

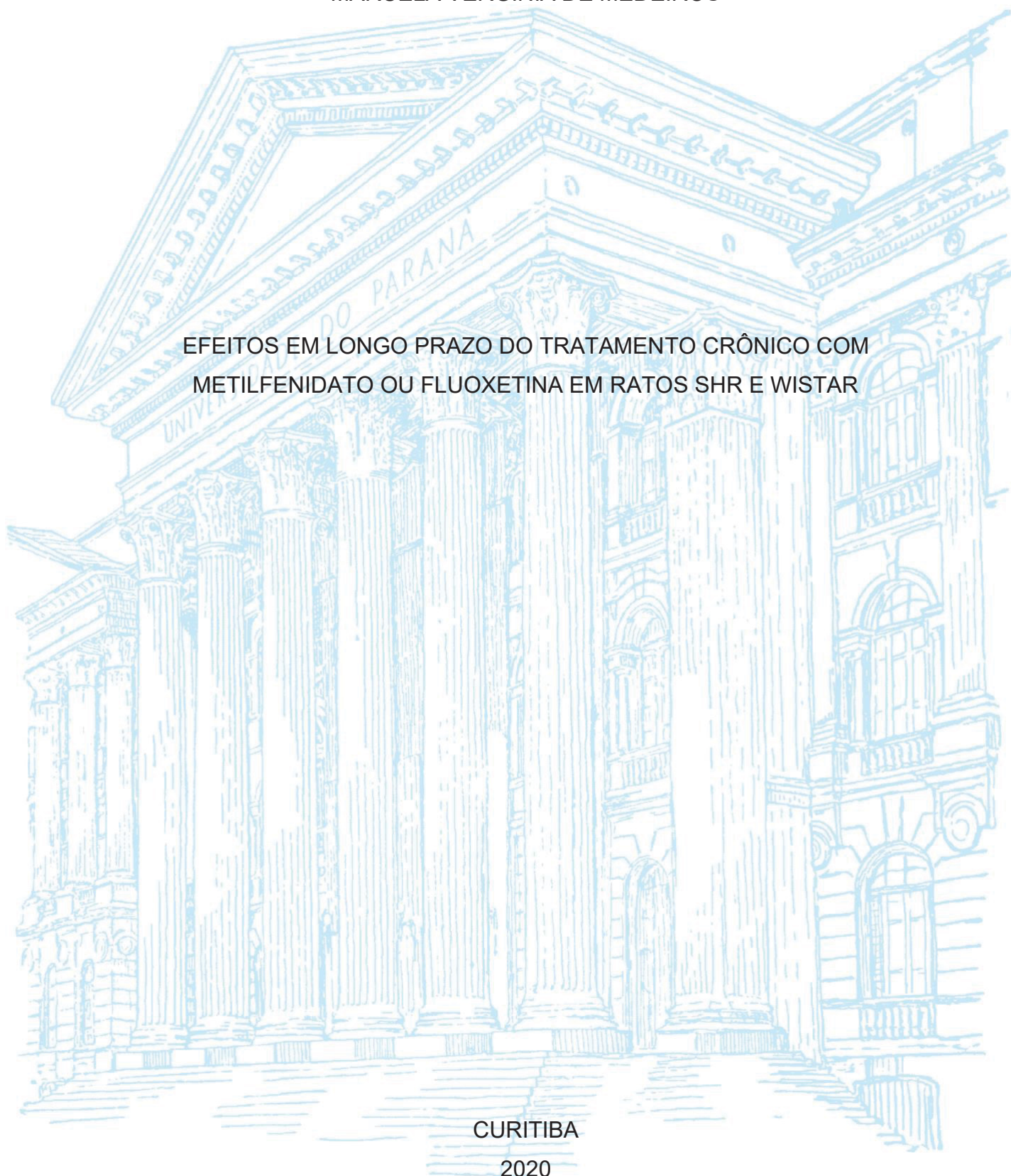
UNIVERSIDADE FEDERAL DO PARANÁ

MARCELA VERGINIA DE MEDEIROS

EFEITOS EM LONGO PRAZO DO TRATAMENTO CRÔNICO COM
METILFENIDATO OU FLUOXETINA EM RATOS SHR E WISTAR

CURITIBA

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METILFENIDATO OU FLUOXETINA EM RATOS SHR E WISTAR

Dissertação apresentada ao Programa de Pós-Graduação em Fisiologia, Setor de Ciências Biológicas, Universidade Federal do Paraná como requisito parcial à obtenção do título de Mestre em Fisiologia.

Orientador: Prof. Dr. Bruno Jacson Martynhak

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ATA DE SESSÃO PÚBLICA DE DEFESA DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE MESTRE EM FISIOLÓGIA.

No dia vinte e oito de fevereiro de dois mil e vinte às 14:00 horas, na sala 103, Auditório do Departamento de Fisiologia da UFPR do Setor de CIÊNCIAS BIOLÓGICAS da Universidade Federal do Paraná, foram instaladas as atividades pertinentes ao rito de defesa da dissertação da mestranda **MARCELA VERGINIA DE MEDEIROS**, intitulada: **EFEITOS EM LONGO PRAZO DO TRATAMENTO CRÔNICO COM METILFENIDATO OU FLUOXETINA EM RATOS SHR E WISTAR**, sob orientação do Prof. Dr. BRUNO JACSON MARTYNHAK. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação da Universidade Federal do Paraná em FISIOLÓGIA foi constituída pelos seguintes Membros: BRUNO JACSON MARTYNHAK (UNIVERSIDADE FEDERAL DO PARANÁ), LUANA FISCHER (UNIVERSIDADE FEDERAL DO PARANÁ), CLAUDIO DA CUNHA (UNIVERSIDADE FEDERAL DO PARANÁ). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga do título de mestre está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, BRUNO JACSON MARTYNHAK, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

Observações: _____

Curitiba, 28 de Fevereiro de 2020.


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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FISIOLÓGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **MARCELA VERGINIA DE MEDEIROS**, intitulada: **EFEITOS EM LONGO PRAZO DO TRATAMENTO CRÔNICO COM METILFENIDATO OU FLUOXETINA EM RATOS SHR E WISTAR**, sob orientação do Prof. Dr. BRUNO JACSON MARTYNHAK, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de Mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 28 de Fevereiro de 2020.


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Presidente da Banca Examinadora


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“Tudo que vale a pena ter, vale a pena o esforço de trabalhar para ter.”

Xaphania, A Luneta Âmbar, Philip Pullman

RESUMO

O Transtorno de Déficit de Atenção e Hiperatividade (TDAH) atinge aproximadamente 5% de crianças e adolescentes em idade escolar. Seus principais sintomas são hiperatividade, desatenção e impulsividade. Estimulantes, principalmente o metilfenidato (MPH), são o tratamento padrão ouro para o transtorno. No Brasil, apenas antidepressivos, como a fluoxetina (FLX), são disponibilizados a pronta entrega nos serviços públicos de saúde para o tratamento do TDAH. Ambos os medicamentos modulam a neurotransmissão, e as crianças que os recebem ainda estão em fase de neurodesenvolvimento. As consequências em longo prazo que tratamento crônico para o TDAH pode causar ainda não estão claras. A maioria dos estudos que investigaram esse aspecto não utilizaram modelos animais validados para o TDAH. No presente estudo, as consequências em longo prazo do tratamento crônico com MPH ou FLX foram avaliados nos ratos espontaneamente hipertensos (SHR, do inglês), um modelo validado de TDAH, e comparado com ratos Wistar (WIS). Primeiramente, diferenças basais entre os SHR e os WIS foram determinadas. Ratos SHR tiveram prejuízos de habituação no teste de campo aberto e apresentaram menor comportamento tipo-ansioso no teste de labirinto em cruz elevado quando comparado com ratos WIS. Com relação ao tratamento crônico, ratos SHR e WIS foram tratados tanto com MPH (2 mg/kg, por gavagem), FLX (5 mg/kg, por gavagem) ou salina durante os DPN 28-55. Após um período de *washout*, os animais passaram pelos testes comportamentais. Tanto o tratamento com MPH quanto com FLX não provocaram efeitos em longo prazo no comportamento emocional, medido nos testes de labirinto em cruz elevado e natação forçada, na cognição, avaliada no teste de reconhecimento de objetos e na locomoção, medida no teste de campo aberto, em ambas as linhagens. Entretanto, após o tratamento tanto com MPH quanto com FLX, não foram observadas diferenças comportamentais entre SHR e WIS nos testes realizados. Dado essas condições, o tratamento crônico e precoce com MPH e FLX parece não levar a prejuízos no comportamento emocional e na cognição na vida adulta após a descontinuação do tratamento.

Palavras-chave: TDAH, metilfenidato, fluoxetina, longo prazo, comportamento emocional, cognição.

ABSTRACT

Attention deficit hyperactivity disorder (ADHD) affects nearly 5% of school-age children and adolescents. It is characterized by hyperactivity, inattention, and impulsivity. Stimulants, mainly methylphenidate (MPH), are the gold-standard treatment for the disorders. In Brazil, only antidepressants, such as fluoxetine (FLX), are promptly delivered in public health services for treating ADHD. Both drugs modulate neurotransmission, and the children receiving them are still under neurodevelopment. The long-term consequences the chronic treatment of ADHD may cause are still unclear. Most studies addressing this question did not use validated animal models of ADHD. In the present study, the long-term consequences of juvenile chronic treatment with either MPH or FLX were evaluated in both spontaneously hypertensive rats (SHR), a validated model of ADHD, and Wistar rats (WIS), a normal strain. First, we determined baseline differences between SHR and WIS. SHR had impaired habituation in the open field test and presented a decreased anxiety-like behavior in the elevated plus maze compared to WIS. Regarding chronic treatment, SHR and WIS rats were treated with MPH (2 mg/kg, by oral gavage), FLX (5 mg/kg, by oral gavage) or saline during PND 28-55. After a washout period, animals went through behavioral tests. Neither treatment with MPH nor FLX provoked any long-term effect in emotional behavior, measured in the elevated plus maze and forced swim test, cognition, evaluated in the object recognition test, and locomotion, measured in the open field test, in both strains. However, differences in behavior between SHR and WIS were not observed after treatment with both drugs tested. Given these results, juvenile chronic treatment with either MPH or FLX did not lead to impairments on emotional behavior and cognition in adulthood after a washout period.

Key words: ADHD. SHR methylphenidate, fluoxetine, long-term, emotional behavior, cognition

LISTA DE SIGLAS

6-OHDA – 6-hidroxidopamina

ADHD – *Attention deficit hyperactivity disorder* (Transtorno de Déficit de Atenção e Hiperatividade)

ANOVA – Análise de Variância

ANVISA – Agência Nacional de Vigilância Sanitária

DAT – *Dopamine Transporter* (Transportador de Dopamina)

DSM-5 – Manual Diagnóstico e Estatístico 5

EPM – *Elevated Plus Maze* (Labirinto em Cruz Elevado)

FDA – *Food Drug Administration*

FLX – Fluoxetina

FST – *Forced Swim Test* (Teste da Natação Forçada)

MPH – Metilfenidato

OFT – *Open Field Test* (Teste de Campo Aberto)

ORT – *Object Recognition Test* (Teste de Reconhecimento de Objetos)

PND – *Post-natal Day* (Dia Pós-natal)

SHR – *Spontaneously Hypertensive rats* (Ratos Espontaneamente Hipertensos)

SUS – Sistema Único de Saúde

TDAH – Transtorno de Déficit de Atenção e Hiperatividade

WIS – Linhagem Wistar

WKY – Linhagem Wistar Kyoto

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1 INTRODUÇÃO

1.1 TRANSTORNO DE DÉFICIT DE ATENÇÃO E HIPERATIVIDADE (TDAH)

O transtorno de déficit de atenção e hiperatividade (TDAH) é uma doença neurocomportamental que atinge de 3 a 5% de crianças em idade escolar no Brasil e no mundo (ARRUDA et al., 2015; POLANCZYK et al., 2015). Apesar de ser mais comum em crianças, adultos também podem apresentar TDAH. A prevalência em adultos é por volta de 2.5% (SIMON et al., 2009). Os sintomas característicos do TDAH são altos níveis de hiperatividade, impulsividade e/ou desatenção, podendo se manifestar como predominantemente hiperativo/impulsivo, predominante desatento ou combinado (APA, 2013). Essas manifestações podem mudar de acordo com o desenvolvimento do paciente: a hiperatividade e impulsividade são mais aparentes durante a infância, enquanto na vida adulta predomina a desatenção (WILLCUTT et al., 2012a).

