0003$) and eyes opening (CPS: $14.66 \pm 1.23/\text{CS}$: 12.58 ± 0.51 , $p < 0.0001$), in the animals submitted to the CP model compared to the control group. CP model did not cause changes in upper or lower incisor eruption. However, the treatment with resveratrol in the experimental CP did not influence the maturation of the evaluated somatic characteristics (Fig. 4).

4.2. Gait analysis on catwalk

Motor behavior after resveratrol treatment was evaluated. For this, the average speed of the animal during the Catwalk test was used as a kinetic parameter. In this analysis, the CPS group had a lower average speed when compared to the CS group (CS: $21.06 \text{ cm/s} \pm 2.13 \text{ cm/s/}$

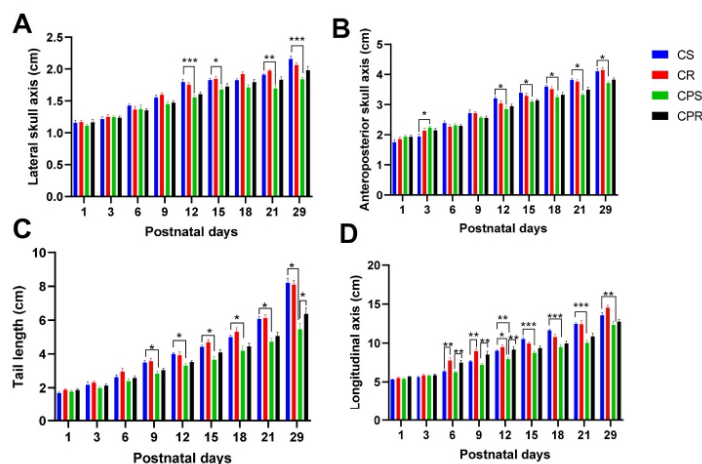


Fig. 3. Somatic growth at P1, P3, P6, P9, P12, P15, P18, P21 and P29 of postnatal life according to the experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 11); CPR: CP + Resveratrol (n = 12). Data were expressed as mean $\pm$ standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

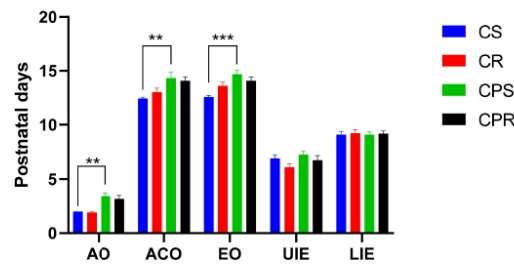


Fig. 4. Physical features between the P1 and P21 according to the experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 11); CPR: CP + Resveratrol (n = 12). AO: auricle opening; ACO: auditory conduit opening; EO: eyes opening UIE: upper incisors eruption; LIR: lower incisors eruption. Data were expressed as mean $\pm$ standard error, $p < 0.05$ (** = $p < 0.01$; *** = $p < 0.001$).

CPS: 18.35 cm/s $\pm$ 3.39 cm/s; $p < 0.05$) (Fig. 5).

In addition to the average velocity, the evaluation of spatial and temporal parameters showed important changes. Regarding the time of the swing phase, the CPS group had a long time when compared to the CS group (CS: 0.26s $\pm$ 0.04s/CPS: 0.32s $\pm$ 0.07s; $p < 0.05$) (Fig. 6B). In the analysis of swing velocity, the CPS group had a lower mean velocity compared to the saline control group (CS: 71.74 cm/s $\pm$ 3.58 cm/s/CPS: 55.61 cm/s $\pm$ 5.52 cm/s; $p < 0.05$) (Fig. 6C). The analysis of the maximum contact area showed that the CPS group showed a reduction in this area when compared to the CS group (CS: 0.78 cm² $\pm$ 0.04 cm²/CPS: 0.52 cm² $\pm$ 0.05 cm²; $p < 0.05$) (Fig. 6D). About the print length, the animals in the CPS group had a reduction in this length compared to the CS group (CS: 1.73 cm $\pm$ 0.04 cm/CPS: 1.27 cm $\pm$ 0.06 cm; $p < 0.0001$) (Fig. 6E). The print width analysis showed that the CPS group had a reduced print width when compared to the saline control group (CS: 1.43 cm $\pm$ 0.04 cm/CPS: 1.18 cm $\pm$ 0.06 cm; $p < 0.0001$), but it showed no statistical difference between the groups CPS and CPR (Fig. 6F).

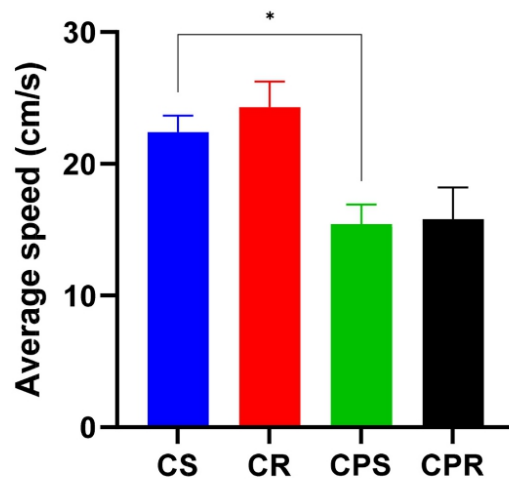


Fig. 5. Average speed during active locomotion on the platform. Experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 10); CPR: CP + Resveratrol (n = 10). Data were expressed as mean $\pm$ standard error, $p < 0.05$ (* = $p < 0.05$).

4.3. Forelimb grip strength and postural analysis

In the muscle strength analysis with the grip test, at P22 and P28, the CS group showed greater strength compared to the CPS group (P22: CS: 160.97g $\pm$ 6.22g/CPS: 113.58g $\pm$ 10.85g; $p < 0.01$; P28: CS: 235.05g $\pm$ 6.51g/CPS: 187.05g $\pm$ 8.5g; $p < 0.01$) (Fig. 6A and B). At the same ages, CP animals treated with RES showed greater strength compared to the CPS group (P22: CPR: 175.6g $\pm$ 9.17g/CPS: 113.58g $\pm$ 10.85g; $p < 0.001$; P28: CPR: 225.19g $\pm$ 11.72g/CPS: 187.05g $\pm$ 8.5g; $p < 0.05$) (Fig. 7A and B). No significant difference was seen between the control groups ($p > 0.05$).

For the postural analysis, at P28 the animals in the CPS group presented greater trunk angulation when compared to the CPR group (CPR: 159.33° $\pm$ 1.44°/CPS: 167.38° $\pm$ 2.87°; $p < 0.05$). Likewise, the CS group presented a greater angulation compared to the CR group (CR: 153.98° $\pm$ 2.51°/163.95° $\pm$ 0.75°; $p < 0.05$) (Fig. 8A). The analysis of the head alignment showed that, from P14 to P28, the CPS animals presented greater angulation compared to the CS group (P14: CS: 45.34° $\pm$ 2.2°/CPS: 54.01° $\pm$ 2.28°; P21: CS: 48.4° $\pm$ 1.55°/57.65° $\pm$ 1.71°; P28: CS: 46.5° $\pm$ 1.46°/CPS: 62.06° $\pm$ 1.93°; $p < 0.05$) (Fig. 8B). Also, at P28, the CP animals submitted to the treatment with resveratrol presented smaller head angulation when compared to the pups of the CPS group (P28: CPR: 46.87° $\pm$ 2.09°/CPS: 62.06° $\pm$ 1.93°; $p < 0.05$) (Fig. 8B).

4.4. Oxidative state in the somatosensory cortex

After our previous analyzes, with the intent to understand how resveratrol treatment could improve the mechanical properties, we evaluate the oxidative stress biomarkers. Our data showed that treatment with resveratrol decreased MDA and Carbonyl markers in the animals with CP compared to the CPS group (MDA: CPR: 4.66 $\pm$ 1.27/CPS: 10.98 $\pm$ 1.30 μ mol/mg prot; $p < 0.001$; Carbonyls: CPR: 13.81 $\pm$ 2.85/CPS: 29.14 $\pm$ 5.08 μ mol/mg prot; $p < 0.01$) (Fig. 9A and B). Also, the control animals treated with resveratrol showed a decrease in MDA compared to the CS group (CR: 5.47 $\pm$ 1.04/CS: 12.12 $\pm$ 1.61 μ mol/mg prot; $p < 0.001$) (Fig. 9A), while the CPS group showed an increase in Carbonyls compared to the CS group (CPS: 29.14 $\pm$ 5.08/CS: 13.55 $\pm$ 2.52 μ mol/mg prot; $p < 0.01$) (Fig. 9B).

In addition, we performed several assays to investigate antioxidant capacity. Superoxide dismutase (SOD) and Catalase (CAT) activity in control animals treated with resveratrol were decreased when compared to the CS group (SOD: CR: 45.44 $\pm$ 4.34/CS: 72.81 $\pm$ 6.19 U/mg prot; $p < 0.05$; CAT: CR: 223.1 $\pm$ 45.78/CS: 405.3 $\pm$ 47.02 U/mg prot; $p < 0.05$) (Fig. 9C and D). No significant difference was seen in the others assays ($p > 0.05$) (Fig. 9E, F, 9G, 9H and 9I).

4.5. Mitochondrial biogenesis and citrate synthase activity in the somatosensory cortex

After the observations in oxidative stress biomarkers, we further analyze some parameters linked with oxidative status. First, we measure the mitochondrial biogenesis mRNA levels. Our data showed a decrease in PGC-1 α in the group of animals with cerebral palsy treated with resveratrol compared to the untreated (CPR: 0.6 $\pm$ 0.1/CPS: 1.4 $\pm$ 0.15; $p < 0.01$) (Fig. 10A) associated with an increase in TFAM levels (CPS: 0.3 $\pm$ 0.1/CPR: 0.9 $\pm$ 0.15; $p < 0.05$). In addition, we observed in CPS decreased levels of TFAM compared to CS (CPS: 0.3 $\pm$ 0.1/CS: 1.0 $\pm$ 0.1; $p < 0.05$) (Fig. 10B).

When we evaluated the mitochondrial complexes II and V, we observed in CR group an increase in both complexes' subunits compared to the CS group (Complex II: CR: 1.7 $\pm$ 0.2/CS: 1.0 $\pm$ 0.2; $p < 0.05$; Complex V: CR: 5.3 $\pm$ 0.25/CS: 1.0 $\pm$ 0.08; $p < 0.0001$). The CPS group showed an increase in V complex when compared with the CPR and CS groups (CPS: 2.8 $\pm$ 0.17/CPR: 0.51 $\pm$ 0.09/CS: 1.0 $\pm$ 0.08; $p < 0.0001$) (Fig. 10C and D).

