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FERNANDA MARIA ARAUJO DE SOUZA

VALIDAÇÃO DE UM NOVO MODELO ANIMAL NO ESTUDO DA ANSIEDADE E DEPRESSÃO EM CAMUNDONGOS INDUZIDO POR UMA DOSE ÚNICA DE PILOCARPINA

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VALIDAÇÃO DE UM NOVO MODELO ANIMAL NO ESTUDO DA ANSIEDADE E DEPRESSÃO EM CAMUNDONGOS INDUZIDO POR UMA DOSE ÚNICA DE PILOCARPINA

Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde (PPGCS), da Universidade Federal de Alagoas (UFAL), em cumprimento às exigências para a obtenção do título de Doutora.

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“Sempre que alimentamos a alma, ela garante a expansão”.

Clarissa Pinkola Estés, 1992

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RESUMO

Os modelos animais utilizados para o estudo dos transtornos de ansiedade e depressão são ferramentas importantes para a compreensão da fisiopatologia e desenvolvimento de novas terapias farmacológicas. Recentemente, estudos têm demonstrado que doses subconvulsivantes de pilocarpina (PILO; agonista não seletivo dos receptores muscarínicos) produzem um comportamento do tipo ansiogênico de curta e longa duração em ratos *Wistar* machos. Nosso grupo de pesquisa estendeu estes dados ao demonstrar que camundongos *Swiss* de ambos os sexos apresentam comportamento do tipo ansiogênico e depressivo após o tratamento com PILO (75 mg/Kg); sendo as fêmeas mais responsivas que os machos aos efeitos a longo prazo. Este estudo teve por objetivo a validação farmacológica deste novo modelo animal de ansiedade e depressão, além de investigar a morte neuronal no hipocampo e os níveis sorológicos de corticosterona após a administração de uma única dose de PILO em camundongos fêmeas. Para a validade farmacológica, camundongos *Swiss* fêmeas foram tratados com PILO (75 mg/Kg, i.p.) e 24 horas após ou por 30 dias consecutivos tratados com diazepam (DZP; 1,5 mg/Kg, i.p.) ou fluoxetina (FLU; 10 mg/Kg, i.p.). Passados 30 minutos do último tratamento, os animais foram submetidos aos testes do labirinto em cruz elevado (LCE) e teste do nado forçado (TNF) a fim de avaliar os efeitos a curto (24h) e a longo (30d) prazo nos comportamentos relacionados com ansiedade e depressão, respectivamente. A atividade locomotora espontânea dos animais foi avaliada no teste do campo aberto (CA). Posteriormente aos testes, o sangue e o cérebro foram coletados para as análises dos níveis sorológicos de corticosterona e da neurodegeneração hipocampal (por Fluoro-Jade C; FJ-C). Como resultados, observamos que o tratamento com DZP bloqueou o comportamento do tipo ansiogênico a curto e a longo prazo (*e.g.*, aumentou o tempo e o número de entradas nos braços abertos no LCE), mas não o comportamento do tipo depressivo no TNF induzido por PILO. O tratamento com FLU foi capaz de bloquear somente a longo prazo o comportamento do tipo ansiogênico (*e.g.*, aumentou o tempo nos braços abertos no LCE) e depressivo (*e.g.*, diminuiu o tempo de imobilidade no TNF) induzidos por PILO. Os animais tratados com PILO e posteriormente com DZP ou FLU não apresentaram alteração na locomoção espontânea no CA. O tratamento com PILO aumentou os níveis sorológicos de corticosterona a curto prazo. Após 24 h ou 30 d, os animais tratados com PILO não apresentaram neurodegeneração (FJ-C-) hipocampal. Estes resultados sugerem que os comportamentos do tipo ansiogênico e depressivo induzidos pela PILO são bloqueados pelos tratamentos com fármacos ansiolítico e antidepressivo padrão, contribuindo para a validação farmacológica deste novo modelo. Além disso, observou-se que as alterações comportamentais provocadas pela administração de PILO não estão relacionadas com a neurodegeneração hipocampal, distanciando esta dose da convulsiva, que tem como característica a morte neuronal nesta região cerebral. Em resumo, a administração de uma única dose de PILO em camundongos fêmeas mostrou ser, após validação comportamental e farmacológica, um bom e promissor modelo animal para o estudo da ansiedade e depressão.

Palavras-chave: Modelo animal. Ansiedade. Depressão. Camundongos. Pilocarpina.