O diagnóstico é feito por observação comportamental. Segundo o Manual Diagnóstico e Estatístico 5 (DSM-5), é necessário que o paciente apresente pelo menos seis sintomas de desatenção e/ou seis sintomas de hiperatividade/impulsividade que impactem negativamente o desempenho pessoal, acadêmico e profissional por pelos menos seis meses, sendo manifestados antes dos 12 anos, no caso de TDAH infantil, em pelo menos dois contextos diferentes (por exemplo na escola e em casa) e devem causar prejuízos funcionais significativos (APA, 2013). Em crianças e adolescentes, meninos geralmente são mais diagnosticados com o transtorno que meninas, já que estas apresentam comportamento predominantemente desatento, um comportamento menos externalizado e de diagnóstico mais difícil (WILLCUTT, 2012b). Por serem menos diagnosticadas, meninas recebem menos o tratamento necessário que meninos (MOWLEM et al., 2019).

Ainda não se sabe qual a etiologia do TDAH. Porém, sabe-se que é um transtorno multifatorial, ou seja, tanto fatores ambientais quanto genéticos estão associados ao seu desenvolvimento. A herdabilidade do TDAH é de aproximadamente 70%, sendo um dos transtornos psiquiátricos com maior influência genética (FARAONE et al., 2005). Apesar disso, nenhum único gene foi associado ao transtorno, indicando que vários genes devem participar do seu desenvolvimento,

cada um com uma pequena contribuição (THAPAR et al., 2013). Vários fatores ambientais também foram ligados ao TDAH, como uso cigarro durante a gravidez, nascimento prematuro ou com baixo peso, deficiência de nutrientes, como zinco e ferro, e negligência parental (TARVER et al., 2014).

Além disso, a estrutura encefálica também parece influenciar o desenvolvimento do transtorno. Estudos de neuroimagem observaram que pacientes com TDAH têm redução global do volume encefálico e redução de volume em regiões específicas, como o núcleo caudado, o corpo caloso, o cerebelo e o córtex pré-frontal quando comparados com pacientes saudáveis (NAKAO et al., 2011; VALERA et al., 2007). Ainda, pacientes com o transtorno apresentaram atraso na maturação em regiões do córtex pré-frontal envolvidas em funções cognitivas e planejamento motor, que estão comprometidas no TDAH (SHAW et al., 2007). Outras regiões que também podem estar envolvidas são o córtex parietal e o córtex cingulado anterior dorsal, apesar de não haver muitas evidências do envolvimento dessas regiões no distúrbio. Entretanto, são regiões muito importantes na neurobiologia da atenção e cognição, e fazem conexões com outros locais envolvidos no TDAH, como o córtex pré-frontal (BUSH, 2010) (Figura 1). Pacientes com TDAH também apresentam menor espessura cortical, menor conectividade do pedúnculo cerebelar e do feixe cingulado e menor fortalecimento de conexões frontoestriatais e do fascículo longitudinal superior direito quando comparado com pacientes controles (SHAW et al., 2006; ASHTARI et al., 2005; LISTON et al., 2011).

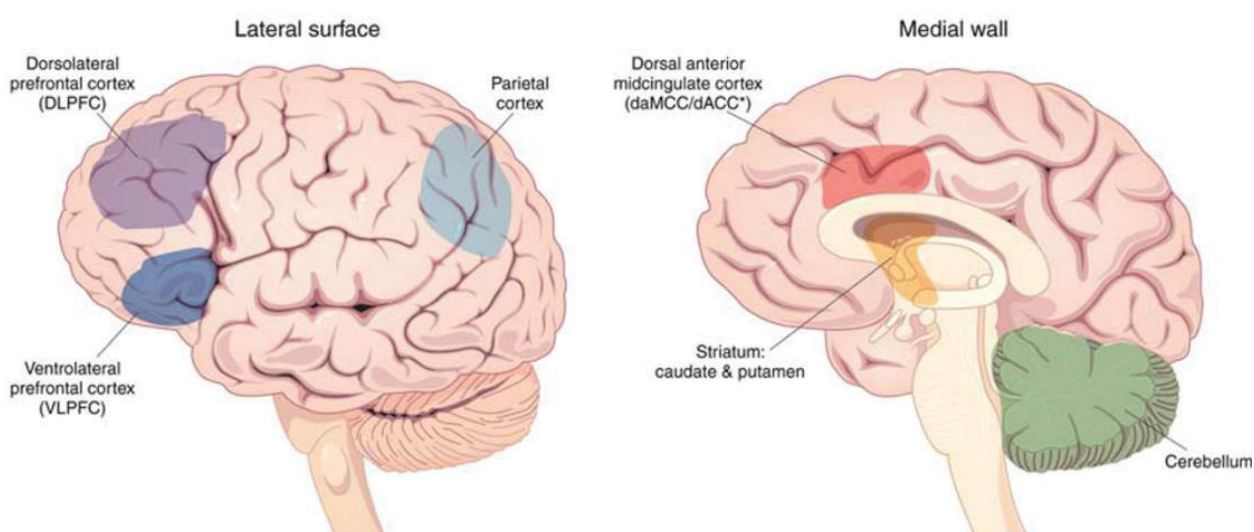


Figura 1 – Regiões encefálicas envolvidas no TDAH. Estão aqui representadas o córtex pré-frontal (roxo e azul escuro), o córtex parietal (azul claro), o córtex cingulado anterior dorsal (vermelho), o estriado (amarelo) e o cerebelo (verde), todas regiões importantes nas interações de atenção e cognitivas. Retirado de Bush, 2010.

Catecolaminas, como dopamina e noradrenalina, são importantes moduladores das funções do córtex pré-frontal e estão envolvidas no estado de alerta dos indivíduos. Tanto a falta quanto o excesso desses neurotransmissores no córtex pré-frontal pode levar a disfunções de comportamento, sendo que a falta de catecolaminas pode levar aos sintomas de TDAH (ARNSTEN & PLISZKA, 2010). Estudos já observaram que pacientes com TDAH apresentam menor densidade de receptores para dopamina em várias regiões encefálicas quando comparados com indivíduos saudáveis (SHARMA & COUTURE, 2014) e que o bloqueio do receptor α_{2A} de noradrenalina em macacos leva a comportamentos tipo-TDAH (MA et al., 2003; MA et al., 2005). Esses resultados sugerem que a patofisiologia do TDAH está ligada com a hipofunção de catecolaminas. Os tratamentos utilizados no TDAH, que serão descritos posteriormente, regulam esse balanço de catecolaminas e aliviam os sintomas. Apesar de todas as evidências, não há nenhum marcador bioquímico ou estrutural para o TDAH.

Além dos sintomas característicos do TDAH, os pacientes podem apresentar diversas comorbidades com outros transtornos psiquiátricos e não-psiquiátricos (ANGOLD et al., 1999). As doenças que apresentam mais comorbidades com o TDAH são o autismo, transtorno de aprendizagem, transtorno de tique, transtornos disruptivos de conduta, abuso de substâncias, ansiedade, depressão, transtorno bipolar e prejuízos motores (FRANKE et al., 2018; TARVER et al., 2014). As comorbidades mudam durante a trajetória da doença. Crianças tendem a ter mais comorbidade com transtornos disruptivos de conduta, enquanto adolescentes e adultos apresentam mais comorbidades com abuso de substâncias, transtornos de humor e prejuízos somáticos, como enxaquecas (TAURINES et al., 2010; INSTANES et al., 2018).

1.2 TRATAMENTO PARA TDAH

O tratamento do TDAH pode ser feito por meios farmacológicos e não-farmacológicos, ambos atuando para amenizar os sintomas dos pacientes.

Dentre os tratamentos não-farmacológicos, são recomendados psicoterapia, intervenções parentais e escolares e mudança na dieta dos pacientes (NICE, 2008).

O tratamento farmacológico pode ser feito com estimulantes ou não-estimulantes. Os estimulantes são os fármacos de primeira escolha para o tratamento,

sendo o metilfenidato o mais utilizado deles (WILENS, 2008). O metilfenidato age inibindo a recaptação de dopamina e noradrenalina no córtex pré-frontal e no estriado, aumentando a quantidade desses neurotransmissores na fenda sináptica dessas regiões (BYMASTER et al., 2002) (Figura 2A).

Com relação aos fármacos não-estimulantes, a atomoxetina é um dos mais utilizados. O mecanismo proposto para a atomoxetina é a recaptação seletiva de noradrenalina no córtex pré-frontal, aumentando a sinalização noradrenérgica, que é importante para a manutenção da atenção e aprendizagem (GARNOCK-JONES & KEATING, 2009) (Figura 2B).

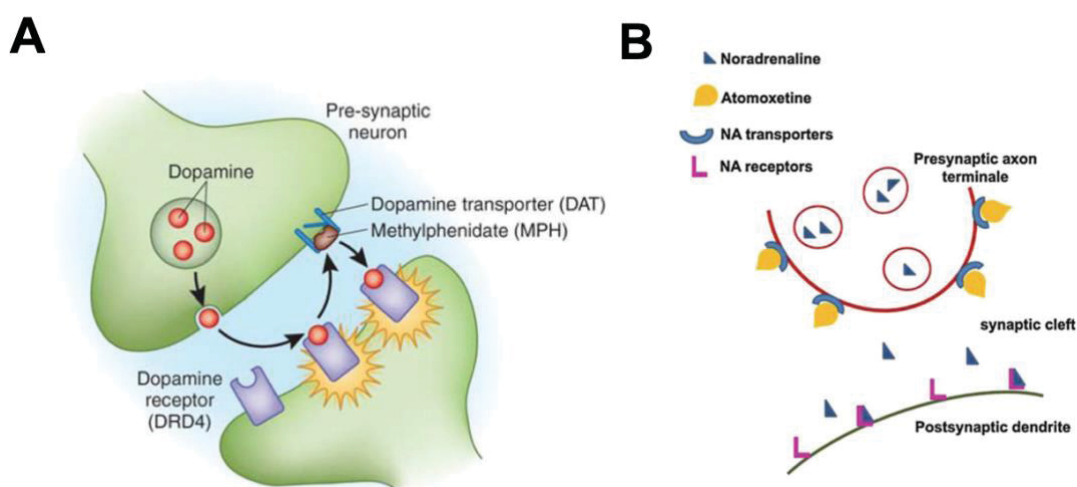


Figura 2 – Mecanismos de ação do metilfenidato (A) e da atomoxetina (B). Em (A), o metilfenidato (MPH) atua bloqueando o transportador de dopamina (DAT, do inglês), presente no neurônio pré-sináptico, o que aumenta a concentração de dopamina na fenda sináptica para atuar em seus receptores no neurônio pré-sináptico. O metilfenidato também pode bloquear o transportador de noradrenalina (NET), não representado na figura. Em (B), a atomoxetina (em amarelo) se liga aos transportadores de noradrenalina (em azul) no neurônio pré-sináptico, aumentando a concentração de noradrenalina para atuar em seus receptores no neurônio pós-sináptico. Retirado de (A) Bush, 2010 e (B) Altunkaynak et al., 2016.

A eficácia de estimulantes e não-estimulantes para o tratamento do TDAH já está bem estabelecida na literatura (CLEMOW et al., 2017; HANWELLA et al., 2011; MAIA et al., 2017; WIGAL et al., 2017). Além disso, esses são os fármacos aprovados pelo *Food Drug Administration* (FDA) no Estados Unidos e pela Agência Nacional de Vigilância Sanitária (ANVISA) no Brasil para o tratamento do transtorno (DE SOUSA & KALRA, 2012; BRATS, 2014).

Embora eficazes, esses tratamentos ainda apresentam uma série de efeitos colaterais, sendo os mais comuns perda de apetite, problemas no sono, náusea e vômito, mudanças de humor, fadiga, sedação, e aumento da pressão arterial (FREDRIKSEN et al., 2013). Contudo, os efeitos adversos podem ser controlados e

geralmente são tolerados quando o tratamento consegue reduzir os sintomas (CORTESE et al., 2013). A taxa de não-aderência ao tratamento é relativamente alta, variando de 13,2% a 64%; porém, está melhorando com o advento de tratamentos de liberação prolongada, no qual não é necessário a utilização do fármaco várias vezes ao dia (ADLER & NIERENBERG, 2010).

Apesar de não serem aprovados para o tratamento do TDAH, antidepressivos tricíclicos, que bloqueiam a recaptação de noradrenalina e serotonina, e a bupropiona, um antidepressivo que inibe a recaptação de dopamina e noradrenalina, também podem ser utilizados, especialmente em pacientes não-responsivos ao tratamento padrão (OTASOWIE et al., 2014; NG, 2017).