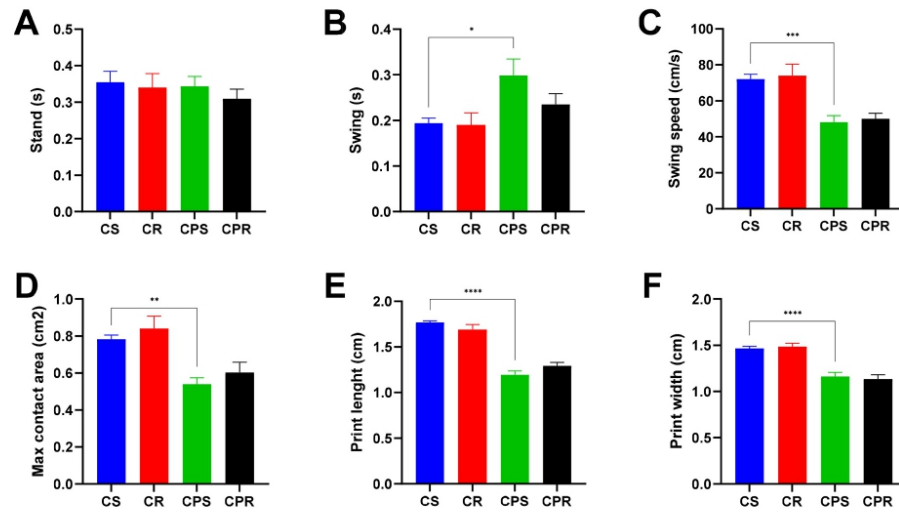


Fig. 6. Evaluation of temporal and spatial parameters of gait at P28. A – Paw support time during locomotion. B – Time during the swing phase on the paws. C – Velocity of the paws during the swing phase. D – Measurement of the area of the paws in contact with the platform during locomotion; E – paw print length during locomotion; F – paw print width during locomotion. Experimental groups: CS: Control Saline (n = 12); CR: Control Resveratrol (n = 10); CPS: CP + Saline (n = 10); CPR: CP + Resveratrol (n = 10). Data were expressed as mean $\pm$ standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

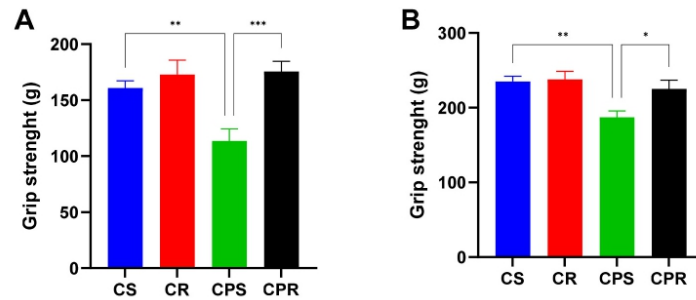


Fig. 7. Grip strength test at P22 (A) and P28 (B) according to the experimental groups: CS: Saline Control (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 11); CPR: CP + Resveratrol (n = 10). Data were expressed as mean $\pm$ standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

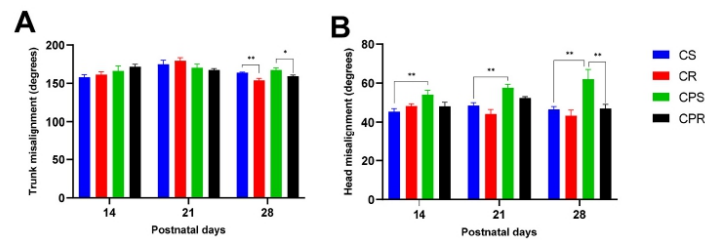


Fig. 8. Analysis of posture based on trunk (A) and head alignment (B) at P14, P21 and P28 according to the experimental groups: CS: Control Saline (n = 12); CR: Resveratrol Control (n = 10); CPS: CP + Saline (n = 10); CPR: CP + Resveratrol (n = 12). Data were expressed as mean $\pm$ standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$).

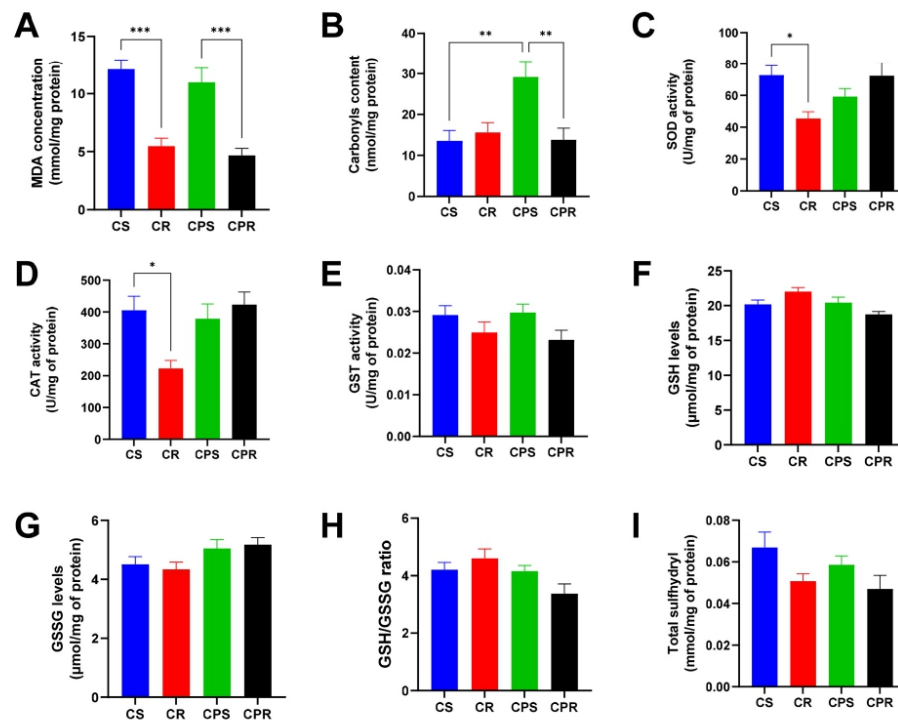


Fig. 9. Analysis of oxidative stress in the somatosensory cortex at P29 according to the experimental groups: CS: Control Saline ($n = 8$); CR: Resveratrol Control ($n = 7$); CPS: CP + Saline ($n = 7$); CPR: CP + Resveratrol ($n = 8$). Data were expressed as mean $\pm$ standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

After our data related to the mRNA of important genes related to mitochondrial biogenesis, we further analyze the citrate synthase activity, since is the first enzyme in Krebs cycle a crucial enzyme for mitochondrial function. Our data showed an increase in the activity of this enzyme in the animals with CP treated with resveratrol compared to the CPS group (CPR: 21.83 ± 1.14 /CPS: 13.43 ± 1.36 ; $p < 0.05$). Similarly, the CPS group showed a decrease in citrate synthase when compared to the CS group (CPS: 13.43 ± 1.36 /CS: 25.0 ± 1.63 ; $p < 0.01$). Our data also showed that the resveratrol-treated control group had an increase in enzyme compared to the saline-treated control group (CR: 39 ± 3.66 /CS: 25.0 ± 1.63 ; $p < 0.001$) (Fig. 11).

5. Discussion

The findings of this study demonstrated that neonatal treatment with resveratrol was able to improve muscle strength and posture in the animals, in addition to acting as a modulator of brain function, reducing markers of oxidative stress and increasing gene expression related to mitochondrial biogenesis in the somatosensory cortex of rats submitted to cerebral palsy. Furthermore, we observed an increase in the activity of the enzyme citrate synthase in the somatosensory cortex of the CPR group, which demonstrates an increase in mitochondrial function.

We analyzed the body weight of the experimental groups over the 28 days and significant differences were found between the groups from P14, in which the animals submitted to experimental CP showed lower weight gain compared to the control animals. Previous studies with this model of CP observed impairments in the musculoskeletal system, verifying a reduction in muscle fibers in the soleus, EDL muscles, and

tibial bone mass associated with impairments in locomotion (Pereira et al., 2021a; Visco et al., 2023). Thus, it is understood that delays in locomotion can impair the animals' eating behavior, which would explain the low weight found in our study. In addition, findings point to a reduction in masseter muscle fibers (Lacerda et al., 2017), which is one of the main muscles involved in mastication, suggesting that there are chewing impairments present in this CP model. In addition, the association of sensorimotor restriction with anoxia exposes the animals to situations of early stress that can delay the weight development of the animals (Pereira et al., 2021a; Strata et al., 2004). However, treatment with resveratrol showed significant changes in the animal's body weight only in the P21. We suggest that more studies be done to better understand how RES influences on weight gain in cerebral palsy.

In addition to body weight analysis, we also evaluated the development of physical features and somatic growth. We observed alterations in the development of the physical features of the animals in the CP group, being possible to notice a delay in the auricle opening, auditory conduit opening and eyes opening in relation to the control animals. In a study carried out by Toso et al. (2005) a delay in eyes opening of animals submitted to a CP model was also observed. However, these authors used a different model of CP through the application of liposaccharides (LPS) during pregnancy, suggesting that brain injuries during pregnancy and after birth can harm the development of animals. Furthermore, the PC model delayed the animals' somatic growth, evidenced by the evaluations of the head axes, longitudinal axis, and tail length. Neonatal exposure to resveratrol was able to attenuate the delay in the growth of the longitudinal axis, from the P6 to the P12, and tail length, in the P29. Visco et al. (2023), using the same CP model, found a reduced body

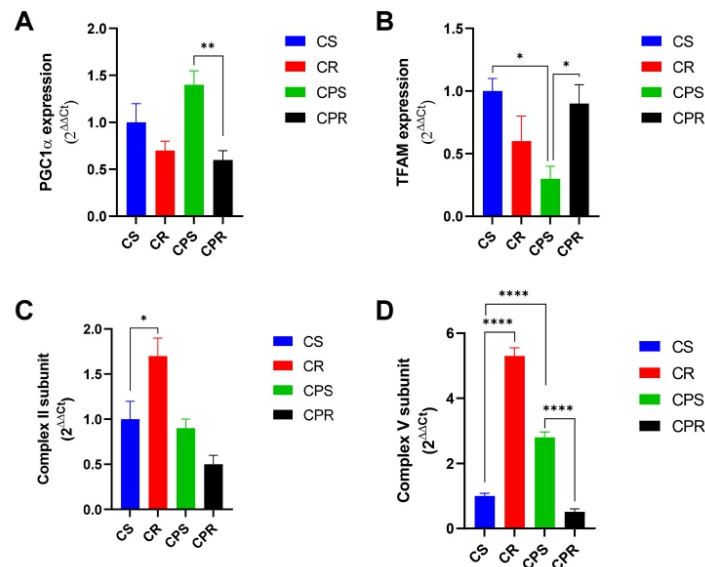


Fig. 10. Analysis of mitochondrial biogenesis genes in the somatosensory cortex at P29 according to the experimental groups: CS: Control Saline (n = 8); CR: Resveratrol Control (n = 7); CPS: CP + Saline (n = 7); CPR: CP + Resveratrol. Data were expressed as mean $\pm$ standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$).