ABSTRACT

Animal models used to study anxiety and depression disorders are important tools for understanding the pathophysiology and developing new pharmacological therapies. Recently, studies have shown that subconvulsant doses of pilocarpine (PILO; non-selective muscarinic receptor agonist) produce short- and long-term anxiogenic-like behavior in male Wistar rats. Our research group extended these data by demonstrating that Swiss mice of both sexes exhibit anxiogenic- and depressive-like behavior after treatment with PILO (75 mg/kg); where females are more responsive than males to long-term effects. This study aimed to pharmacologically validate this new animal model of anxiety and depression, in addition to investigating neuronal death in the hippocampus and serological levels of corticosterone after the administration of a single dose of PILO in female mice. For pharmacological validity, female Swiss mice were treated with PILO (75 mg/kg, ip) and 24 hours after or for 30 consecutive days treated with diazepam (DZP; 1.5 mg/kg, ip) or fluoxetine (FLU; 10 mg/kg, ip). After 30 minutes of the last treatment, animals were submitted to the elevated plus maze (EPM) and forced swim (FST) tests to evaluate the short- (24h) and long-effects (30d) on related behaviors with anxiety and depression, respectively. The spontaneous locomotor activity of the animals was evaluated in the open-field (OF). After the tests, animals the blood and brain were collected for the analysis of serological levels of corticosterone and hippocampal neurodegeneration (by Fluoro-Jade C; FJ-C). Our results demonstrated that DZP blocked short- and long-term anxiogenic-like behavior, but not depressive-like behavior in PILO-induced FST. While FLU only blocked long-term anxiogenic and depressive-like behavior induced by PILO. Animals treated with PILO and later with DZP or FLU showed no change in spontaneous locomotion in the OF. Treatment with PILO increased serum corticosterone levels in the short-term. After 24 h or 30 d, animals treated with PILO did not show hippocampal neurodegeneration (FJ-C-). Taken together, these results suggest that PILO-induced anxiogenic- and depressive-like behaviors are blocked by standard anxiolytic and antidepressant drugs, contributing to the pharmacological validation of this new model. Furthermore, the behavioral changes caused by PILO are not related to hippocampal neurodegeneration, distancing this dose from the convulsive dose, which is characterized by neuronal death in this brain region. In summary, the administration of a single dose of PILO in female mice proved to be, after behavioral and pharmacological validation, a good and promising animal model for the study of anxiety and depression.

Keywords: Animal model. Anxiety. Depression. Mice. Pilocarpine.

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LISTA DE ABREVIATURAS

AChE	Acetylcholinesterase
ACTH	Adrenocorticotropic hormone
CRH	Corticotropin Releasing Hormone
CRO	Crossing
DZP	Diazepam
ECA	Entries on closed arms
EPM	Elevated plus-maze
FJ-C	Fluoro-Jade C
FLU	Fluoxetine
FST	Forced swim test
GD	Glucocorticoid receptor
GM	Mineralocorticoid receptor
GRO	Grooming
HPA	Hypothalamic-pituitary-adrenal axis
i.p.	Intraperitoneal
OAT	Open arms times
OAE	Open arms entry
OF	Open-field
PILO	Pilocarpine
PT	Pretreatment
pSAP	protected Stretch-attend posture
SAL	Saline solution
REA	Rearing
T	Treatment
uHD	unprotected Head-dipping
VAP	Vasopressine

SUMÁRIO

1 INTRODUÇÃO

Dentre os maiores desafios da saúde pública, os transtornos de ansiedade e depressão chamam a atenção em virtude da alta prevalência mundial, principalmente nas mulheres (World Health Organization, 2017). De forma simultânea, os indivíduos experimentam uma sobreposição dos sintomas de ambos os transtornos (de até > 60 %; Lamers et al., 2011) e este fato, sugere o compartilhamento das bases neurobiológicas entre as patologias consideradas comorbidades (Gorman, 1996; Montgomery and Judge, 2000; Taporoski et al., 2015). A ausência de resposta farmacológica aos fármacos atuais e ainda, em muitos casos, a impossibilidade da continuação do tratamento farmacológico por conta de seus efeitos indesejados/adversos, possibilitando a progressão destas patologias (Griebel and Holmes, 2013; Tiller, 2012). Além disso, os impactos negativos gerados por estes transtornos não se limitam apenas ao paciente, se expandem ao seu ambiente social, aos sistemas de saúde e a economia; com a diminuição significativa da produtividade em escala global (Chisholm et al., 2016).