No Brasil, os estimulantes e a atomoxetina não constam na Relação Nacional de Medicamentos Essenciais do Sistema Único de Saúde (SUS) (MINISTÉRIO DA SAÚDE, 2020). Para conseguir os medicamentos aprovados, as famílias devem entrar com mandados judiciais ou processos administrativos junto ao Programa de Dispensação de Medicamentos em Circunstâncias Especiais (MAIA et al., 2015). Contudo, é um processo demorado e dispendioso. Em contrapartida, o SUS oferece a pronta entrega para os pacientes com TDAH antidepressivos tricíclicos, como a desipramina, antidepressivos inibidores seletivos da recaptação de serotonina, como a fluoxetina, antipsicóticos, como o haloperidol, e benzodiazepínicos ansiolíticos, como o diazepam, todos fármacos não regulamentados pela ANVISA para o tratamento de TDAH (MINISTÉRIO DA SAÚDE, 2012). Embora não sejam aprovados pela ANVISA para o tratamento de TDAH, alguns estudos já demonstraram a eficácia dos antidepressivos para o tratamento de TDAH (BARRICKMAN et al., 1991; OTASOWIE et al., 2014; QUINTANA et al., 2007).

1.3 MODELOS ANIMAIS DE TDAH

Existem vários modelos já validados para estudar o TDAH em roedores, como animais *knock-out* para o transportador de dopamina (DAT, do inglês), o modelo de lesão neonatal com 6-hidroxidopamina (6-OHDA), ratos espontaneamente hipertensos (SHR, do inglês), dentre outros (FAN et al., 2012). Desses, os ratos SHR são os mais utilizados (RUSSELL, 2011). A linhagem SHR foi primeiramente desenvolvida como um modelo animal de hipertensão, através do cruzamento de ratos Wistar Kyoto (WKY) que apresentavam pressão arterial elevada até chegar na

linhagem isogênica que possuía hipertensão de maneira espontânea (OKAMOTO et al., 1966). Contudo, foi observado que os SHR apresentavam hiperatividade, desatenção e impulsividade quando comparado com o controle WKY, indicando uma validade de face dos SHR como modelo de TDAH (SAGVOLDEN, 2000). Os SHR também apresentaram validade preditiva, já que os tratamentos utilizados para transtorno também amenizaram os comportamentos tipo-TDAH dos SHR (MYERS et al., 1982).

Além de aspectos comportamentais, os SHR também apresentaram redução da expressão de tirosina hidroxilase no estriado (AKIYAMA et al., 1992). Alguns estudos reportaram que há diminuição basal nas quantidades de dopamina no estriado dos SHR (FUJITA et al., 2003; LINTHORST et al., 1991). Contudo, foi observado aumento de dopamina estriatal em estudos com técnicas de quantificação mais avançadas (HEAL et al., 2008). Ratos SHR também apresentaram menor expressão do receptor de dopamina D4 no córtex pré-frontal (LI et al., 2007). Essas evidências levam a crer que há uma modulação dopaminérgica diferenciada nos SHR, o que corrobora com a hipótese dopaminérgica do TDAH e reforça a validade desses animais como modelo de TDAH.

1.4 EFEITOS EM LONGO PRAZO DO TRATAMENTO FARMACOLÓGICO

O tratamento farmacológico para o TDAH atua modulando a neurotransmissão monoaminérgica em várias regiões encefálicas, o que poderia modificar as conexões neurais nos indivíduos recebendo essa medicação, principalmente durante a infância e adolescência, podendo levar a consequências duradoras na vida adulta desses indivíduos.

Muitos estudos avaliaram os efeitos em longo prazo do tratamento em pacientes com TDAH. Já foi visto que o tratamento crônico com metilfenidato antes dos 16 anos pode aumentar as chances de depressão, porém não influencia no aparecimento de ansiedade (SCHRANTZ et al., 2018). Entretanto, o uso de estimulantes durante a infância também diminuiu o risco de desenvolvimento de depressão e transtorno bipolar em paciente diagnosticados com TDAH (BIEDERMAN et al., 2009; LEE et al., 2016; WANG et al., 2016). Também já foi demonstrado que o tratamento crônico com metilfenidato não altera aspectos emocionais em pacientes com TDAH após um período sem usar o medicamento (SCHWERNEN et al., 2019).

Logo, ainda não está claro se o tratamento de pacientes com TDAH durante a infância com estimulantes pode alterar o comportamento emocional desses indivíduos. Com relação ao abuso de substâncias, vários estudos concordam que o uso precoce de metilfenidato reduz as chances de desenvolvimento de adição (CHANG et al., 2014; GROENMAN et al., 2013; MANNUZZA et al., 2008; MCCABE et al., 2016). Contudo, quanto mais tarde o tratamento é iniciado, maiores as chances de envolvimento com abuso de drogas (MANNUZZA et al., 2008).

Em roedores, foi visto que o tratamento crônico com metilfenidato durante a infância e/ou adolescência aumentou comportamentos tipo-depressivo e ansioso (BOLAÑOS et al., 2003; BRITTON & BETHANCOURT, 2009; CARLEZON et al., 2003), aumentou déficits de memória no teste de reconhecimento de objetos (LEBLANC-DUCHIN & TAUKULIS, 2007), aumentou o consumo de drogas de abuso (GILL et al., 2014; WHEELER et al., 2013) e alterou a transmissão dopaminérgica no mesencéfalo (BRANDON et al., 2003) na vida adulta. Contudo, esses estudos não utilizaram modelos animais de TDAH; logo, esses efeitos em longo prazo podem não ser representativos para a população diagnosticada com o transtorno. Em estudos que utilizaram modelos animais de TDAH, foi observado que o tratamento crônico com metilfenidato durante a infância e/ou adolescência aumentou o comportamento tipo-ansioso e diminuiu a resposta a recompensa (VENDRUSCOLO et al., 2008), aumentou a impulsividade (SOMKUWAR et al., 2016), aumentou o consumo de drogas de abuso (HARVEY et al., 2011; JORDAN et al., 2014) e alterou a transmissão dopaminérgica (SOMKUWAR et al., 2013) na vida adulta. Os efeitos em longo prazo do tratamento crônico com metilfenidato no comportamento emocional e na cognição foram pouco avaliados utilizando modelos de TDAH.

Com relação aos antidepressivos, não há estudos avaliando seus efeitos em longo prazo em pacientes com TDAH. Contudo, já foi observado que crianças expostas à antidepressivos durante a gestação apresentaram prejuízos psicomotores (CASPER et al., 2003) e aumento de comportamentos internalizados e do comportamento ansioso (HANLEY et al., 2015; OBERLANDER et al., 2010) durante a primeira infância e aumento do risco de depressão na adolescência (MALM et al., 2016). Já em roedores, o uso de antidepressivos na infância levou a prejuízos emocionais e neofobia na adolescência e na vida adulta (ANSORGE et al., 2004; HANSEN et al., 1997; IÑIGUEZ et al., 2010; SIMPSON et al., 2011; WARREN et al., 2011). Nenhum desses estudos utilizou modelos animais de TDAH.

2 JUSTIFICATIVA

Muitos dos estudos que avaliaram os efeitos em longo prazo do metilfenidato não utilizaram modelos animais de TDAH. Logo, esses resultados podem não ser significativos quando se trata da população diagnosticada com o transtorno, apenas para a população que utiliza esses medicamentos de forma indevida. A maioria dos pacientes diagnosticados com TDAH são crianças que ainda estão em fase de neurodesenvolvimento. Por isso, é necessário entender quais são as consequências do tratamento farmacológico durante a infância na vida adulta. Além disso, aqui no Brasil, apenas antidepressivos são disponibilizados gratuitamente pelo SUS, fármacos que não são aprovados pela ANVISA para o tratamento do TDAH. Por isso, entender os efeitos desse tratamento é essencial para caso necessário, mudar as políticas públicas da distribuição de medicamentos para o TDAH no nosso país.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Analisar os efeitos em longo prazo do tratamento crônico com metilfenidato, fármaco padrão para TDAH, e fluoxetina, fármaco distribuído gratuitamente pelo SUS, no comportamento emocional e na cognição de ratos SHR, modelo animal validado de TDAH, e ratos Wistar, usados como grupo controle.

3.2 OBJETIVOS ESPECÍFICOS

- Investigar as diferenças basais no comportamento entre ratos SHR e ratos Wistar utilizando os testes de campo aberto, reconhecimento de objetos e labirinto em cruz elevado;
- Avaliar e comparar os efeitos em longo prazo do tratamento crônico com metilfenidato ou fluoxetina durante a infância e adolescência nos comportamentos tipo-depressivo e tipo-ansioso com os testes de natação forçada e labirinto em cruz elevado;
- Avaliar e comparar os efeitos em longo prazo do tratamento crônico com metilfenidato ou fluoxetina durante a infância e adolescência na cognição com o teste de reconhecimento de objetos;

4 ARTIGO CIENTÍFICO

Long-term effects of juvenile chronic treatment with methylphenidate or fluoxetine in SHR and Wistar rats

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ABSTRACT

Attention deficit hyperactivity disorder (ADHD) affects nearly 5% of school-age children and adolescents. It is characterized by hyperactivity, inattention, and impulsivity. Stimulants, mainly methylphenidate (MPH), are the gold-standard treatment for the disorders. In Brazil, only antidepressants, such as fluoxetine (FLX), are promptly delivered in public health services for treating ADHD. Both drugs modulate neurotransmission, and the children receiving them are still under neurodevelopment. The long-term consequences the chronic treatment of ADHD may cause are still unclear. Most studies addressing this question did not use validated animal models of ADHD. In the present study, the long-term consequences of juvenile chronic treatment with either MPH or FLX were evaluated in both spontaneously hypertensive rats (SHR), a validated model of ADHD, and Wistar rats (WIS), a normal strain. First, we determined baseline differences between SHR and WIS. SHR had impaired habituation in the open field test and presented a decreased anxiety-like behavior in the elevated plus maze compared to WIS. Regarding chronic treatment, SHR and WIS rats were treated with MPH (2 mg/kg, by oral gavage), FLX (5 mg/kg, by oral gavage) or saline during PND 28-55. After a washout period, animals went through behavioral tests. Neither treatment with MPH nor FLX provoked any long-term effect in emotional behavior, measured in the elevated plus maze and forced swim test, cognition, evaluated in the object recognition test, and locomotion, measured in the open field test, in both strains. However, differences in behavior between SHR and WIS were not observed after treatment with both drugs tested. Given these results, juvenile chronic treatment with either MPH or FLX did not lead to impairments on emotional behavior and cognition in adulthood after a washout period.

Key words: ADHD. SHR methylphenidate, fluoxetine, long-term, emotional behavior, cognition

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder that affects 3 to 5% of children and adolescents in school-age [1,2]. The core symptoms of ADHD are hyperactivity, impulsivity, and inattention [3]. As these symptoms start during childhood, they lead to learning impairments at school [4]. Moreover, ADHD impacts directly the quality of life of these patients [5].

Stimulants, such as methylphenidate (MPH), are the gold standard treatment for ADHD [6]. MPH acts blocking the reuptake of dopamine and norepinephrine in the pre-frontal cortex and striatum [7]. In Brazil, stimulants are not distributed free of charge by the public health system. Instead, other drugs, such as the antidepressant fluoxetine (FLX), are provided prompt delivery to ADHD patients [8]. In North America, tricyclic antidepressants and the antidepressant bupropion may also be prescribed, mainly to patients that are unresponsive to the gold standard treatment, though they are not FDA-approved for ADHD treatment [9,10].

All these treatments act in neurotransmission, and most of the patients are children, who are still going through neurodevelopment. However, it is unclear if these drugs can cause long-term consequences. It has been observed that children diagnosed with ADHD treated with stimulants during childhood had a lesser risk of developing depression or anxiety later in life [11]. Contradicting these results, it has been reported that chronic juvenile treatment of ADHD patients with stimulants increased risk for depression later in life [12]. However, no beneficial or adverse effect of stimulant usage was also observed in ADHD patients after a washout period [13]. Childhood use of stimulants seems to decrease drug abuse in patients with ADHD [14–16]. In studies using rodents, chronic treatment with MPH during childhood and/or adolescence increased anxiety and depressive-like behavior [17–19], caused memory impairments [20] and increased drug abuse [21,22] later in life. However, these aforementioned studies did not use a validated animal model of ADHD; hence, these findings might not be true for the ADHD population. In studies that used animal models of ADHD, it was observed that rats treated with MPH during childhood and/or adolescence increased impulsivity [23], increased anxiety-like behavior and decreased reward response [24], and increased drug abuse [25,26] in adulthood. Regarding antidepressants, there is no study investigating long-term effects of these drugs in

patients with ADHD. Studies with both mice and rats showed that early-life exposure to antidepressants could also lead to emotional impairments and neophobia in adolescence and adulthood; however, these studies did not use validated models of ADHD [27–31].