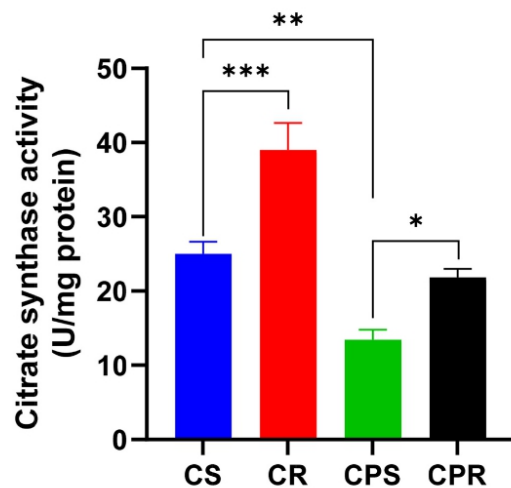


Fig. 11. Analysis of citrate synthase activity in the somatosensory cortex at P29 according to the experimental groups: CS: Control Saline (n = 8); CR: Resveratrol Control (n = 7); CPS: CP + Saline (n = 7); CPR: CP + Resveratrol (n = 8). Data were expressed as mean $\pm$ standard error, $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

growth in animals with CP, which was attenuated with a neonatal treatment with kaempferol, another polyphenol. These results show that polyphenols have an important positive action on development. Thus, it is understood that cerebral palsy delays body growth and development, in addition to delaying the motor development of these children. To

date, we are not aware of other studies evaluating the effects of resveratrol on these outcomes in models of cerebral palsy. We suggest that future studies be carried out to obtain more information about the effectiveness of RES in improving the somatic growth and physical features of animals with CP.

In the Catwalk analysis, we demonstrated that the induction of CP altered the gait pattern of the animals. Pups submitted to CP model showed lower average speed during walking and lower speed during the swing phase, resulting in a longer duration of this phase. In addition, a decrease in the contact area and the length and width of the paw print of these animals was observed, suggesting dysfunctions in the gait of the animals of the CP group. These findings are similar to those found in children with cerebral palsy, such as incoordination of locomotion, lack of precision in foot positioning, and reduced walking ability (Cappellini et al., 2016; Prosser et al., 2010). A previous study using the same PC model found similar results, in addition to altered gait cadence, with interlimb incoordination, and an increased base of support for the forepaws during active locomotion (Visco et al., 2023). The use of sensorimotor restriction was able to change the gait pattern, reducing the maximum contact area of the rodents' paws (Delcours et al., 2018), corroborating the findings of this study. A recent review showed that models that associate perinatal anoxia and sensorimotor restriction lead to a greater impairment of locomotion and motor coordination in animals, similar to what happens in children with CP (Pereira et al., 2021b). Animals with CP develop locomotor characteristics similar to the equinus foot, possibly because they undergo ankle and knee extension during sensorimotor restriction (Delcours et al., 2018), which explains the abnormal gait pattern observed in our results.

In an unprecedented way, this is the first study to evaluate the postural outcomes present in an experimental model of CP. From these results, postural alterations evidenced by the misalignment of the head and trunk were found. However, neonatal treatment with RES attenuated these damages, where the animals in the CPR group had better postural control, similar to the pups in the control group. Regarding the

evaluation of the muscular strength of the front paws, the animals submitted to the CP model showed a reduction in strength. Treatment with resveratrol was able to reverse these losses, where the animals in the CPR group demonstrated an increase in strength, similar to the animals in the control group. Here, we demonstrate that neonatal treatment with resveratrol prevented the impact of CP on postural development and strength, suggesting that this polyphenol may act by modifying motor function.

It is already well established that muscle weakness is the predominant negative characteristic in people with cerebral palsy (Multani et al., 2019), as observed in the experimental model used in our study. Children with CP have skeletal muscle abnormalities such as weakness and decreased muscle thickness and volume (Elder et al., 2003; Graham and Selber, 2003; Multani et al., 2019). Studies are currently being carried out with natural compounds that can act on skeletal muscle, such as polyphenols (resveratrol, curcumin, urolithin A) and flavonoids (quercetin, apigenin) (Hil et al., 2015; Nikawa et al., 2021; Yadav et al., 2022). These are well known for increasing muscle strength and mass, promoting muscle stem cell differentiation and mitochondrial biogenesis, and reducing hydrogen peroxide production as well as inflammation in skeletal muscles (Yadav et al., 2022). Suggesting possible ways of action of resveratrol in the musculoskeletal system, which may explain the improvement in posture and muscle strength of the animals.

In addition to motor function outcomes, we further investigate the effects of resveratrol on the somatosensory cortex. It is well known in the literature that several injuries are associated to the oxidative stress, because of this we evaluate several parameters, and we notice a significant increase in carbonyls levels, an important OS biomarker, in rats submitted to CP model. Furthermore, we continue analyzing the effect of CP and we observed a significant decrease in MDA and carbonyls in CP rats treated with resveratrol. There are no reports in the literature that show the neuroprotective and antioxidant effects of resveratrol in our model of cerebral palsy. Using a different models, previous authors showed results that indicated that RES had an antioxidant effect, being able to decrease MDA in brain tissue as we observed in CR group (Gao et al., 2018; Orsu et al., 2013). These results are similar to those of our study and support the antioxidant role of resveratrol as evidenced by significantly reducing the levels of MDA and carbonyls. Gao et al. (2018) suggested that RES can scavenge a variety of free radicals owing to its phenol rings and three free hydroxyl group, and thus RES treatment can concomitantly abrogate the ROS production under ischemic conditions.

After the promising finding observed in MDA and carbonyls levels in the CPR group, we further analyzed the antioxidant enzymes, but to our surprise we observed a decrease in SOD and CAT activity in CR group, compared to the control animals. Raising the hypothesis that the antioxidant enzymes had a decrease activities, due to the potent antioxidant effect of RES. Resveratrol is a remarkable antioxidant (Miguel et al., 2021) and here it can act in the healthy body; we hypothesized that it acts preventively against the insults. In this context, we suggest that future studies be carried out to elucidate the molecular and biochemical mechanisms whereby resveratrol beneficially affects the nervous system of healthy animals.

In sequence, with the intent to understand more the mechanistic effect of RES we evaluated mitochondrial biogenesis markers, measuring the levels of transcription factors and mitochondrial complexes subunits. Our data showed that PGC-1 α expression is reduced in CPR animals when compared to the CPS group. However, resveratrol-treated CP animals showed upregulation of mitochondrial transcription factor A (TFAM) compared to the CPS group. PGC-1 α is mainly expressed in energy-demanding tissues, such as the brain and skeletal muscle (Di et al., 2018), and is an important coordinator of mitochondrial metabolism and function (Finck and Kelly, 2006; Gabrielson et al., 2014). In addition, it participates in the regulation and TFAM transcription (Gabrielson et al., 2014). These data suggest that PGC-1 α and TFAM were positively modulated by RES treatment in animals submitted to CP. Therefore, at mRNA levels, we can speculate that RES

treatment in CP animals might increase the number of mitochondria. As a result, the increase in the number may decrease the levels of free radicals released by mitochondria that CP damaged. Corroborating our results, Zhou et al. (2021) highlight that RES increased the expression of PGC-1 α , NRF1, and TFAM in the temporal cortex in animals submitted to early brain injury after subarachnoid hemorrhage (Zhou et al., 2021), improving the mitochondrial biogenesis.

Oxidative stress is closely linked to mitochondrial function, with the mitochondrial respiratory chain being the main source of ROS (Chen et al., 2021). Dysfunctions in the mitochondrial respiratory chain, such as changes in the protein complexes, which are responsible for the functionality of this system, prevents electrons from being transferred, resulting in increased production of reactive oxygen species (Chen et al., 2021). The lack of oxygen in the developing brain leads to the depletion of cellular energy stores and triggers various pathophysiological responses, including suppression of mitochondrial respiration (Jacobsson and Hagberg, 2004; Thornton and Hagberg, 2015). Here, we observed a marked decrease in complex V subunits and a tendency to reduce the complex II subunits in the CPR group compared to the animals of the CPS group. With our results, RES treatment decreases the levels of these mRNA, which might lead to a decrease in the electron leak and ROS production.

On the other hand, in healthy animals, RES treatment affects the cells differently. In the control group treated with resveratrol, we observed an increase in complexes II and V subunits, compared the CS group, which suggest that RES treatment increase the flow of electrons at mitochondrial respiratory chain, improving the mitochondrial function as a whole. Due to these results, we decided to analyze the activity of the citrate synthase, the first enzyme in Krebs cycle and highly used as an indicator of mitochondrial function. Indeed, in CR group, the citrate synthase activity increased significantly compared to CS, corroborating with our data related to the complex subunits. However as expected in CPS the activity of the citrate synthase was decreased compared to CS, and the treatment with RES could restore the activity capacity in CPR similarly to the levels of CS. Citrate synthase is an important component of energy metabolism: it is necessary for the catalysis and condensation of Acetyl CoA with oxaloacetate for the formation of citrate (Fernandes et al., 2020; Meng et al., 2021). With the increase in citrate synthase activity in the groups treated with resveratrol, we can speculate that this polyphenol was able to substantially improve the mitochondrial function of cells after CP injury.

Finally, our study demonstrated that resveratrol improved posture and muscle strength, reduced carbonyls and MDA, the main markers of oxidative stress, and increased the expression of the TFAM gene and the enzyme citrate synthase in animals submitted to cerebral palsy. The findings of this work give a new perspective on the use of RES in the treatment of cerebral palsy, through its antioxidant properties. More studies are needed to determine the mechanisms of action involved and to establish the best form of administration to obtain a beneficial and prolonged effect.