Most of the previously mentioned studies did not use animal models of ADHD, and the ones that used did not evaluate emotional behavior and cognition after juvenile chronic treatment. Since many of the users of these drugs are children diagnosed with ADHD, it is essential to evaluate the long-term consequences of the treatment in a validated model of ADHD. One of the most used models of ADHD is the spontaneously hypertensive rats (SHR), a model that presents ADHD-like behavior spontaneously [32]. Thus, the aim of this study was to evaluate the long-term effects on emotional behavior and cognition of juvenile chronic treatment with MPH and the antidepressant FLX in SHR, in both males and females. Wistar rats (WIS) were used for comparison. For this purpose, SHR and WIS were treated with MPH, FLX or saline during adolescence and they were tested for emotional behavior and cognition in adulthood. Investigating the potential long-term consequences of the current forms of treatment for ADHD is essential to better treat children and adolescents with ADHD.

MATERIALS AND METHODS

Animals:

Wistar and SHR rats were obtained from the breeding stock of Federal University of Parana (UFPR). SHR rats were originally obtained from Federal University of Santa Catarina (UFSC). Pregnant female WIS and SHR were individually housed and maintained in our animal facility. Delivery day was considered post-natal day (PND) 0. Animals were weaned and separated by sex on PND 24. All animals were kept in the animal facility, in a 12h:12h light-dark cycle (lights on at 7 am), at controlled temperature and humidity, with water and food *ad libitum*. We chose to use WIS rats over the most common control strain Wistar Kyoto (WKY) because WKY are used as a model of depression [33] and depressive-like behavior was one of the outcomes to be evaluated. All procedures were approved by the Ethics Committee on the Use of Animals of the Biological Division of the Federal University of Parana (#993).

Drugs and treatment:

Methylphenidate chloride (Ritalin®, 10 mg, Norvartis, Biociências SA, São Paulo, Brazil) (MPH) and fluoxetine (Alchimia, Curitiba, Brazil) (FLX) were dissolved in 0,9% saline at a dose of 2 mg/kg for MPH and 5 mg/kg for FLX. Both drugs were administered at a volume of 2 ml/kg by oral gavage. The dose of MPH was chosen because it reaches similar plasma concentrations as those found in humans [34]. The dose of FLX was chosen because falls into the range of clinically relevant doses for rats (3-9 mg/kg) and is the commonly chosen dose for rat studies [35]. Control groups received saline at the same volume. Drugs were administered every day, during the morning, from PND 28 to 55, which represents early to late adolescence [36].

Behavioral assessments:*Open field test (OFT):*

This test was conducted in a square arena (58 cm x 58 cm x 40 cm). The test was performed on three consecutive days, with two 5-min sessions each day. Traveled distance was estimated using a tracking software (OptiMouse, version 3.0, available at: <https://github.com/yorambenshaul/optimouse>). The sessions were also used as arena habituation for the object recognition test.

Object recognition test (ORT):

This test was divided in two sessions: training and testing. In the training session, animals were allowed to explore freely two identical objects for 3 minutes (i.e. familiar object). The animals went to the testing session 1 h or 24 h, in Experiment 1, and 1h, in Experiments 2 and 3, after the ending of the training session. In the testing, one of the familiar objects was replaced by a new object. Animals could explore both objects for 3 minutes. Both training and test sessions were recorded with a video camera. Time exploring the objects in both sessions was analyzed by a trained observer blind to the experimental groups. Animals that explored less than 20 seconds in the training session were excluded from the statistical analysis, except in Experiment 1, because all SHR rats explored less than 20 seconds.

Elevated plus maze (EPM):

EPM apparatus consisted of two opposite closed arms (50 cm x 10 cm x 40 cm), and two opposite open arms (50 cm x 10 cm x 0.5 cm), connected by a central platform (10 cm x 13 cm). The maze was elevated 50 cm above the floor. Animals were placed in the central platform facing one of the closed arms and were allowed to explore the maze for 5 minutes. The test was recorded with a video camera and analyzed by a trained observer blind to the experimental groups. The parameters evaluated were the percentage of time spent in the open arms, calculated as follows: % time OA = (time in the open arms / (time in the open arms + time in the closed arms))*100 and the percentage of entries in the open arms, calculated as: % entries OA = (entries OA / (entries OA + entries CA))*100. In Experiment 1, % of time in the open arms and % of entries in the open arms were used to evaluate risk-taking behavior instead of anxiety-like-behavior, as seen elsewhere [37].

Forced swim test (FST):

Cylinders (61 cm x 25 cm) were filled with 2/3 of water, at a temperature of 24°C ± 1°C. The FST was performed in two sessions: the training and the testing sessions. In the training session, animals were put in the cylinders filled with water for 15 minutes. The testing session began 24h after the training. During the testing, animals spent 5 minutes in the cylinders. Both training and testing sessions were recorded with a video camera. Immobility time was analyzed by a trained observer blinded to the experimental groups.

Experimental design:

Experiment 1: Behavioral comparison between SHR and WIS:

This experiment aimed to verify if SHR and WIS showed basal differences in behavior. To do so, 20 male SHR and 15 male WIS, 60 days old, were used. Only male rats were used in this experiment. We used the OFT to evaluate hyperlocomotion, ORT to evaluate cognitive deficits, and the EPM to evaluate anxiety-like and risk-taking behaviors. All behavioral procedures were performed between 08:00 a.m. and 12:00 p.m. (Fig. 1A).

Experiment 2: Long-term effects of juvenile chronic treatment with methylphenidate in adulthood:

Nineteen male SHR, 10 female SHR, 20 male WIS, and 11 female WIS received MPH (2 mg/kg) or SAL during PND 28-55. Then, animals went through a washout period of 17 to 24 days, in which they were maintained in the animal facility. After this period, the behavioral assessments began. OFT was used to evaluate locomotor activity, ORT for cognitive performance, EPM for anxiety-like behavior, and FST for depressive-like behavior. All behavioral procedures were performed between 08:00 a.m. and 14:00 p.m. (Fig. 1B).

Experiment 3: Long-term effects of juvenile chronic treatment with fluoxetine in adulthood:

Fifteen male SHR, 14 female SHR, 13 male WIS, and 14 female WIS received FLX (5 mg/kg) or SAL during PDN 28-55. After the treatment, animals went through a washout period of 10 to 18 days. Behavioral assessments began after the washout period. The tests used in this experiment were the same as in Experiment 2. All behavioral procedures were performed between 08:00 a.m. and 14:00 p.m. (Fig. 1B).

Statistical analysis:

All data were represented as mean $\pm$ SEM. In experiment 1, OFT was analyzed using repeated measures ANOVA, ORT was analyzed by two-way ANOVA and EPM parameters was analyzed by unpaired Student's t-test. In experiments 2 and 3, three-way repeated measures ANOVA was used to analyze the OFT and factorial ANOVA was used to analyze all other parameters. The factors were strain, sex, and treatment for all the parameters, object for analysis of the ORT, and day of the test as the repeated measure in the OFT. Newman-Keuls test was used for post-hoc comparisons. Significance level was set at $p < 0.05$.

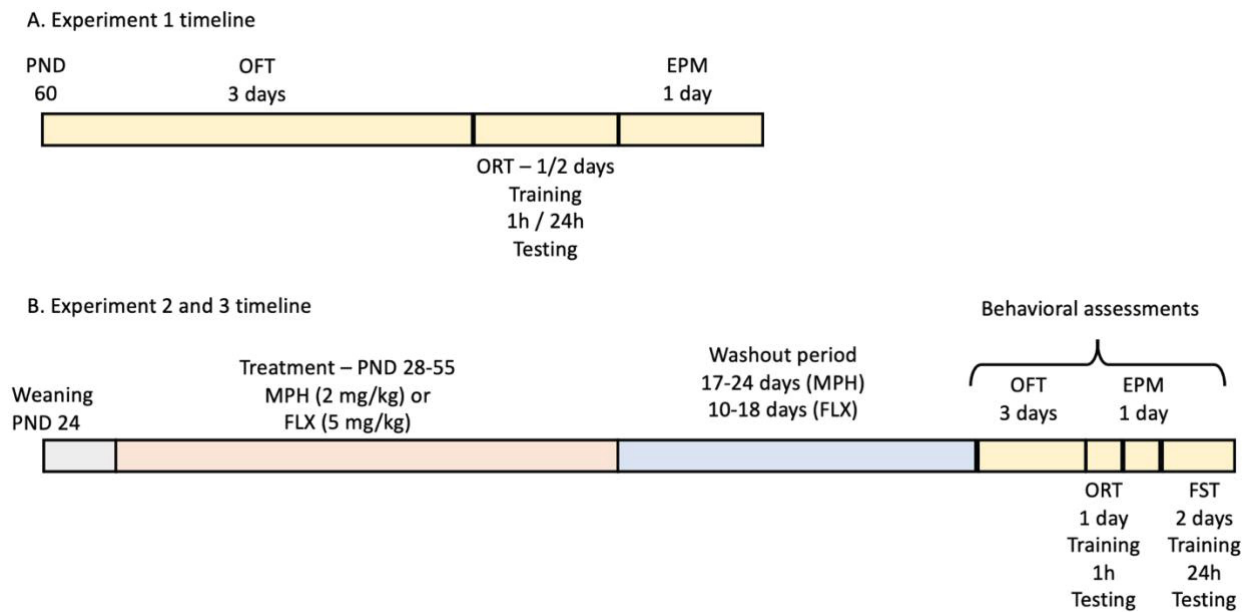


Figure 1. Schematic timeline of the experiments. A. Experiment 1 compared WIS and SHR rats in the open field test (OFT), object recognition test (ORT), and elevated plus maze test (EPM). B. Experiments 2 and 3 evaluated the effects of chronic treatment with methylphenidate (MPH) or fluoxetine (FLX) in WIS and SHR rats in the OFT, ORT, EPM and forced swim test (FST).

RESULTS

Experiment 1 – Behavioral comparison between SHR and WIS:

In the OFT, there was no effect of strain ($F(1, 33) = 0.599$, $p = 0.44$). However, there was an effect of the day of test ($F(2, 66) = 20.344$, $p < 0.001$), and there also was an interaction between days of the test and strain ($F(2, 66) = 5.84$, $p < 0.005$). In the post hoc analysis, it was noted that WIS had a lower traveled distance in both days 2 ($p < 0.001$) and 3 ($p < 0.001$) compared to WIS in day 1, whereas the locomotion of SHR in the first p day of the test did not differ from Days 2 ($p = 0.152$) and 3 (0.080) (Fig. 2A). Also, WIS and SHR did not showed differences in day 1 ($p = 0.474$).

In the ORT, in the 24-h interval, the new object was explored more than the familiar one by both strains ($F(1, 24) = 15.496$, $p < 0.001$). There was a tendency for effect of strain ($F(1, 24) = 3.935$, $p = 0.058$), indicating that SHR explored the objects for more time than WIS. However, there was no interaction between strain and object ($F(1,24) = 0.009$, $p = 0.925$) (Fig. 2B). As both strains were able to recognize the new object in a 24-h interval, we decided to test recognition in a 1-h interval. It was seen in other studies that longer intervals did not impair recognition in SHR, while shorter

intervals did [38]. However, in the 1-h interval, no effect of strain ($F(1, 14) = 1.387$, $p = 0.259$) nor object ($F(1, 14) = 1.021$, $p = 0.329$) was observed. Also, there was no interaction between strain and object ($F(1, 14) = 0.402$, $p = 0.537$) (Fig. 2C).

In the EPM, SHR presented less anxious and more risk-taking behavior than WIS, that is marked by the percentage of time spent in the open arms ($t(22) = 4.269$, $p < 0.005$, Fig. 2D) and percentage of entries in the open arms ($t(22) = 3.724$, $p < 0.005$, Fig. 2E).

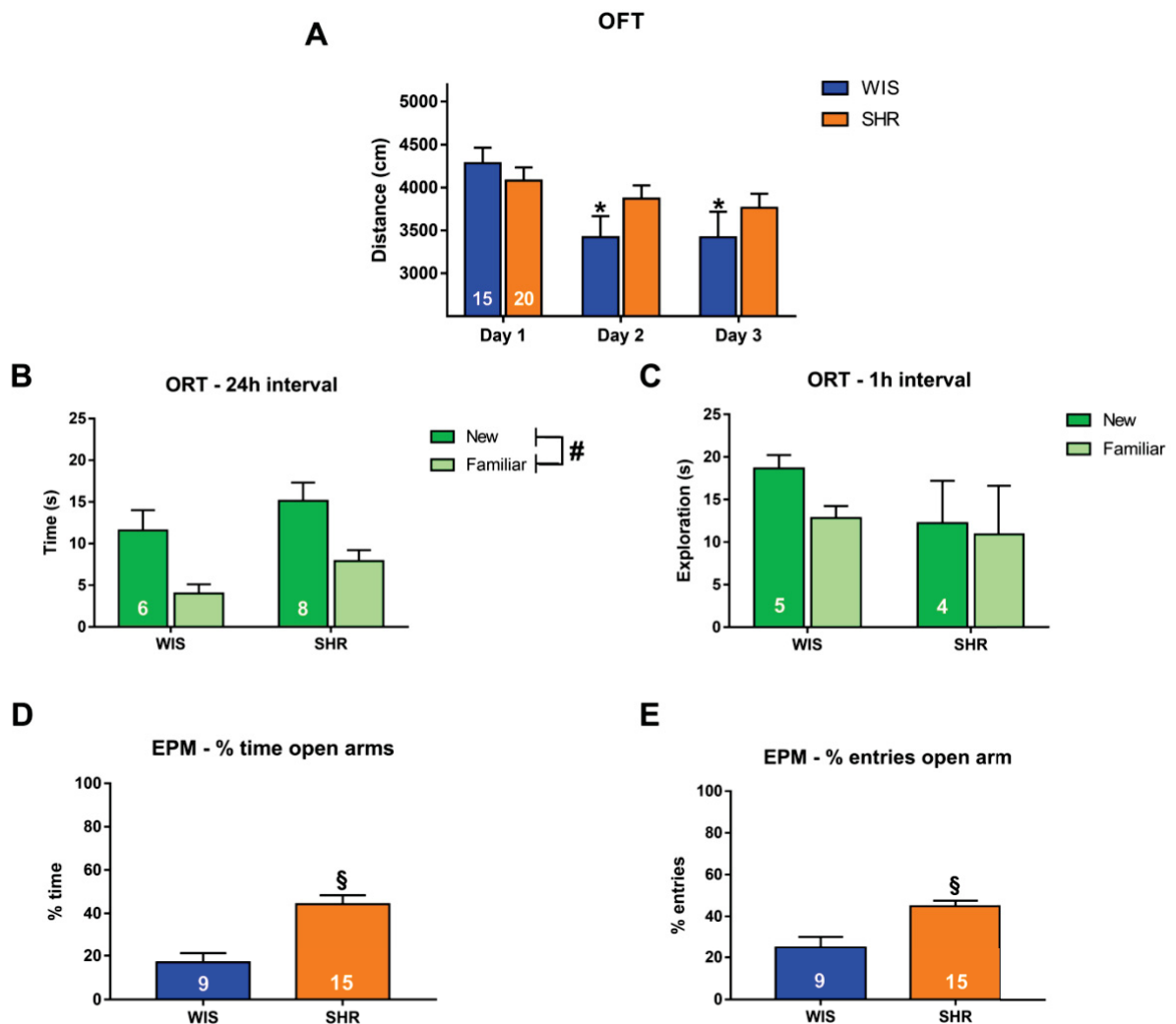


Figure 2. Behavioral comparison between WIS and SHR. A) Distance traveled in the OFT during three days of test (* $p < 0.005$ compared to WIS-Day 1, repeated measures ANOVA). B) Time exploring the new and familiar objects during a 24-h interval ORT. Animals explored more the new object than the familiar one (# $p < 0.001$, two-way ANOVA). C) Time exploring the new and familiar objects during a 1-h interval ORT (two-way ANOVA). D) Percentage of time spent in the open arms in the EPM and E) Percentage of entries in the open arms in the EPM. SHR spent more time and had more entries in the open arm (§ $p < 0.005$, unpaired student's t-test). All data is represented as mean $\pm$ SEM. The numbers inside the bars represent the sample size of each group for each test.

Experiment 2 - Long-term effects of juvenile treatment with methylphenidate in adulthood:

In the OFT, there was not an interaction among treatment, strain and sex ($F(2, 94) = 1,234$, $p = 0.296$). Moreover, no effects of treatment ($F(1, 47) = 1.529$, $p = 0.222$) and strain ($F(1, 47) = 0.849$, $p = 0.361$) were observed. No effect of day of test was observed as well ($F(2, 94) = 1.464$, $p = 0.236$) (Fig. 3A for males and Fig. 3B for females). However, there was an effect of sex ($F(1, 47) = 40.254$, $p < 0.0001$) and an interaction between strain and sex ($F(1, 94) = 4,441$, $p < 0.05$). In the post-hoc analysis, it was noted that both female WIS and SHR showed higher traveled distances than male WIS and SHR ($p < 0.05$). Also, surprisingly, male WIS ambulated more than male SHR ($p < 0.05$). As treatment and day of test did not show any effect in both male and female rats, we grouped these factors to better depict the differences between strain and sex (Fig. 3C).

In the ORT, WIS explored the objects more than SHR in the training session, regardless of sex and treatment ($F_{\text{strain}}(1, 45) = 11.284$, $p < 0.005$; $F_{\text{sex}}(1, 45) = 0.446$, $p = 0.508$; $F_{\text{treatment}}(1, 45) = 0.298$, $p = 0.588$, Fig. 4A). The same was seen for total exploration time in the testing session ($F_{\text{strain}}(1, 45) = 8.115$, $p < 0.001$; $F_{\text{sex}}(1, 45) = 0.576$, $p = 0.452$; $F_{\text{treatment}}(1, 45) = 0.928$, $p = 0.340$, Fig. 4B). There was no interaction among strain, sex, and treatment in exploration of both training ($F(1, 45) = 1,347$, $p = 0.252$) and testing ($F(1, 45) = 1.149$, $p = 0.289$) sessions. Despite that, all groups discriminated between objects. There were effects of strain ($F(1, 90) = 8.173$, $p < 0.01$) and object ($F(1, 90) = 30.473$, $p < 0.0001$), however no effects of MPH treatment ($F(1, 90) = 0.935$, $p = 0.336$) or sex ($F(1, 90) = 0.580$, $p = 0.448$) were observed. Also, no interaction was observed among strain, sex, treatment, and object ($F(1, 90) = 1,139$, $p = 0.289$) (Fig. 4C).

In the EPM, MPH treatment did not change anxiety-like behavior in SHR or WIS, independent of sex (Fig. 5). There was no interaction among strain, sex and treatment in the percentage of time in the open arms ($F(1, 52) = 0.567$, $p = 0.455$, Fig. 5A) nor in the percentage of entries in the open arms ($F(1, 52) = 1,250$, $p = 0.269$, Fig. 5B).

Likewise, MPH did not affect depressive-like behavior in the FST. No interaction among factors was observed ($F(1, 52) = 1.613$, $p = 0.210$). No effects of sex ($F(1, 52) = 0.294$, $p = 0.590$) or treatment ($F(1, 52) = 0.417$, $p = 0.521$) were noted as well. There was, however, an effect of strain ($F(1, 52) = 22.805$, $p < 0.001$), meaning that WIS had

a higher immobility time than SHR. There was also no interaction between strain and sex ($F(1, 52) = 2.803$, $p = 0.100$) (Fig. 6).

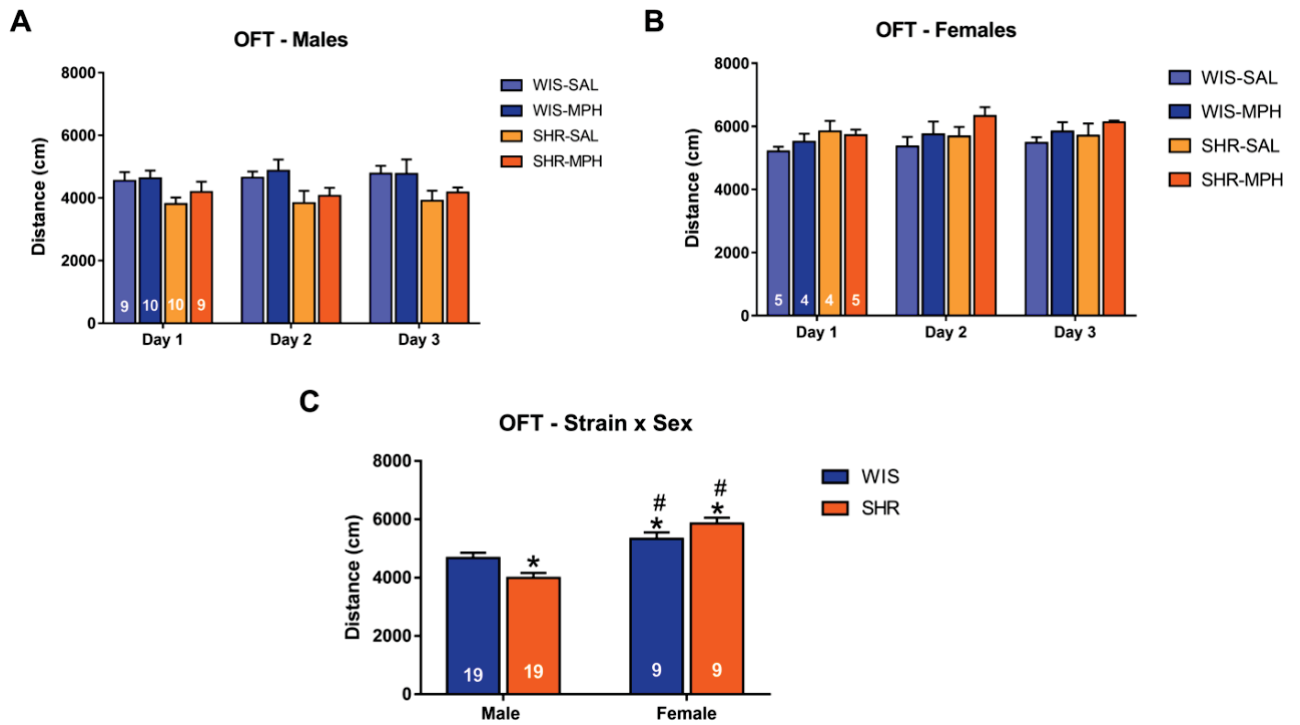


Figure 3. Effects of long-term treatment with MPH on locomotion. A) Distance (cm) traveled in the OFT during 3 days of test by male SHR and WIS. B) Distance (cm) traveled in the OFT during 3 days of test by female SHR and WIS. In both A) and B), there was no effect of treatment of strain, as well as day of the test, indicating a poor habituation along the days. C) As day of test and treatment did not show any effect, these data were grouped to better visualize the differences in strain and sex. Female rats, regardless of the strain, traveled more along the arena than male rats. Also, male SHR ambulated less and male WIS (* $p < 0.05$ compared to male WIS, # $p < 0.05$ compared to male SHR). Five animals were excluded from the final analysis due to technical problems (1 male WIS-MPH, 1 female WIS-SAL, 1 female WIS-MPH, 1 female SHR-SAL, and 1 female SHR-MPH). All data is represented as mean $\pm$ SEM and three-way repeated measures ANOVA was used as statistical test. The numbers inside the bars represent the sample size of each group.