CRediT authorship contribution statement

Vanessa da Silva Souza: Conceptualization, Formal analysis, Investigation, Writing – original draft. **Raul Manhães-de-Castro:** Resources, Writing – original draft. **Sabrina da Conceição Pereira:** Formal analysis, Investigation, Writing – original draft. **Caio Matheus Santos da Silva Calado:** Formal analysis, Investigation, Writing – original draft. **Beatriz Souza de Silveira:** Formal analysis, Investigation, Writing – original draft. **Eulália Rebeca da Silva Araújo:** Formal analysis, Investigation, Writing – original draft. **Severina Cassia de Andrade Silva:** Formal analysis, Investigation, Writing – original draft. **Osmar Henrique dos Santos Junior:** Formal analysis, Investigation, Writing – original draft. **Claudia Jacques Lagranha:** Formal analysis, Investigation, Writing – original draft, Resources. **Luan Kelwyny Thaywā Marques da Silva:** Formal analysis, Investigation, Writing –

original draft. Ana Elisa Toscano: Supervision, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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APÊNDICE E – NEONATAL TREATMENT WITH FLUOXETINE ALTERS LOCOMOTOR ACTIVITY AND THE CORTICAL GLIAL/NEURON INDEX IN RATS WITH CEREBRAL PALSY



Article

Neonatal treatment with fluoxetine alters locomotor activity and the cortical glial/neuron index in rats with cerebral palsy

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Abstract

Cerebral palsy (CP) is characterized by motor disorders, including deficits in locomotor activity, coordination, and balance. Selective serotonin reuptake inhibitors have been shown to play an important role in brain plasticity. This study investigates the effect of neonatal treatment using fluoxetine on locomotor activity and histomorphometric parameters of the primary somatosensory cortex (S1) in rats submitted to an experimental model of CP. CP was found to reduce bodyweight and locomotion parameters and also to increase the glia/neuron index in the S1. Administration of fluoxetine 10 mg/kg reduced bodyweight, impaired locomotor activity parameters, and increased the number of glial cells and the glia/neuron ratio in the S1 in rats with CP. However, treatment with fluoxetine 5 mg/kg was not found to be associated with adverse effects on locomotor activity and seems to improve histomorphometric parameters by way of minor changes in the S1 in animals with CP. These results thus indicate that experimental CP, in combination with the use of a high dose of fluoxetine (10 mg/kg), impairs locomotor and histomorphometric parameters in the S1, while treatment with a low dose of fluoxetine (5 mg/kg) averts the negative outcomes associated with a high dose of fluoxetine in relation to these parameters but produces no protective effect.

Key words: cerebral palsy, locomotor activity, neurons, neuroglia, fluoxetine

1. Introduction

Cerebral palsy (CP) is considered a nervous disorder that is caused by an injury that manifests itself during brain development (Kriger 2020). The injury caused by CP results in neuron loss due to apoptosis and reduced dendritic arborization (Rocha-Ferreira and Hristova 2016). Along with damage to neurons, CP gives rise to an altered neurogenesis response after injury (Visco et al. 2021). Subsequent activation of microglia and oligodendrocytes leads to hypomyelination and neuron loss in important parts of the nervous system (Kannan et al. 2011). Experimentally induced CP causes damage to the nervous system that has a negative impact on the development of locomotor activity (Stigger et al. 2011; Silva et al. 2016) with long-term neuropathological changes (McAdams et al. 2017) and altered organization of the map of the primary somatosensory cortex (S1) (Coq et al. 2008).

The development of the serotonergic system has been shown to be impaired in the CP (Reinebrant et al. 2012). Hypoxia-ischemia-induced CP activates microglia, leading to excitotoxic lesions (Reinebrant et al. 2012). Damage to the

serotonergic system in the S1 thus seems to play an important role in the appearance of sensorimotor deficits typical of CP (Williams et al. 2017). Experimentally induced CP impairs the development of locomotor activity as a consequence of damage to the nervous system (Silva et al. 2016; Buratti et al. 2019). In addition, the manipulation of the serotonergic system seems to contribute to the development of parameters that reflect the integrity of the nervous system and protect neural cells from oxidative stress during brain development (Silva et al. 2014). Studies indicate that selective serotonin reuptake inhibitors may increase the production of brain-derived neurotrophic factor, which plays an important role in survival, neuron remodeling, and differentiation (De Foubert et al. 2004; Karpova et al. 2011; Liu et al. 2014). Selective serotonin reuptake inhibitors can regenerate hippocampal neurons, thereby affecting recovery of neurological function (Lee et al. 2004; Kubera et al. 2009; Li et al. 2009).

Clinical and experimental evidence has shown that selective serotonin reuptake inhibitors affect the neuromotor systems, with effects on the structure, function, and metabolism

of skeletal muscle (Loubinoux et al. 2002). High neonatal doses (>10 mg/kg) of fluoxetine appear to exacerbate functional deficits in adult rats with hypoxic-ischemic brain injury (Chang et al. 2006). The use of fluoxetine in high doses results in neurobehavioral consequences (increased emotional reactivity and changes in the motivational system), in addition to low weight (Cagiano et al. 2008). Conversely, the neonatal use of low doses (5 mg/kg) of fluoxetine provides benefits in terms of neuroplasticity, reducing the functional deficit in rats with hypoxic-ischemic brain injury and with no adverse effects on cognition or long-term behavior (Chang et al. 2006). However, the effects of administration of fluoxetine, especially in low doses, on reducing nerve tissue damage or locomotion parameters in an experimental model of CP remain unclear.

We therefore hypothesized that alterations in the serotonergic system induced by neonatal treatment with high doses of fluoxetine may increase nerve damage in the S1 and aggravate the development of locomotor activity in animals with CP. We also postulated that low doses of fluoxetine may attenuate such effects in animals with induced CP.

2. Materials and methods

2.1. Animals

This controlled and randomized experimental study, developed at the Department of Nutrition of the Federal University of Pernambuco (Brazil), between July 2018 and July 2019 was approved in June 2018 by the Ethics Committee on Animal Use of the Federal University of Pernambuco Biosciences Center (registration 0015/2018). The description of animal experiments was in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, Vol. 1, 2nd ed., 1993).

A total of 107 male albino Wistar rats were obtained from 30 litters. Thirty ($n = 30$) virgin female rats (200–280 g) and ten ($n = 10$) male rats with 90–120 days of life were obtained from the Department of Nutrition, Federal University of Pernambuco, and were mated (two females for one male). The day when spermatozoa were identified in vaginal smear was the conception day. The animals were kept at a temperature of $22 \pm 2^\circ\text{C}$, in an environment with an inverted light-dark cycle of 12/12 h (lights on at 8:00 pm), and given free access to water and food (Purina do Brasil S/A). At birth, male animals were randomly assigned to the following groups: control saline (CS, $n = 19$), control fluoxetine 10 mg/kg (CF10, $n = 16$), control fluoxetine 5 mg/kg (CF5, $n = 19$), CP saline (CPS, $n = 19$), CP fluoxetine 10 mg/kg (CPF10, $n = 16$), and CP fluoxetine 5 mg/kg (CPF5, $n = 18$). Each litter contained eight pups, weighing between 5 and 8 g each, and females were used only to standardize the size of each litter. Weaning was performed at P25. To avoid difficulties in reaching the food, feed was added inside all cages, aiming that all animals, especially the animals in the CP group, had easy access to food. Bodyweight, locomotor activity, and S1 assessments were performed only in male pups.

From P1 to P21 (1st to 21st postnatal day of life), fluoxetine (5 or 10 mg/kg) or saline solution, respectively, were admin-

istered to each experimental group. The drug was administered subcutaneously in the animal's back, on a daily basis, at 9:00 am. The drug was obtained in the form of fluoxetine hydrochloride (Bristol-USA[®]) and dissolved in a control solution of 0.9% sodium chloride solution. The doses used were selected from a previous study that demonstrated the therapeutic potential of low-dose fluoxetine in animals subjected to hypoxia-ischemia (Chang et al. 2006). For the control group, a 0.9% sodium chloride solution was used.

2.2. Experimental model of cerebral palsy

The experimental model of CP (Strata et al. 2004) was based on a combination of perinatal anoxia and sensorimotor restriction of the hind limbs. The animals in the CP groups were subjected to two episodes of anoxia: one on the day of their birth (P0) and another on the first day of life (P1). Rat pups were placed in a chamber with a flow of 9 L/min of 100% N₂ (White Martins, Brazil) for 12 min. Between P2 and P28, sensorimotor restriction was administered for 16 h each day. Sensorimotor restriction was carried out using an orthosis that allowed only limited movements of the hip joint, with hind limbs extended. The elimination of feces and urine and maternal care were not affected during the immobilization period (Strata et al. 2004).

2.3. Measurement of bodyweight

The effects of experimentally induced CP and neonatal treatment with fluoxetine on the physical development of animals was evaluated by measuring postnatal bodyweight on various postnatal days (P1, P8, P14, P17, and P21) using a digital electronic scale (Marte, capacity of 1 kg and sensitivity of 0.01 g).

2.4. Evaluation of locomotor activity

Locomotor activity parameters were evaluated at P8, P14, P17, and P21, during the dark cycle, as previously described by Silva et al. (2016). A circular open field monitoring system (Ø1m) was used, with 30 cm high outer walls, black internal surfaces, and a black ethyl vinyl acetate base. A digital camera (VTR[®] 6638-CCTV System) with an infrared sensor set at a distance of 2.65 m from the ground was fixed to the ceiling. The animals were positioned at the center of the open field and each was filmed for 5 min. After registering the animal, a number of physical measurements were extracted using specific analysis software to obtain information about the animal's behavior. Ulead Video Studio[®] software was used for the filming. After filming, the video was analyzed using the ANY-maze[®] software package to obtain the following parameters: distance traveled (m), average speed (m/s), immobile time (s), average potency (mW), and kinetic energy (J). The parameters of average potency and kinetic energy were calculated based on the animal's mass (g), average speed (m/s), and time traveled (s), as described by Silva et al. (2016).

2.5. Transcardiac perfusion and removal of brain

On P29, the animals were deeply anesthetized with ketamine and xylazine (50 mg/kg) and transcardially perfused,

and 150 mL of saline solution in phosphate buffer was initially infused. The saline solution infusion was followed by an infusion of 200 mL fixative solution (4% paraformaldehyde). Once the fixative had been applied, the brain was removed from the skull and post-fixed in the same fixative solution overnight and subsequently placed in sucrose (20%) for 24–48 h and then stored in a freezer at -80° and cryosectioned.