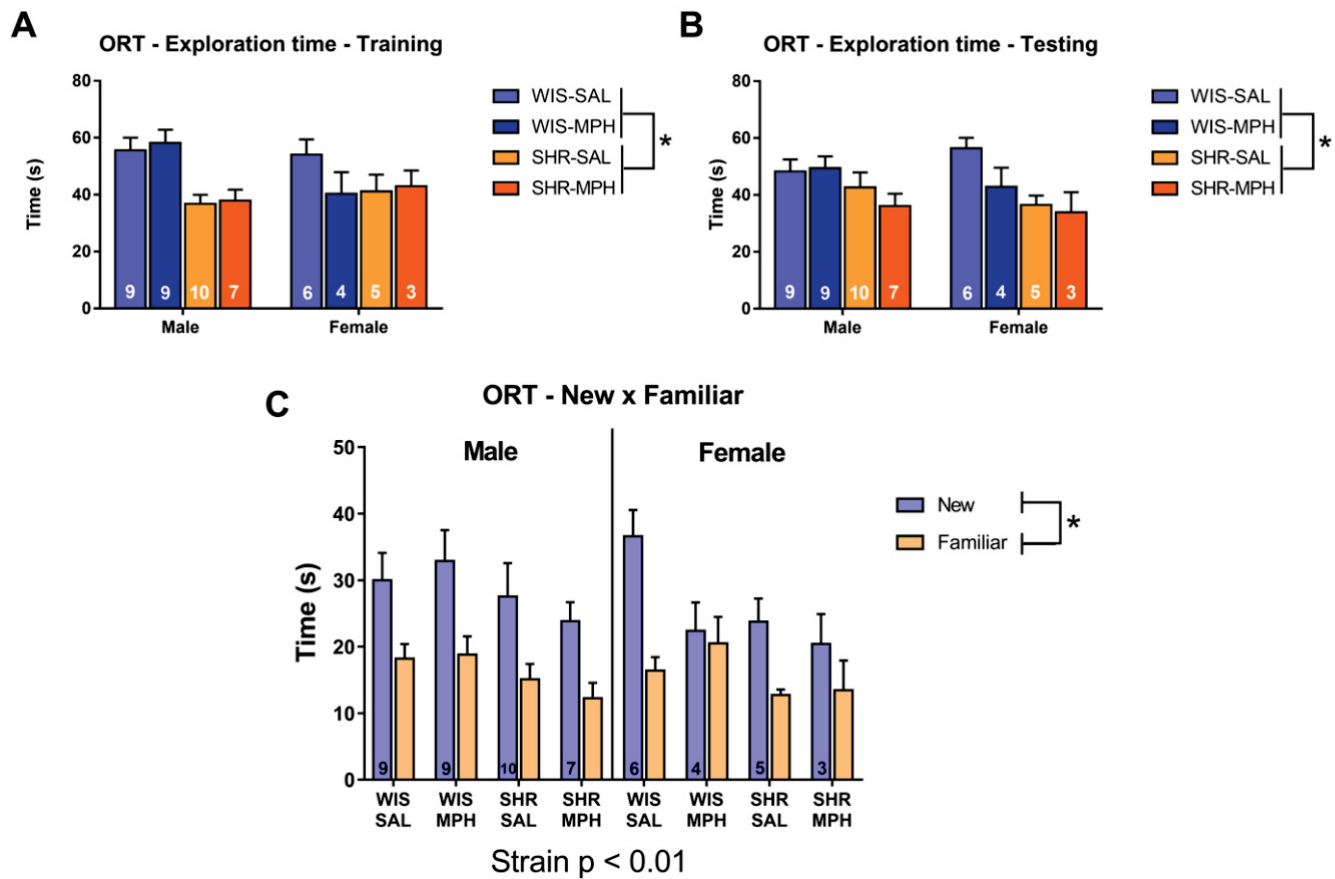


Figure 4. Effects of long-term treatment with MPH on short-term memory. A) Exploration time of both objects during training sessions. WIS spent more time exploring the objects than SHR. B) Exploration time of both objects during testing sessions. Again, WIS spent more time exploring the objects than SHR (* $p < 0.005$). C) Exploration time in the testing sessions separated by new and familiar objects. There was no interaction between strain, sex, and treatment, indicating that long-term MPH did not impair cognition after a washout period (* $p < 0.001$). Seven animals were excluded from the analysis because their exploration time during training was less than 20 s (1 from the group male WIS-SAL, 1 from male WIS-MPH, 2 from male SHR-MPH, 1 from female WIS-MPH, and 2 from female SHR-MPH). All data is represented as mean $\pm$ SEM and factorial ANOVA was used as statistical test for each parameter. The numbers inside the bars represent the sample size of each group.

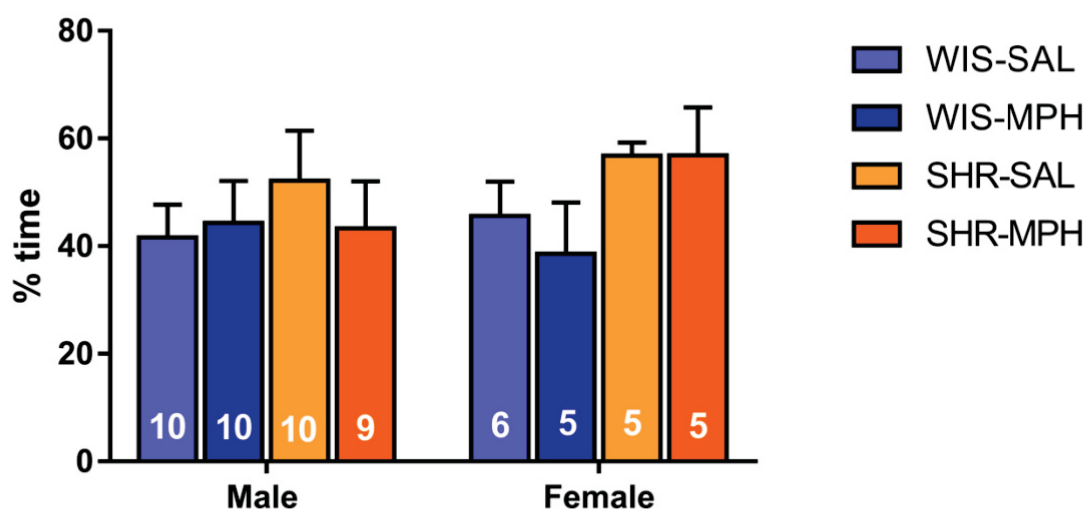
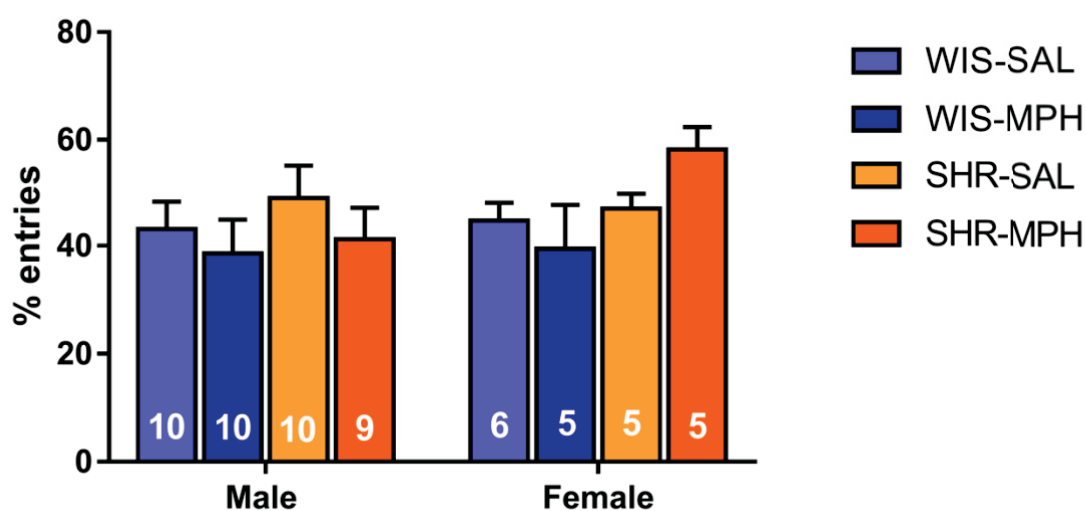
A**EPM - % time open arms****B****EPM - % entries open arms**

Figure 5. Effects of long-term treatment with MPH on anxiety-like behavior. A) Percentage of time spent in the open arms. There was no difference among groups. B) Percentage of entries in the open arms. Also, no difference among groups was observed. All data is represented as mean $\pm$ SEM and factorial ANOVA was used as statistical test for each parameter. The numbers inside the bars represent the sample size of each group.

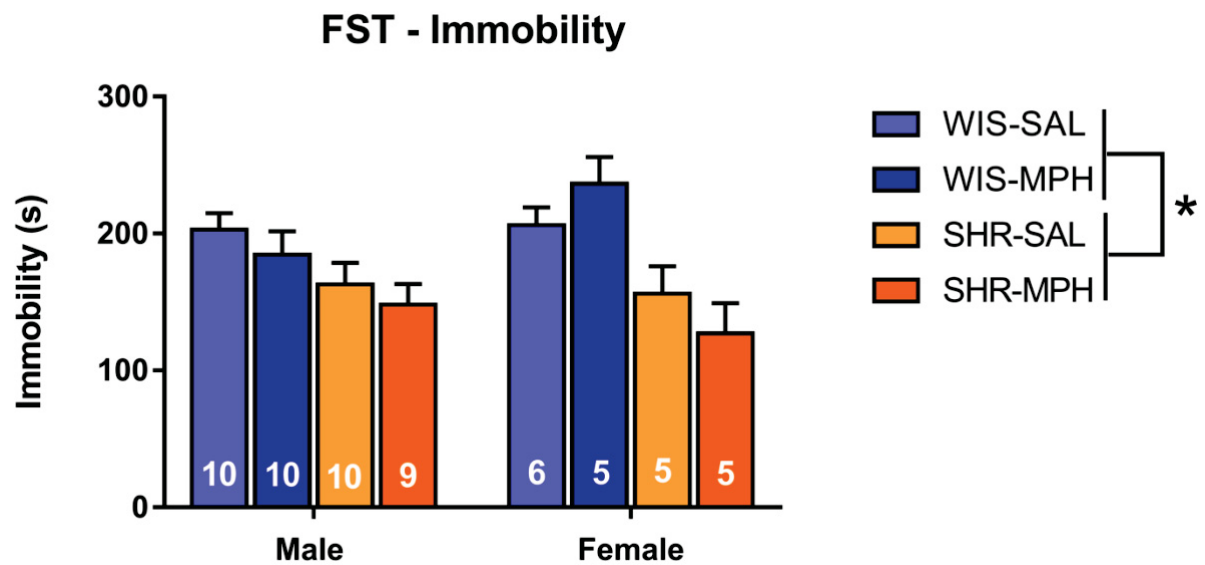


Figure 6. Effects of long-term treatment with MPH on depressive-like behavior. There was no interaction between the factors, indicating that MPH did not cause any impairments in any strain or sex. However, SHR showed decreased depressive-like behavior compared to WIS (* $p < 0.001$). All data is represented as mean $\pm$ SEM and factorial ANOVA was used as statistical test. The numbers inside the bars represent the sample size of each group.

Experiment 3 - Long-term effects of juvenile treatment with fluoxetine in adulthood:

In the OFT, there was no interaction among the factors ($F(2, 88) = 0.343$, $p = 0.711$). No effects of treatment ($F(1, 44) = 0.066$, $p = 0.798$), strain ($F(1, 44) = 0.005$, $p = 0.945$) and day of test ($F(2, 88) = 1.058$, $p = 0.351$) were observed (Fig. 7A for males and Fig. 7B for females). Yet, there was an effect of sex ($F(1, 44) = 6.965$, $p < 0.05$) and an interaction among day of test, strain, and sex ($F(2, 88) = 3.700$, $p < 0.05$). The post-hoc analysis showed that female SHR increased their traveled distance along the days ($p < 0.05$), while female WIS decreased their distance ($p < 0.05$). Also, in the first day of test, male WIS ambulated more than male SHR ($p < 0.05$). Since there was no effect of treatment, this factor was grouped to better visualize the data (Fig. 7C).

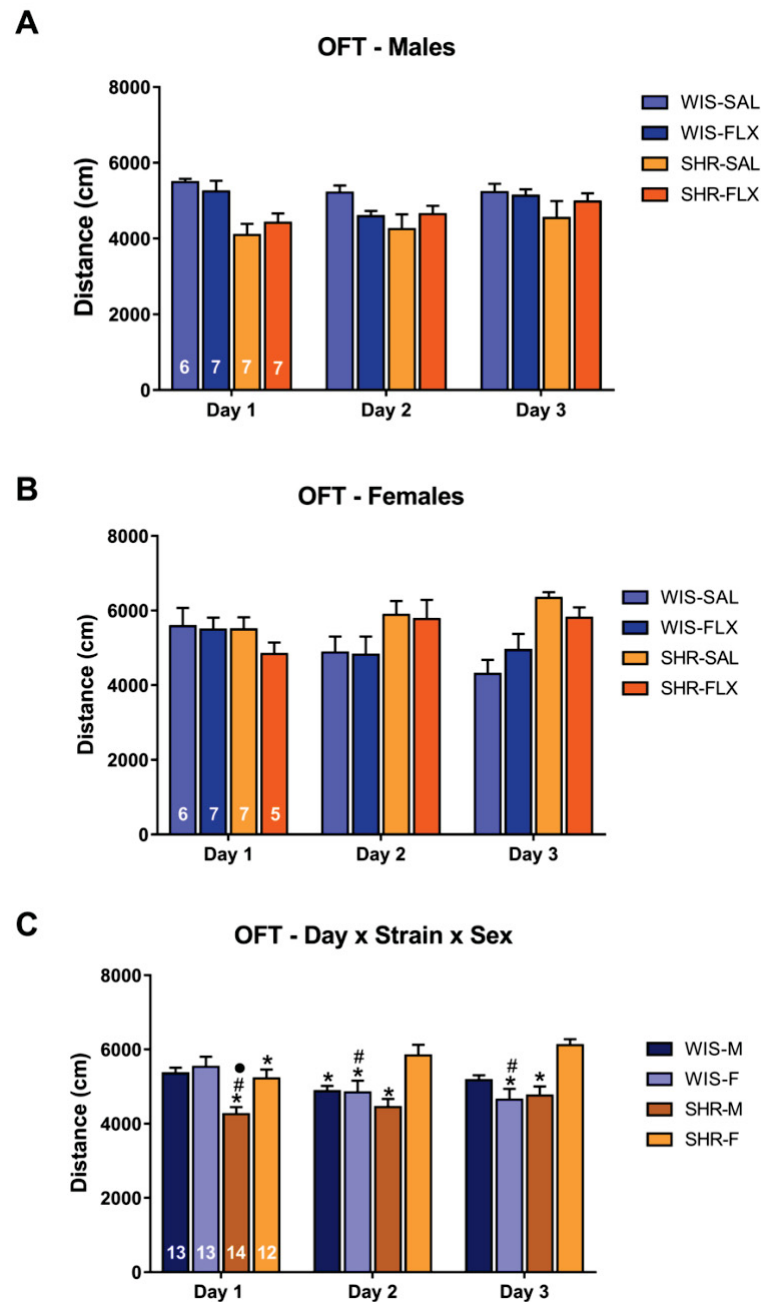


Figure 7. Effects of long-term treatment with FLX on locomotion. A) Distance (cm) traveled for each group along the three days of test by male SHR and WIS. C) Distance (cm) traveled for each group along the three days of test by female SHR and WIS. In both A) and B), there was no interaction between the factors (day, strain, sex, and treatment). C) Since no effect of treatment was observed, this factor was grouped in the graph to better visualize the data. Female SHR had an increase in distance along the days, whereas female WIS had a decrease in distance along the days. Also, male WIS ambulated more than male SHR during the first day of test (* $p < 0.05$ compared to SHR-F Day 2 and SHR-F Day-3, # $p < 0.05$ compared to WIS-F Day 1, • $p < 0.05$ compared to WIS-M Day 1). Five animals were excluded from the analysis due to technical problems (1 male WIS-FLX, 1 female WIS-SAL, 1 male SHR-FLX, and 2 female SHR-FLX). All data is represented as mean $\pm$ SEM three-way repeated measures ANOVA was used as statistical test. The numbers inside the bars represent the sample size of each group.