2.6. Histomorphometric analysis of the S1

The brains were cryosectioned in 20 μ m thick coronal sections using a cryostat (Leica®). The sections obtained were stained with hematoxylin-eosine for the observation of histological characteristics and to show neurons and glial cells. The hematoxylin-eosine is a technique already consolidated in the literature that allows the evaluation of changes in neural tissues (Marcuzzo et al. 2010). Images of the areas of S1 that map to the hind limbs were captured and digitized (in 40 $\times$ for counting of cells in layer V) and identified using stereotaxic coordinates (-2.28 mm from the bregma). The microscopic fields were obtained using an OLYMPUS optical microscope model U-CMAD-2 (40 $\times$ objective) connected to an image capturing program. ImageJ version 1.8.0_172 was used for cell counting. Ten fields were counted in each cut (five in the right hemisphere and five in the left). The count was performed by a single properly trained evaluator. Neurons were distinguished by their size, which is generally larger and non-spherical in outline. Glial cells were distinguished by their smaller size and spherical appearance. The glia-neuron ratio was calculated as the number of glial cells per neuron to ascertain the precise relationship between the number of glial cells and neurons (Marcuzzo et al. 2010).

2.7. Statistical analysis

Data analysis and statistics were performed using R (The R Foundation for Statistical Computing, Institute for Statistics and Mathematics, Vienna, Austria). The results were expressed as mean $\pm$ standard error of the mean and analyzed by two-way analysis of variance (ANOVA) repeated measures and Tukey's post hoc tests for analysis of bodyweight, locomotor activity, and S1 cortex. The *p*-values are uncorrected but were significant by Tukey's HSD at *p* < 0.05. Possible associations between sets of variables within the same animals were assessed using principal component analysis (PCA) to reduce high dimensionality. PCA is defined as an orthogonal linear transformation of the dataset into a reduced subspace with new coordinates so that the variance is maximized on each new coordinate axis or principal component, thereby minimizing loss of information. A normalization method involving adjustment of mean to 0 and standard deviation (SD) to 1 was applied to allow comparisons of data with disparate values and variances. PCA was performed on three sets of variables: (1) bodyweight measured at five points in time; (2) locomotor activity analysis (distance traveled, average speed, average potency, kinetic energy, and immobile time); and (3) S1 analysis (number of neurons, number of glial cells, and glia/neuron index). A score that optimally differentiated the groups of animals was thus obtained for each rat and for each set of variables along the PC1- or *x*-axis. The aim was to cor-

relate the scores for the two variables for each rat, i.e., the scores that optimally characterized each animal for a given set of variables. These optimal scores were used to perform subsequent ANOVA for all six groups using R (Lacerda et al. 2019; Gouveia et al. 2020).

3. Results

3.1. Low-dose fluoxetine does not exacerbate CP-induced weight loss compared to high-dose fluoxetine

PCA was performed to quantify individual scores along the PC1-axis for the five variables dealing with bodyweight analysis during the lactation period (P1, P8, P14, P17, and P21) [$F(5, 132) = 10.78$; *p* < 0.005]. The score for the CPS group (-4 ± 0.5) differed from that of the CS group (10 ± 0.1). The score for the CPF10 group (-19 ± 0.2) also differed from that for the animals in the CPS group. No difference, however, was observed between the CPF5 group (0.5 ± 0.4) and the CPS group. These data suggest that CP and fluoxetine at a dose of 10 mg/kg reduced bodyweight (Tukey's post hoc test: *p* < 0.01; Fig. 1) but fluoxetine at a dose of 5 mg/kg had no specific impact. To complement the PCA data, the parameters were evaluated individually.

The animals' bodyweight increased gradually in all experimental groups from P1 to P21. Both experimentally induced CP and pharmacological manipulation with fluoxetine (10 mg/kg) reduced bodyweight gain when compared with the respective control groups (Tukey's post hoc test: *p* < 0.01; Fig. 2). The bodyweight of the CPS animals was significantly lower after P8 when compared to the CS group (Tukey's post hoc test: *p* < 0.01). The bodyweight of CF10 animals was lower compared with those in the CS group, from P14 onwards (Tukey's post hoc test: *p* < 0.01). No difference was found, however, between CF5 and CS (Tukey's post hoc test: *p*: N.S.), suggesting that 10 mg/kg of fluoxetine was needed to reduce bodyweight gain. Furthermore, between P8 and P21, the bodyweight of CPF10 rats was significantly lower than that of those in the CPS group (Tukey's post hoc test: *p* < 0.01), with no statistically significant difference between CPF5 and CPS (Tukey's post hoc test: *p*: N.S.). Experimentally induced CP and (or) fluoxetine administration at a dose of 10 mg/kg thus reduced bodyweight gain, while fluoxetine at a dose of 5 mg/kg had no specific impact. These data suggest that a low dose of fluoxetine (5 mg/kg) did not result in loss of bodyweight in these animals.

3.2. Low-dose fluoxetine is not associated with adverse effects on locomotion in animals with CP while high doses exacerbate these parameters

PCA was performed to quantify the individual scores along the PC1-axis for the five variables related to locomotor activity (distance traveled, average speed, average potency, kinetic energy, and immobile time). Experimentally induced CP delayed the development of locomotor activity on days P8 [$F(5, 100) = 2.868$; 0.01; Fig. 3A], P14 [$F(5, 100) = 7.631$; 0.001;

Fig. 1. PCA of animals' bodyweight at P1, P8, P14, P17, and P21 postnatal days of life in experimental groups CS (green; $n = 19$), CF10 (black; $n = 16$), CF5 (red; $n = 19$), CPS (brown; $n = 19$), CPF10 (blue; $n = 16$), and CPF5 (purple; $n = 18$).

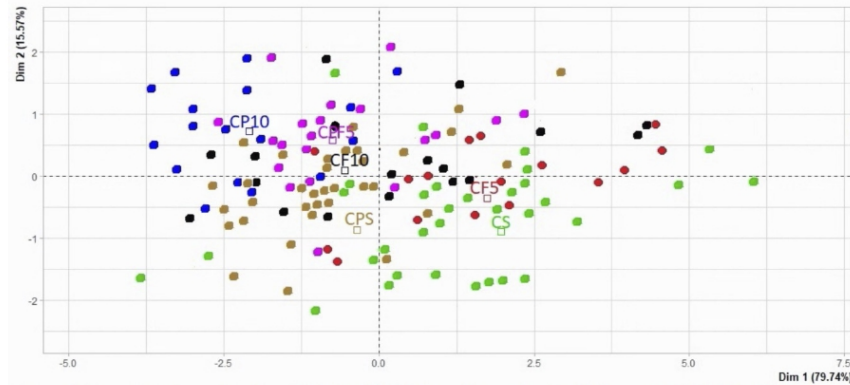


Fig. 2. Effect of CP and fluoxetine on animals' bodyweight (g) after birth in CS ($n = 19$), CF10 ($n = 15$), CF5 ($n = 19$), CPS ($n = 19$), CPF10 ($n = 16$), and CPF5 ($n = 18$) rats at P1, P8, P14, P17, and P21 postnatal days of life. Data expressed as mean and SEM. Two-way ANOVA followed by Tukey's test was used ($^*p < 0.01$).

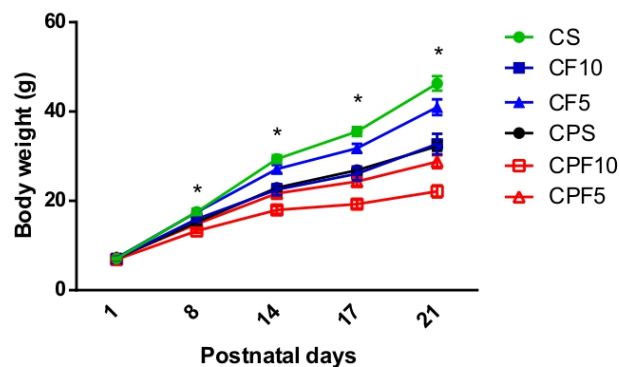


Fig. 3B], P17 [$F(5, 100) = 2.495$; 0.05 ; Fig. 3C], and P21 [$F(5, 100) = 5.396$; 0.001 ; Fig. 3D]. Treatment with a high dose of fluoxetine (10 mg/kg) further aggravated locomotor activity deficits in CP rats, especially on day P21 (-18.2 ± 9.1 ; Table 1). However, low-dose fluoxetine treatment (5 mg/kg) attenuated the deficits in locomotion in CP animals (-3.1 ± 17.2 ; Table 1), thereby highlighting the effectiveness of using a low dose of fluoxetine in rats with CP. Moreover, PCA showed that the variable that contributed most to the variability of the results on all days analyzed was average potency (Table 1).

The distance traveled and the average speed were lower in the CPS animals compared to the CS ones at P21 (Tukey's post hoc test: $p = 0.036$ and $p = 0.034$, respectively; Table 2). There was no significant difference between the control groups for these parameters (Tukey's post hoc test: p : N.S.). The administration of fluoxetine at a high dose (10 mg/kg) in CP ani-

mals reduced distance and average speed compared to the CPS group at P17 (Tukey's post hoc test: $p = 0.009$) and P21 (Tukey's post hoc test: $p = 0.032$ and $p = 0.029$, respectively). Treatment with 5 mg/kg of fluoxetine had no impact on distance and average speed parameters in animals with CP, suggesting no adverse effect of a low dose on these parameters (Tukey's post hoc test: p : N.S.).

Average potency did not show any significant difference between CPS and CS or between controls on any of the days covered by the study (Tukey's post hoc test: p : N.S.; Table 2). The CPF10 group showed lower potency at P17 and P21 compared to the CPS (Tukey's post hoc test: $p < 0.01$ and $p < 0.05$, respectively). Similarly, kinetic energy data suggest that groups that received 10 mg/kg doses registered a more greatly impaired response with regard to this parameter. The CF10 group exhibited lower kinetic energy compared

Fig. 3. PCA composed of five variables' characterizing of animals' locomotor activity at (A) P8, (B) P14, (C) P17, (D) P21 postnatal day of life in experimental groups CS (green; $n = 19$), CF10 (black; $n = 16$), CF5 (red; $n = 19$), CPS (brown; $n = 19$), CPF10 (blue; $n = 16$), and CPF5 (purple; $n = 18$).

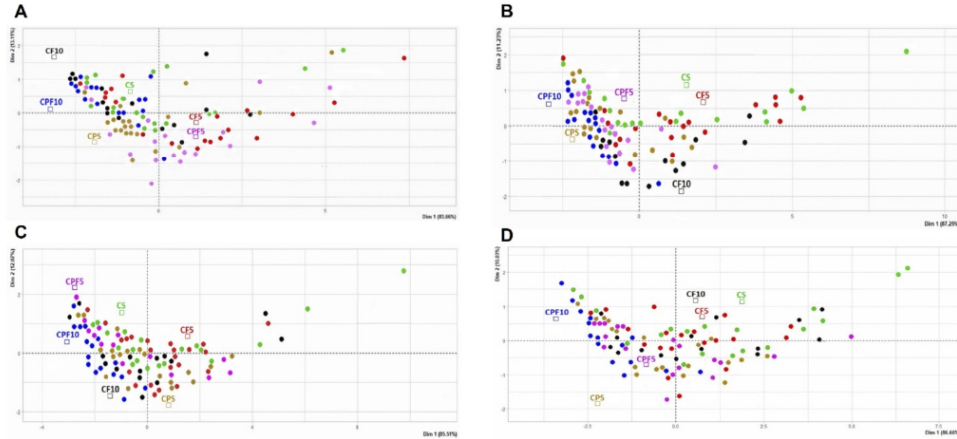


Table 1. PCA of the effects of fluoxetine on the locomotor activity of offspring.