In the ORT, WIS explored more the objects than SHR in both training ($F(1, 38) = 27.634, p < 0.0001$) and testing ($F(1, 38) = 20.218, p < 0.0001$) sessions, regardless of sex ($F_{\text{training}}(1, 38) = 0.001, p = 0.977$; $F_{\text{testing}}(1, 38) = 0.017, p = 0.897$) or treatment ($F_{\text{training}}(1, 38) = 0.049, p = 0.825$; $F_{\text{testing}}(1, 38) = 0.038, p = 0.843$). Also, there was no interaction among sex, strain, and treatment both in the training ($F(1, 38) = 0.11, p = 0.742$) and testing ($F(1, 38) = 0.472, p = 0.496$) sessions. Despite differences in exploration time, all groups discriminated between the objects, which is indicated by an effect of object ($F(1, 74) = 25.227, p < 0.0001$), meaning that animals explored the new object more than the familiar one. There also was an effect of strain ($F(1, 74) = 26.721, p < 0.0001$). Nevertheless, no effect of FLX ($F(1, 74) = 0.079, p = 0.779$) and sex ($F(1, 74) = 0.041, p = 0.839$) was detected. In addition, no interaction among strain, sex, treatment, and object were observed ($F(1, 74) = 0.237, p = 0.628$) (Fig. 8C).

Long-term treatment with FLX did not have any effect on anxiety-behavior as well. There was no interaction among factors neither in percentage of time in the open arms ($F(1, 49) = 0.032, p = 0.869$, Fig. 9A) nor in the percentage of entries in the open arms ($F(1, 49) = 0.002, p = 0.960$, Fig. 9B). There was a trend for the strain factor in the percentage of time in the open arms ($F(1, 49) = 3.761, p = 0.058$), which corroborates with Experiment 1. Yet, no other effect was observed in both parameters.

No effect of treatment ($F(1,49) = 1,418, p = 0.241$) and sex ($F(1, 49) = 1.454, p = 0.233$) was observed in the FST. However, as in Experiment 2, WIS had a higher immobility time than SHR ($F(1, 49) = 10.611, p < 0.005$). No interaction among all the factors was observed ($F(1, 49) = 0.006, p = 0.940$). Also, there was no interaction between strain and sex ($F(1, 49) = 2,835, p = 0.098$) and between sex and treatment ($F(1, 49) = 3,076, p = 0.086$) (Fig. 10).

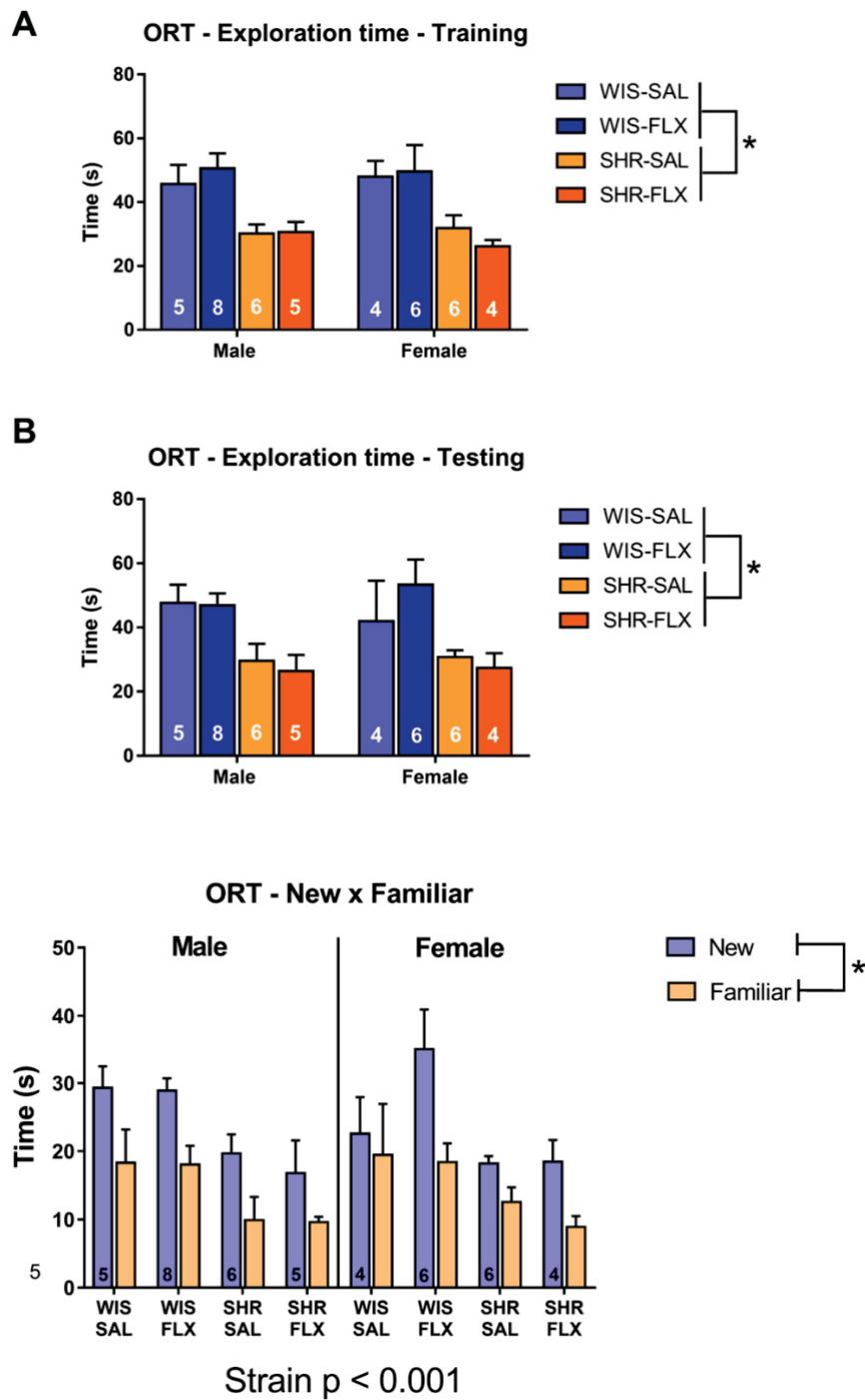


Figure 8. Effects of long-term treatment with FLX on short-term memory. A) Exploration time of both objects in the training sessions. WIS explored the objects for more time than SHR. B) Exploration time of both objects in the testing sessions. Likewise, WIS explored the objects for more time than SHR (* $p < 0.0001$). C) Exploration in the testing session, separated by exploration of new and familiar objects. All groups discriminated between objects, exploring the new object for more time (* $p < 0.001$). Eleven animals were excluded from the analysis because they explored less than 20 seconds in the training phase (1 male WIS-SAL, 2 female WIS-SAL, 1 female WIS-FLX, 2 male SHR-SAL, 1 male SHR-FLX, 1 female SHR-SAL, and 2 female SHR-FLX). All data is represented as mean $\pm$ SEM and factorial ANOVA was used as statistical test for each parameter. The numbers inside the bars represent the sample size of each group.

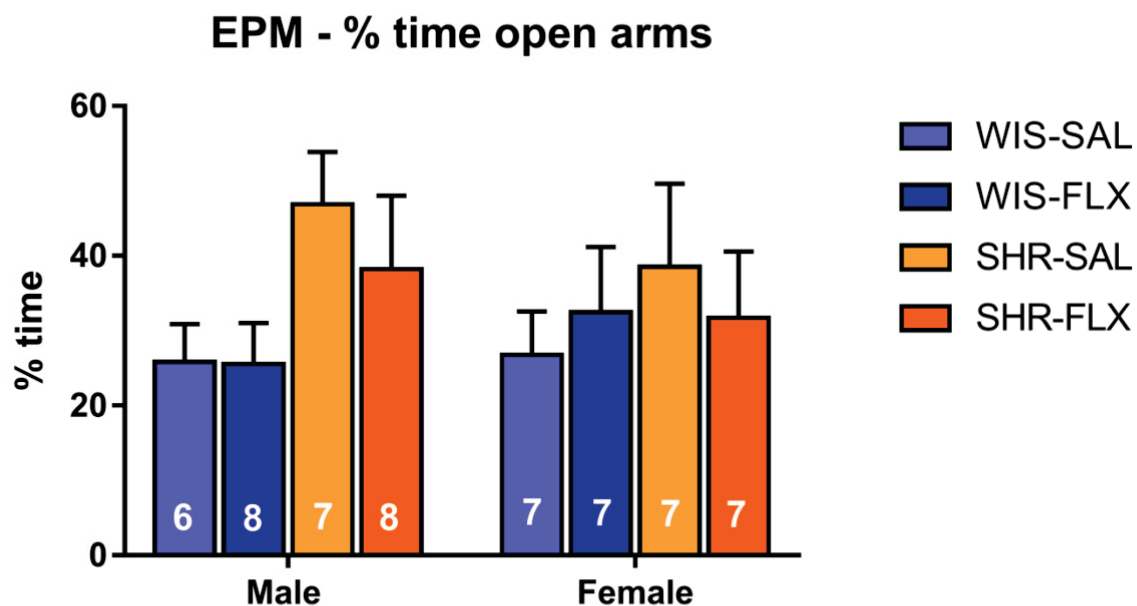
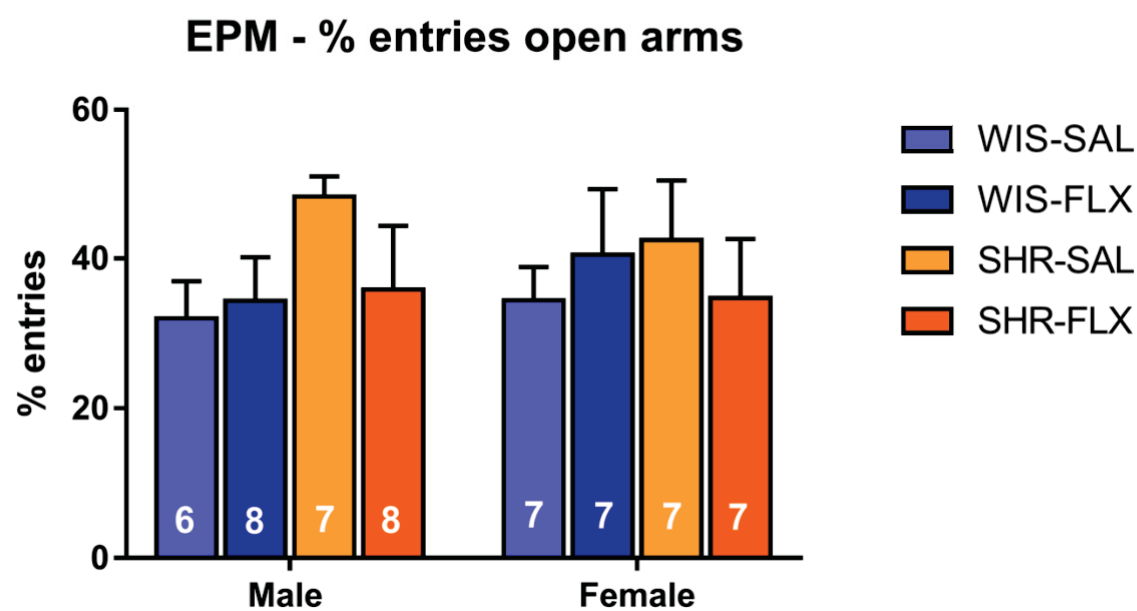
A**B**

Figure 9. Effects of long-term treatment with FLX on anxiety-like behavior. A) Percentage of time spent in the open arms. There was a tendency for the strains to differ ($p = 0.058$). Though, no other effect or interaction were seen. B) Percentage of entries in the open arms. Again, there was no difference among groups. All data is represented as mean $\pm$ SEM and factorial ANOVA was used as statistical test for each parameter. The numbers inside the bars represent the sample size of each group.