	Initial eigenvalue						
Component	Total	% of variance	Cumulative %	Groups	Mean	SD	N
Day 8				Day 8			
Average speed (m/s)	41.828	836.559	836.559	CS	2.2	± 19.5	19
Traveled distance (cm)	0.6556	131.113	967.672	CF10	-4.6	± 15.1	15
Kinetic energy (J)	0.1103	22.054	989.726	CF5	10.8	± 25.8	19
Average potency (mW)	0.027	0.5405	995.131	CPS	-2.1	± 17.4	19
Immobile time (s)	0.0243	0.4869	100	CPF10	-10.9	± 9.9	16
				CPF5	5.2	± 19.8	18
Day 14				Day 14			
Average speed (m/s)	43.625	872.498	872.498	CS	10.6	± 21.5	19
Traveled distance (cm)	0.5614	112.282	98.478	CF10	3.6	± 16.4	15
Kinetic energy (J)	0.076	15.195	999.976	CF5	14.3	± 21.6	19
Average potency (mW)	0.0001	0.0023	999.999	CPS*	-5.8	± 11.3	19
Immobile time (s)	0	0.0001	100	CPF10	-11.4	± 6.9	16
				CPF5	-6.9	± 10.8	18
Day 17				Day 17			
Average speed (m/s)	42.753	855.051	855.051	CS	7.5	± 26.4	19
Traveled distance (cm)	0.6036	120.715	975.766	CF10	1.1	± 21.3	15
Kinetic energy (J)	0.1199	23.976	999.742	CF5	6.5	± 15.4	19
Average potency (mW)	0.0012	0.0249	999.991	CPS	-1.4	± 15.9	19
Immobile time (s)	0	0.0009	100	CPF10	-11.0	± 9.4	16
				CPF5	-2.9	± 14	18
Day 21				Day 21			
Average speed (m/s)	43.333	866.607	866.607	CS	11.3	± 27.9	19
Traveled distance (cm)	0.5015	100.295	966.902	CF10	6.7	± 21.2	15
Kinetic energy (J)	0.1186	23.714	990.616	CF5	4.1	± 13.3	19
Average potency (mW)	0.0469	0.9384	100	CPS	-3.3	± 15.2	19
Immobile time (s)	0	0	100	CPF10	-18.2	± 9.1	16
				CPF5	-3.1	± 17.2	18

Note: CS, control saline; CF10, control fluoxetine 10 mg/kg; CF5, control fluoxetine 5 mg/kg; CPS, CP saline; CPF10, CP fluoxetine 10 mg/kg; CPF5, CP fluoxetine 5 mg/kg. Data expressed as mean and SD ANOVA two-way RM. Tukey's test *CPS versus CS; $p < 0.05$.

Table 2. Evolution of locomotion parameters to P8, P14, P17, and P21 of offspring.

Component	Groups					
	CS (n = 19)	CF10 (n = 15)	CF5 (n = 19)	CPS (n = 19)	CPF10 (n = 16)	CPF5 (n = 18)
Day 8						
Average speed (m/s)	0.00826 (0.00119)	0.00740 (0.00134)	0.0112 (0.00119)	0.00737 (0.00119)	0.00481 (0.00129)	0.00963 (0.00122)
Traveled distance (cm)	2.478 (0.349)	2.207 (0.393)	3.322 (0.349)	2.171 (0.349)	1.439 (0.381)	3.098 (0.359)
Kinetic energy (J)	0.000884 (0.000234)	0.000602 (0.000263)	0.00147 (0.000234)	0.000621 (0.000234)	0.000202 (0.000255)	0.000953 (0.000240)
Average potency (mW)	0.00000295 (0.000000780)	0.00000201 (0.000000878)	0.00000491 (0.000000780)	0.00000207 (0.000000780)	0.000000674 (0.000000850)	0.00000318 (0.000000801)
Immobile time (s)	187.695 (15.641)	179.960 (17.603)	124.653 (15.641) ^a	155.047 (15.641)	216.081 (17.044) ^b	96.028 (16.069) ^d
Day 14						
Average speed (m/s)	0.0343 (0.00401)	0.0249 (0.00452)	0.0371 (0.00401)	0.0177 (0.00401)	0.00937 (0.00437)	0.0157 (0.00412)
Traveled distance (cm)	10.300 (1.200)	7.456 (1.350)	11.137 (1.200)	5.320 (1.200)	2.787 (1.307)	4.698 (1.233)
Kinetic energy (J)	0.0266 (0.00439)	0.0119 (0.00495) ^c	0.0254 (0.00439)	0.00559 (0.00439)	0.00106 (0.00479)	0.00368 (0.00451)
Average potency (mW)	0.0000888 (0.0000146)	0.0000395 (0.0000165)	0.0000848 (0.0000146)	0.0000186 (0.0000146)	0.00000353 (0.0000160)	0.0000123 (0.0000150)
Immobile time (s)	125.742 (12.716)	77.033 (14.311) ^e	98.963 (12.716)	141.889 (12.716)	137.756 (13.857)	134.061 (13.064)
Day 17						
Average speed (m/s)	0.0572 (0.00618)	0.0447 (0.00696)	0.0541 (0.00618)	0.0408 (0.00618)	0.0130 (0.00673) ^c	0.0389 (0.00635)
Traveled distance (cm)	17.134 (1.858)	13.432 (2.091)	16.212 (1.858)	12.216 (1.858)	3.910 (2.025) ^c	11.707 (1.909)
Kinetic energy (J)	0.0781 (0.0128)	0.0429 (0.0145)	0.0556 (0.0128)	0.0306 (0.0128)	0.00239 (0.0140) ^c	0.0275 (0.0132)
Average potency (mW)	0.000260 (0.0000428)	0.000143 (0.0000482)	0.000185 (0.0000428)	0.000102 (0.0000428)	0.00000797 (0.0000467) ^c	0.0000917 (0.0000440)
Immobile time (s)	122.111 (13.525)	77.033 (15.222)	121.195 (13.525)	118.874 (13.525)	137.756 (14.739)	122.506 (13.896)
Day 21						
Average speed (m/s)	0.0704 (0.00721)	0.0668 (0.00812)	0.0576 (0.00721) ^f	0.0484 (0.00721)	0.0207 (0.00786) ^b	0.0524 (0.00741)
Traveled distance (cm)	21.114 (2.194)	20.051 (2.469)	19.095 (2.194) ^f	14.522 (2.194)	6.220 (2.391) ^b	15.743 (2.254)
Kinetic energy (J)	0.145 (0.0177)	0.0967 (0.0199)	0.0797 (0.0177)	0.0480 (0.0177)	0.00845 (0.0193) ^b	0.0526 (0.0182)
Average potency (mW)	0.000483 (0.0000589)	0.000322 (0.0000663)	0.000266 (0.0000589)	0.000160 (0.0000589)	0.0000282 (0.0000642) ^b	0.000175 (0.0000605)
Immobile time (s)	113.363 (13.464)	115.273 (15.153)	123.395 (13.464)	118.979 (13.464)	160.900 (14.672)	118.533 (13.833)

Note: CS, control saline; CF10, control fluoxetine 10 mg/kg; CF5, control fluoxetine 5 mg/kg; CPS, CP saline; CPF10, CP fluoxetine 10 mg/kg; CPF5, CP fluoxetine 5 mg/kg. Data expressed as mean and SEM. ANOVA two-way RM. Tukey's test: CF5 versus CS: ^a $p < 0.01$. CPF10 versus CPS: ^b $p < 0.05$; ^c $p < 0.01$. CPF5 versus CPS: ^d $p < 0.05$. CF10 versus CS: ^e $p < 0.05$. CPS versus CS: ^f $p < 0.05$.

to the CS group at P14 (Tukey's post hoc test: $p < 0.01$). The CPF10 group also presented decreased kinetic energy at P17 and P21 compared to the CPS groups (Tukey's post hoc test: $p < 0.01$ and $p < 0.05$, respectively). These data suggest that the use of fluoxetine at a high dose (10 mg/kg) has a negative impact on potency and kinetic energy in CP animals, while the use of a low dose (5 mg/kg) does not have a negative impact on such parameters (Tukey's post hoc test: p : N.S.).

There was no difference in immobile time between the CPS and CS groups (Tukey's post hoc test: p : N.S.; Table 2), although the CS group exhibited a longer immobile time than either the CF5 group at P8 (Tukey's post hoc test: $p = 0.015$) or the CF10 at P14 (Tukey's post hoc test: $p = 0.033$). Administration of 10 mg/kg of fluoxetine increased immobile time at P8 in animals with CP compared to those in the CPS group (Tukey's post hoc test: $p = 0.026$). Treatment with fluoxetine 5 mg/kg reduced immobile time in CP animals in compari-

son with the CPS group at P8 (Tukey's post hoc test: $p = 0.026$), demonstrating that experimental CP and fluoxetine 10 mg/kg increased immobile time, while CP with fluoxetine 5 mg/kg reduced the time that the animals remained immobile.

3.3. Low-dose fluoxetine does not have negative effects like high-dose fluoxetine on histomorphometric parameters of the S1 in animals with CP

Neurons and glial cells were individually counted in both hemispheres of the S1, with neurons identified by their larger size and glial cells identified by their smaller size and spherical shape. Figure 4 shows the images obtained by microscopic examination of each area of the S1 hemispheres in the (A) CS, (B) CF10, (C) CF5, (D) CPS, (E) CPF10, and (F) CPF5 groups.

PCA was performed to quantify the individual scores along the PC1-axis involving the three variables related to histomorphometric analysis of the S1 cortex (number of neurons, number of glial cells, and glia/neuron index) [$F(5, 43) = 32.15$; $p < 0.005$]. The scores for animals in the CF10 group (18 ± 0.1) differed from that of those in the CS (-1 ± 0.2) and CF5 (-17 ± 0.2) groups. In the case of administration of fluoxetine in animals with CP, the score of the animals in the CPS group (6 ± 0.1) differed from that of those in the CPF10 (21 ± 0.6) and CPF5 (-9 ± 0.8) groups. These data suggest that treatment with fluoxetine at a dose of 10 mg/kg exacerbated the deleterious effects of CP on histomorphometric parameters of S1, while the same treatment at a dose of 5 mg/kg attenuated these same effects (Tukey's post hoc test: $p < 0.01$; Fig. 5). PCA further showed that the variable that contributed most to the variability of the results was the glia/neuron ratio (Fig. 5).

The number of neurons was higher in the CF5 group compared to the CS group (2216.6 ± 121 , 5, $p = 0.029$). However, no significant difference was observed comparing the animals with CP (Tukey's post hoc test: p : N.S.; Fig. 6A). These data suggest that low-dose fluoxetine stimulated neuron survival in the S1 in the control animals.

The number of glial cells was significantly higher in the CF10 group compared to the CS group (4312 ± 120.8 versus 3034.6 ± 162.9 ; $p = 0.003$). The number of glial cells was also greater in the CPF10 group than in the CPS group (3544.6 ± 303.5 ; $p = 0.004$). Conversely, the CPF5 group showed a smaller number of glial cells compared to the CPS group (Tukey's post hoc test: $p = 0.002$; Fig. 6B). These results suggest that the increase in the number of glial cells on treatment with fluoxetine (10 mg/kg) was the result of reactive astrogliosis, which may be associated with neuroinflammation, while a low dose of fluoxetine (5 mg/kg) attenuated this effect.

The glia/neuron ratio was calculated as the quotient of the number of glial cells and the number of neurons. The CPS group (1.73 ± 0.11) presented a higher glia/neuron ratio compared to the CS group (1.38 ± 0.05), showing the influence of CP on this parameter (Tukey's post hoc test: $p < 0.001$; Fig. 6C). Furthermore, a high dose of fluoxetine CF10 (2.18 ± 0.06) increased the glia/neuron ratio compared to the CS group (Tukey's post hoc test: $p < 0.001$). Conversely, this parameter was lower in the CF5 (0.88 ± 0.07) compared to the CS group

(Tukey's post hoc test: $p < 0.001$). In those animals with CP, the CPF10 group (2.28 ± 0.03) presented a higher glia/neuron index compared to the CPS group, while this parameter was lower in the CPF5 group (1.08 ± 0.07) compared to the CPS one (Tukey's post hoc test: $p < 0.001$). These results suggest that low-dose fluoxetine (5 mg/kg) induced a protective response by reducing the glia/neuron ratio in the S1.

4. Discussion

The present study is the first to evaluate the therapeutic potential of fluoxetine in minimizing S1 impairments and locomotor activity in a rat model of CP. Our main findings show that experimental CP leads to limited bodyweight, delayed development of locomotor activity, and a higher glia/neuron ratio. The data obtained from control animals treated with fluoxetine showed that (i) a high dose (10 mg/kg) limited bodyweight, impaired locomotor activity parameters, and increased the number of glial cells, and the glia/neuron ratio; (ii) a low dose (5 mg/kg) did not affect bodyweight, did not impair locomotor activity, increased the number of neurons and reduced the number of glial cells, and the glia/neuron index. The data obtained from CP animals treated with fluoxetine showed that (i) a high dose (10 mg/kg) limited bodyweight, aggravated locomotor activity development, increased the number of glial cells, and glia/neuron index; and (ii) a low dose (5 mg/kg) was not associated with adverse effects in relation to these parameters. These findings demonstrate that pharmacological manipulation of serotonin by administration of a low dose of fluoxetine (5 mg/kg) may avoid the negative outcomes of high-dose fluoxetine in relation to histomorphometric and locomotion parameters, while producing no protective effect in rats with CP.

CP limited bodyweight gain during the lactation period. Previous studies have shown that the CP model used impaired somatic growth and bodyweight gain (Marcuzzo et al. 2010). Structural changes in the musculoskeletal system, such as muscle atrophy and low bone density resulting from sensorimotor restriction, have been found to influence bodyweight gain during somatic development (Delcour et al. 2018; Buratti et al. 2019). Furthermore, changes in the control of orofacial muscles in animals with CP models also contribute to impairment of sucking and swallowing, reducing food intake and causing low nutritional status (Lacerda et al. 2017). We, therefore, suggest that induction of CP leads to a decline in food consumption and consequent reduced bodyweight.

We found that the administration of a high dose of fluoxetine (10 mg/kg), whether or not in combination with CP, reduced bodyweight gain in comparison to the CPS, CPF5, and CS groups. These results are corroborated by those of another study, in which the administration of selective serotonin reuptake inhibitor at a high dose (over 10 mg/kg) reduced the bodyweight of newborn rats (Deiró et al. 2006; Silva et al. 2015). Previous studies have shown that the use of selective serotonin reuptake inhibitors reduces food intake in animals due to the inhibitory action of serotonin on satiety (Ishii et al. 2003; Deiró et al. 2006). Serotonin then acts on the arcuate nucleus by increasing pro-opiomelanocortin synthesis (anorectic neuropeptide) through serotonin 5-HT2C

Fig. 4. Microscopic images of S1 area stained with hematoxylin-eosine showing neurons (blue) larger and nonspherical in shape and glial cells (yellow) identified by their spherical shape and smaller size. (A) CS ($n = 11$), (B) CF10 ($n = 5$), (C) CF5 ($n = 8$), (D) CPS ($n = 13$), (E) CPF10 ($n = 4$), and (F) CPF5 ($n = 8$). Scale bar = 100 μm .

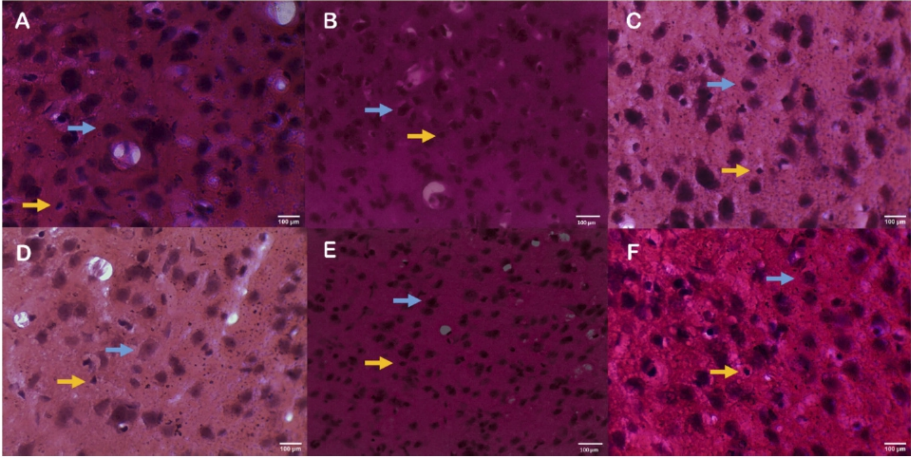


Fig. 5. PCA of number of neurons, number of glial cells, and glia/neuron ratio at P29 postnatal day of life. Groups: CS (green; $n = 11$), CF10 (black; $n = 5$), CF5 (red; $n = 8$), CPS (brown; $n = 13$), CPF10 (blue; $n = 4$), and CPF5 (purple; $n = 8$).

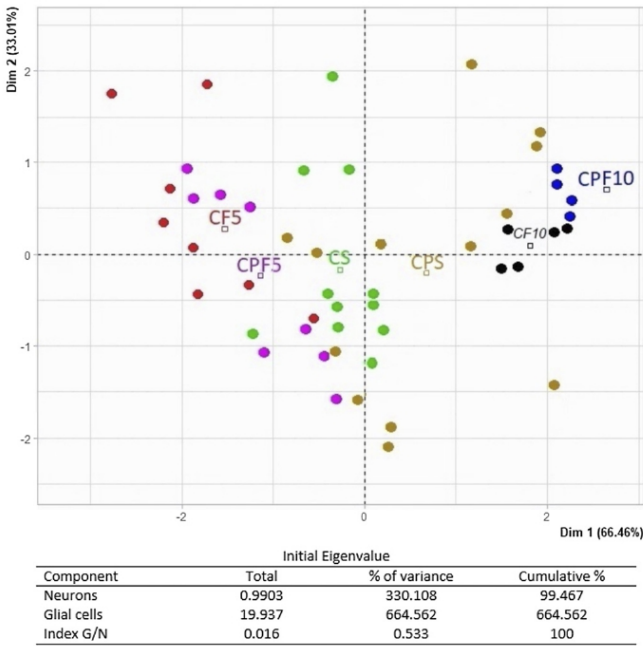
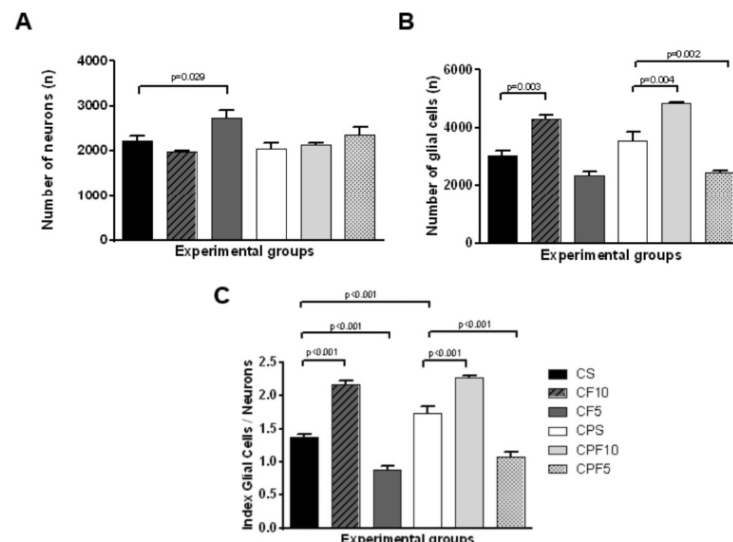


Fig. 6. (A) Number of neurons per unit area, (B) number of glial cells per unit area, and (C) glia/neuron ratio in experimental groups CS ($n = 11$), CF10 ($n = 5$), CF5 ($n = 8$), CPS ($n = 13$), CPF10 ($n = 4$), and CPF5 ($n = 8$) at P29 postnatal day of life. Data expressed as mean and SEM. Two-way ANOVA followed by Tukey's test was used.



receptor and inhibiting the action of neuropeptide Y/agouti-related protein (orexigenic neuropeptides) through serotonin 5-HT_{1B} receptor, thereby reducing food intake (Barros et al. 2018). The hypothalamus is thus an important region for food intake (Lima et al. 2020) and acts by regulating the activity of the hypothalamic-pituitary-adrenal axis through stressors (Stamper et al. 2017). It is possible that the CP model used in our study had a direct impact on the modulation of the serotonergic system in the hypothalamus, although further studies are needed to assess the effect of CP on this system.