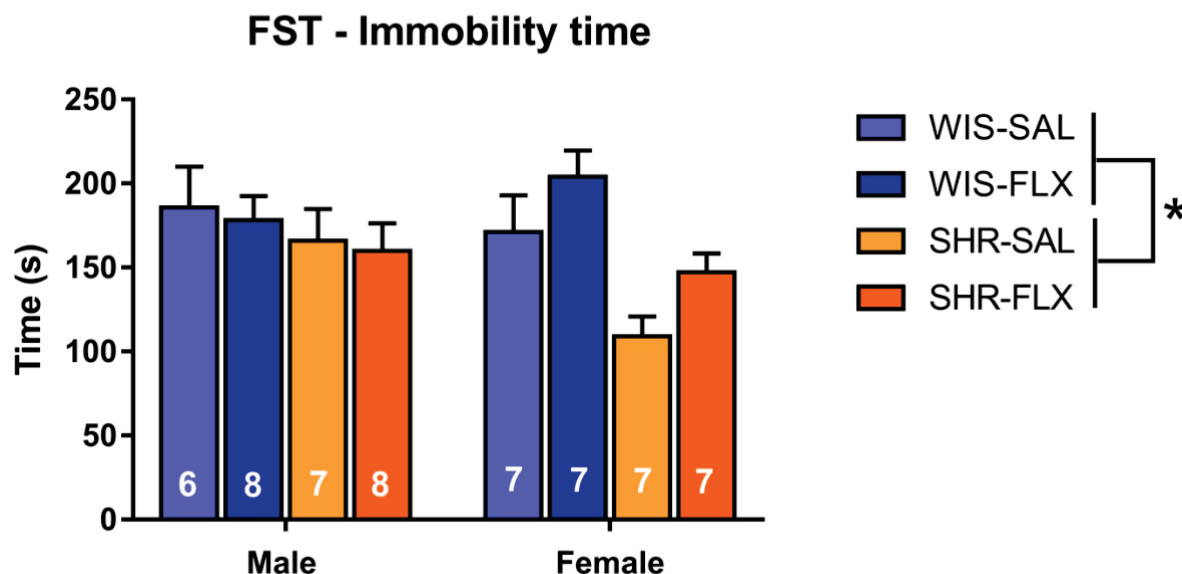


Figure 10. Effects of long-term treatment with FLX on depressive-like behavior. FLX treatment did not change immobility time in any strain nor sex. Yet, SHR swam more than WIS (* $p < 0.005$). All data is represented as mean $\pm$ SEM and factorial ANOVA was used as statistical test. The numbers inside the bars represent the sample size of each group.

DISCUSSION

The present study aimed to understand possible long-term consequences of juvenile chronic treatment with MPH or FLX using SHR, a validated animal model of ADHD. In order to do so, we initially assessed baseline differences between SHR and WIS. It was observed that male SHR failed to habituate in the OFT compared to male WIS. It was reported before that SHR and WIS did not have differences in locomotion in a non-habituated arena, but WIS rats ambulated less than SHR in a habituated one [39], corroborating the results found in this study. We also found that SHR were less anxious in the EPM than WIS. Anxious behavior in SHR is still a little controversial in the literature. Some studies found no difference in the EPM between SHR and WIS and in the light-dark box [39,40]. However, other studies suggested that SHR presented a decreased anxiety-like behavior when compared to the control strain [40,41]. The present study supports the finding that SHR tend to have less anxiety-like behavior and more risk-taking behavior than WIS. Regarding cognition, SHR and WIS could discriminate between a new and a familiar object in the ORT after a long interval (24 h). Other studies also observed that SHR did not show impairments in

discrimination of objects after 24-h interval [42] and after a 72-h interval, SHR had a better recognition of the new object than both Wistar Kyoto (WKY) and WIS [39], indicating that SHR did not have impairments in long-term memory. However, at 30-min intervals, SHR could not discriminate between objects [43,44]. Thus, compared to WIS, SHR seem to have impaired recognition memory at short intervals. So, we decided to evaluate differences between SHR and WIS in a short interval (1 h). We observed that neither WIS nor SHR discriminated between objects after a short interval. We could not detect differences in discrimination in WIS rats in a short interval probably due to the small sample size in this test.

Juvenile chronic treatment with MPH did not have any identifiable beneficial or harmful effect in emotion and cognition during adulthood in any of the strains. Other studies observed that chronic treatment with MPH reduced locomotion and increased anxiety-like behavior in SHR [19,45] and increased impulsivity and disrupted spatial and working memory in other strains [46–48]. However, these studies performed the behavioral tests right after the end of the treatment. In our study, animals went through a washout period before starting the behavioral tests. Other studies failed to find differences between treated and non-treated animals after washout in different parameters as well, such as locomotion [49], recognition of novel object [49,50], anhedonia [51], anxiety [19], and reversal learning [52]. Moreover, a 1-week washout period seemed to decrease depressive and anxiety-like behaviors, while a 5-week washout these differences are no longer observed [53], meaning that the effects of MPH did not last for longer periods of time. By contrast, other studies found that, even after a washout period, rats treated with MPH had increased depressive-like behavior [18], increased anxiety-like behavior [17], and impaired recognition memory [20,54]. These studies, however, used mainly WIS and Sprague Dawley rats, which are not animal models of ADHD; therefore, these findings could be better informative of MPH misuse. In SHR, the effects of chronic treatment after a washout period are still controversial. It has been observed that juvenile chronic treatment with MPH increased anxiety-like behavior (VENDRUSCOLO et al., 2008) and impulsivity (SOMKUWAR et al., 2016) after washout. In contrast, it was reported that juvenile chronic treatment with MPH did not impact in locomotion after cocaine sensitization [23] after washout. Hence, it is still unclear if chronic MPH treatment has a harmful long-term impact in the behavior of both ADHD models and control strains, and how long could these effects

last. This study corroborates that, in both WIS and SHR rats, juvenile chronic MPH does not impair emotion and cognition in adulthood in the performed tests.

Chronic treatment with FLX during adolescence did not seem to impair emotional behavior and cognition in adult SHR and WIS as well. Few studies investigated the effects of FLX treatment in SHR. It was observed that chronic FLX did not impair anxiety-like behavior in SHR, but did in the control strain, WKY [55]. However, it was also observed decreased time spent in open arms in both strains in another study [56]. In both studies, behavioral assessments were performed right after the end of treatment and chronic treatment were performed in adult animals. No study investigating long-term effects of fluoxetine in SHR in juvenile rats or after a washout period was found, reinforcing the importance of the present study. Other studies, using Sprague-Dawley, rats found that chronic treatment with fluoxetine during childhood led to increased anxiety-like behavior and decreased depressive-like behavior after a washout period [30,31]. Also, in mice, fluoxetine treatment during childhood increased anxiety-like behavior in adulthood [27], which goes against the findings in our study. This might have happened because treatment began in younger rats than it did in our study [27,31], indicating that the age of the treatment might influence in long-term consequences and younger rodents are more vulnerable to these consequences than older ones. Rats treated with fluoxetine in a similar period of time to the one used in our study (PND 35-49) showed impaired behavior in adulthood [30]; however; they used a higher dose (10 mg/kg), which might have contributed to impair behavior later in life. A 10 mg/kg dose is considered a less clinically relevant dose in rodents than a 5 mg/kg dose [35]. So, it seems that, in a clinically relevant dose, fluoxetine did not impair emotional behavior and cognition in adolescent SHR and WIS. Nevertheless, the treatment might impair behavior if administered in other ages.

SHR rats had lower immobility time than WIS rats in the FST test, independently of treatment. These differences were not observed before [57], although there are not many studies addressing differences in depressive-like behavior between SHR and WIS. So, this difference in immobility could be an intrinsic difference in behavior between these strains, indicating that SHR rats show less depressive-like behavior than WIS rats. Alternatively, it also might indicate that SHR rats present memory impairments, since this test was performed on two consecutive days and studies that demonstrates that there are learning processes involved in FST performance [58–60].

Moreover, MPH or FLX did not modify this behavior in both strains, which point out that these drugs were not able to alter immobility in this test in the long-term.

We could observe that females from both strains had a higher traveled distance than males from both strains in the two experiments. This hyperactivity in females compared to males was observed before in both SHR and WIS [24,61–64]. We did not evaluate estrous cycle phases in the present studies; however, different phases of the cycle did not seem to alter locomotion in female from both strains [65–68][65–68]. In addition, in both Experiments 2 and 3, male WIS treated with saline did not habituate, while female WIS treated with saline habituated only in Experiment 3. This goes against our baseline results. It is possible that habituation in the OFT is linked to how much time animals spent in the arena [69]. However, in our baseline experiment, habituation was observed in 5-min sessions. So, time in the arena might not have influenced lack of habituation in Experiments 2 and 3. Habituation might not be detected in the treated experiments due to the oral gavage process, that might be stressful, or due to intrinsic differences among animals from different experiments.

Furthermore, in Experiment 2 and 3, SHR were not hyperactive in the OFT and did not show impairments in the ORT compared to WIS. Since SHR is an animal model of ADHD, it is expected that they show hyperactivity and cognitive impairments. Many studies were able to find these impairments when comparing SHR behavior to Wistar-Kyoto (WKY) rats, the strain that originated SHR rats [70–75]. However, when comparing SHR to other strains, mainly WIS and Sprague-Dawley, no differences in locomotion [39,61,76,77], inattention [70,78], and impulsivity [47,76] were observed. Moreover, WKY is a validated animal model of depression [33,79]. Also, it has been demonstrated that they were hypoactive in comparison to many other strains [80]. So, using WKY as control group for hyperactivity is not adequate, since other strains also are hyperactive and more impulsive when compared to WKY [39,47,70,76]. Thus, the validity of SHR as an animal model of ADHD should be reviewed.

In conclusion, the present study did not find any long-term effect juvenile chronic treatment with MPH or FLX on emotional behavior and cognition after a washout period both in SHR and WIS, independent of sex. It is important to point out that these findings are true for the dose and treatment regimen used. Other doses or ages might produce different results. Moreover, the animals performed a battery of tests in each experiment, which might impact their behavior. Thus, it is essential to evaluate long-

term effects of the current treatment for ADHD using other doses and treating in other periods of time, as well as performing other behavioral tests to better understand the consequences of the treatment.

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5 CONCLUSÃO

Esse trabalho teve como objetivo avaliar as possíveis consequências do tratamento crônico durante a infância e adolescência com MPH e FLX, dois fármacos utilizados para o tratamento de TDAH, na vida adulta. Nenhum dos fármacos causou efeito persistentes, nas doses utilizadas, no comportamento emocional e na cognição de ratos SHR, um modelo de TDAH, e ratos WIS, usados como controle.

No basal, machos SHR apresentaram prejuízos na habituação no campo aberto e menor comportamento-tipo ansioso. Contudo, essas diferenças não foram mais observadas após o tratamento. Na verdade, SHR e WIS se comportaram de forma semelhante nos testes utilizados, independente do tratamento. SHR ficaram menos imóveis que WIS no teste de natação forçada e exploraram menos os objetos no teste de reconhecimento de objetos, mas ainda assim não apresentaram déficits de memória. Também foi observado que fêmeas de ambas as linhagens são mais ativas no campo aberto que os machos e ratos SHR não apresentam hiperatividade quando comparados com ratos WIS, o que vai contra o esperado para os SHR como modelo de TDAH. Esses resultados sugerem que os ratos SHR podem não ser um modelo adequado para avaliar comportamento tipo-TDAH em roedores, já que eles não apresentaram hiperatividade e déficits cognitivos quando comparados com os ratos WIS.

Em suma, o tratamento crônico com MPH e FLX, no período e nas doses utilizados nesse estudo, não levou a alterações no comportamento emocional e na cognição de ratos SHR e WIS após um período sem receber a medicação, o que pode indicar que o uso desses fármacos durante a infância é seguro. Porém, ainda é preciso investigar essas possíveis consequências utilizando outros regimes de tratamento e dose e avaliar outros aspectos comportamentais e neuroquímicos que poder ser alterados devido ao tratamento crônico com esses fármacos.

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