On the other hand, some previous studies have found that the administration of citalopram or fluoxetine at a low dose (5 mg/kg) in neonatal rats did not produce any significant difference compared to the control groups, similar to our findings (Deiró et al. 2004, 2006). Furthermore, L-tryptophan (50 mg/kg), a precursor of serotonin, also produced no difference in bodyweight and food consumption in animals subjected to the same CP model (Lacerda et al. 2019). In view of this, we suggest that L-tryptophan acts similarly to a low dose of fluoxetine (5 mg/kg), since only a small portion of the L-tryptophan absorbed, about 1%–2%, is converted into serotonin (Le Floch et al. 2011). These data thus corroborate our findings, demonstrating that manipulation of the serotonergic system using a low dose of fluoxetine (5 mg/kg) maintains the bodyweight of animals by preserving food consumption.

In combination with decrease in bodyweight, exposure to CP resulted in deficits in functional aspects of motor performance. Our study found a reduction in the distance traveled and average speed in animals subjected to anoxia with senso-

rimotor restriction. Previous studies have also demonstrated the impact that CP has on these parameters and the impaired locomotor activity that ensues (Sab et al. 2013; Stigger et al. 2011; Kim et al. 2017). The use of sensorimotor restriction showed that the animals tend to develop an extensor pattern in the joints of the hindlimbs associated with plantar flexion (Strata et al. 2004; Delcour et al. 2018). Furthermore, muscle atrophy may contribute to the alterations in motor function, especially atrophy of the soleus muscle and a decrease in the quantity of type-I fibers, as found in CP (Stigger et al. 2011; Silva et al. 2016).

The combination of high-dose fluoxetine and CP results in decreases in relation to speed, distance, potency, kinetic energy, and immobile time compared to the CPS and CPF5 groups. One recent systematic review reports that administration of 10 mg/kg of fluoxetine reduces the number of myocytes and sectional area of muscle fiber in young and adult rats (Visco et al. 2018). It can thus be suggested that experimentally induced CP in combination with administration of a high dose of fluoxetine may cause musculoskeletal disorders, affecting parameters related to locomotion. A reduction in muscle weight and soleus muscle area has also been observed in male rats receiving 10 mg/kg of fluoxetine (Visco et al. 2020). According to our findings, high neonatal doses (>10 mg/kg) of fluoxetine exacerbated functional deficits in adult rats with hypoxic-ischemic brain injury (Chang et al. 2006).

Neonatal administration of low doses (5 mg/kg) of fluoxetine did not impair the development of locomotor activity in

CP rats except with respect to immobile time at eight days. A previous study has found that low-dose fluoxetine (5 mg/kg) has no effect on locomotion in the open field in rats and has no adverse effects on long-term behavior. Furthermore, low-dose fluoxetine mitigated the HI effect in another behavioral test (Morris water maze) in rats (Chang et al. 2006). The use of selective serotonin reuptake inhibitors, however, does appear to influence the musculoskeletal system by altering its function, structure, and metabolism (Visco et al. 2018). Exposure to fluoxetine during early life has been observed to increase mitochondrial respiratory activity and the presence of oxidative stress (Visco et al. 2018). On the other hand, exposure to fluoxetine may have adverse effects such as agitation and anxiety (Perez-Caballero et al. 2014), which may have an effect on locomotion. A low dose of fluoxetine seems to be involved in the process of restoration of this system, mitigating the motor deficits found in CP. However, since our findings showed no effect of a low dose of fluoxetine (5 mg/kg) on locomotor activity and only minor effects on S1 parameters, future studies are needed to provide more exact identification of the most effective dose for reducing locomotor activity impairment.

The CP model used in the present study did not affect the number of neurons, although it did increase the number of the glia/neuron index in the S1 cortex. Our data corroborate those of previous studies that examined the effects of CP based on sensorimotor restriction in the S1 and found no differences in the number of neurons (Marcuzzo et al. 2010; Santos et al. 2017). The increase in the number of glial cells observed in CP may be related to the anoxic insult that stimulates neuroinflammation. Several models of CP have shown changes in the nervous system, such as early activation of microglia and oligodendrocytes, resulting in hypomyelination and neuron loss in crucial parts of the nervous system, including the S1 (Rocha-Ferreira and Hristova 2016; Lacerda et al. 2017). Furthermore, the glia/neuron index showed a significant increase in the number of glial cells per neuron in CPF10 animals, thereby reaffirming the pathological findings were more severe when both CP and fluoxetine were present.

The number of neurons did not change in the CF10 group but increased in the CF5 group compared to CS, indicating that a low dose of fluoxetine promotes neuron survival. Corroborating our results, another study found that the administration of high doses of fluoxetine produced an adverse effect on neurogenesis, with consequent exacerbation of functional deficits in hypoxic-ischemic animals (Chang et al. 2006). Lower doses (<5 mg/kg), however, tend to induce neurogenesis and neuroplasticity in hypoxic-ischemic rats (Chang et al. 2006; Wang et al. 2010). Many studies corroborate the hypothesis that fluoxetine plays an important role in angiogenesis and axonal and dendritic reorganization (Benninghoff et al. 2012; Sun et al. 2017). We therefore suggest that, in low doses, fluoxetine is capable of stimulating the serotonergic system in animals with CP, producing a protective response in the S1. These histomorphometric findings were not, however, found to be associated with improvements in locomotion in animals with CP.

Insults to the central nervous system during early life trigger early immune activation, which can lead to neuronal and

cognitive changes (Turano et al. 2017). An increase was thus observed in the number of glial cells and in the glia/neuron index in groups CPF10 and CF10 in relation to the respective controls. Previous studies have also demonstrated that experimental CP based on sensorimotor restriction increased the number of glial cells (Marcuzzo et al. 2010; Santos et al. 2017). Other studies that replicate CP lesions using inflammation (lipopolysaccharide administration) during pregnancy found an increase in the number of astrocytes in the brains of the young rats (Zhengwei et al. 2000; Kim et al. 2014). In view of this, we suggest that the combination of CP lesions with a high dose of fluoxetine exacerbates the number of glial cells, causing reactive astrogliosis, which is a proinflammatory phenomenon, while a low dose of fluoxetine plays non-harmful effects.

Currently, manipulation of the serotonergic system with selective serotonin reuptake inhibitors (Pariente et al. 2001; Chollet et al. 2011) is used to increase the quantity of serotonin in the synaptic cleft and this seems to improve the motor function of individuals affected by stroke (Batsikadze et al. 2013). We, therefore, suggest that treatment with a low dose of fluoxetine may have positive repercussions for the neuromotor system in CP. It is worth mentioning as a limitation of the present article the fact that the hematoxylin-eosine stain used here for histomorphometric analysis of the S1 is a sub-optimal technique for visualizing nerve tissue. The hematoxylin-eosine technique is also similar to other techniques already well covered in the literature that obtained similar relevant information on how CP induction can damage nerve tissue (Marcuzzo et al. 2010; Gouveia et al. 2020). There is a need, however, to conduct further studies using the most effective methods and the specific gold standard for analysis of nerve tissue structures.

The use of selective serotonin reuptake inhibitors, including fluoxetine, constitutes a promising CP treatment strategy and demonstrates the important neuroprotective, anti-inflammatory, and neuroregenerative properties of these substances (Budhdeo and Rajapaksa 2011; McCann et al. 2014; Sun et al. 2017). We found that a low dose of fluoxetine (5 mg/kg) was not associated with adverse effects on locomotor activity, while it tended to provide only slight protection of the S1, as evaluated by histomorphometric parameters. It should be noted that the protective effects on the S1 may influence other parameters modulated by the S1, including sensorimotor reflexes, spontaneous activity, processing of sensory information, and behavioral sensorial skills. Our study thus presents a pioneering investigation into the therapeutic potential of fluoxetine in CP and has produced data that could be extrapolated to clinical settings. We should stress that there is still no evidence from studies investigating the safety of different doses of fluoxetine in human patients with CP in a neonatal intensive care unit. Individuals with CP may be more vulnerable to excess serotonin in the nervous system. Therefore, it is important to be careful when recommending the use of combined drugs, as they can trigger serotonin syndrome (Schindzielorz 2022). Thus, it is necessary to determine the effective dose needed to induce neuroprotection and minimize impairment caused by CP.

It is essential to mention that our study was the first to investigate the effect of different doses of fluoxetine in terms of attenuating brain and locomotor deficits in an experimental model of CP. To date, there are no studies evaluating the effects of fluoxetine in CP animals; therefore, it is necessary to develop future studies that explore different types of manipulation of the serotonergic system using other dosages and intervention periods. In addition, a comprehensive analysis of the biology of muscle tissue and other areas of the brain, such as the cortex axis motor and hypothalamus, is essential to clarify the possible signaling pathways involved and the behavioral findings. Finally, this study demonstrates its relevant potential for human therapy, providing valuable information that expresses the importance of investigating neuroprotective strategies, such as manipulation of the serotonergic system, which may suggest alternatives for better functional results in children with CP.

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Data availability

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Author information

Author contributions

Conceptualization: AET

Formal analysis: DCL, ATBdM, FMM

Investigation: GLdA, CMSdSC, ATBdM

Project administration: GLdA, AET

Resources: RM, AET

Supervision: RM, AET

Validation: CMSdSC

Visualization: DBV

Writing – original draft: GLdA, DCL

Writing – review & editing: DBV

Competing interests

The authors declare there are no competing interests.

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ANEXO A- CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA



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Recife, 04 de outubro de 2022

Ofício nº 73/22

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE

Prof. Ana Elisa Toscano Meneses da Silva Castro

Centro Acadêmico de Vitória

Processo nº0082/2022

Certificamos que a proposta intitulada “**Efeitos do tratamento neonatal com resveratrol sobre o desenvolvimento neurocognitivo e o comportamento emocional de ratos submetidos à paralisia cerebral**”. Registrado com o nº 0082/2022 sob a responsabilidade da **Prof. Ana Elisa Toscano Meneses da Silva Castro** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 27/09/2022

Finalidade	() Ensino (x) Pesquisa Científica
Vigência da autorização	27/09/2022 a 25/06/2026
Espécie/linhagem/raça	Rato heterogênico wistar
Nº de animais	105 animais
Peso/Idade	220-250g (90-120 dias) 5-80g (1-29 dias)
Sexo	(30 fêmeas) e (75 machos)
Origem: Biotério de Criação	Biotério de criação do Departamento de Nutrição da UFPE.
Destino: Biotério de Experimentação	Biotério de criação do Departamento de Nutrição da UFPE.

Atenciosamente

Vice-presidente CEUA

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