Os modelos animais constituem uma importante ferramenta para mimetizar os transtornos mentais em laboratório (van der Staay, 2006). Embora o estado interno ou a experiência de um animal não possam ser mensurados nestes modelos, os circuitos que coordenam as respostas comportamentais e/ou neurobiológicas frente às condições manipuladas podem ser identificados e utilizados como medidas correspondentes às alterações em humanos (Calhoon and Tye, 2015; Campos et al., 2013). De fato, os modelos animais podem prever resultados terapêuticos importantes, incrementando novas e prováveis opções de tratamentos farmacológicos na clínica, ou ainda a melhor compreensão da fisiopatologia de determinada doença (Belzung and Lemoine, 2011; McGonigle, 2014; van der Staay, 2006).

Atualmente, os modelos animais no estudo dos transtornos neuropsiquiátricos têm como base as seguintes características: manipulação genética (*e.g.*, animais transgênicos), modulação de neurotransmissores (*e.g.*, administração de fármacos), exposição a determinados fatores ambientais (*e.g.*, submissão as condições estressantes e aversivas), alteração elétrica cerebral (*e.g.*, por meio de estimulação elétrica e danos mecânicos) e seleção de cepas (Griebel and Holmes, 2013; van der Staay, 2006). Entretanto, existem relevantes limitações associadas aos modelos animais, uma vez que a maioria destes avaliam os efeito ansiogênico a curto prazo, tem baixo poder preditivo e utilizam apenas animais machos sendo as fêmeas negligenciadas nos protocolos

experimentais (Griebel and Holmes, 2013; Palanza, 2001). Além disso, faz-se necessário avaliar os efeitos crônicos da manipulação farmacológica nestes modelos animais.

De fato, as mulheres sofrem de transtornos de ansiedade e/ou depressão cerca de 2 vezes mais que os homens (National Institute of Mental Health, 2017). A flutuação de hormônios ao longo da vida da mulher parece ser a principal hipótese que justifica estas diferenças entre os sexos (Kaczkurkin et al., 2016). Os ensaios clínicos e pré-clínicos têm demonstrado alterações neurobiológicas-comportamentais significativas com o sexo feminino apresentando um aumento da atividade do eixo hipotálamo-pituitária-adrenal (HPA) e dos níveis plasmáticos de corticosterona (Goel and Bale, 2010), do comportamento do tipo ansiogênico (Johnston and File, 1991) e depressivo (Goodwill et al., 2019), como também, na expressão diferencial de genes hipocampais, *e.g.*, heat-shock protein (Bundy et al., 2017).

Recentemente, uma série de estudos têm proposto a administração intraperitoneal de uma única dose subconvulsivante de pilocarpina (PILO; agonista não seletivo de receptores muscarínicos) como um novo modelo animal no estudo da ansiedade traço. De fato, os estudos demonstraram que a injeção de PILO, em ratos *Wistar* machos promoveu um comportamento do tipo ansiogênico de curta e longa duração (Duarte et al., 2013, 2010; Hoeller et al., 2016, 2013), alterou a atividade theta hipocampal (Hoeller et al., 2013), como também, a neurogênese e expressão de microRNAs no hipocampo (Ramos Costa et al., 2019). Estendendo estes dados, nosso grupo de pesquisa investigou os comportamentais relacionados com ansiedade e depressão após a administração de PILO (75, 150 e 300 mg/Kg) em camundongos *Swiss* de ambos os sexos. Nossos resultados demonstraram que a administração única de PILO (75 mg/Kg) induziu um comportamento do tipo ansiogênico e depressivo de curta e longa duração nos animais; sendo que as fêmeas foram mais responsivas que os machos (manuscrito submetido para publicação).

Dessa forma, neste estudo buscamos validar farmacologicamente a administração única de PILO como um novo modelo animal de ansiedade traço e de depressão em camundongos *Swiss*. Além de estender a validação comportamental como modelo de depressão, dosando os níveis sorológicos de corticosterona e o padrão de neurodegeneração hipocampal.

2 OBJETIVOS

2.1 Objetivo geral

Validar farmacologicamente a administração única de PILO como um novo modelo animal de ansiedade traço e de depressão em camundongos *Swiss* fêmeas.

2.2 Objetivos específicos

- Avaliar se o tratamento agudo ou crônico com o fármaco diazepam bloqueia o efeito do tipo ansiogênico induzido por uma única injeção de PILO (75 mg/Kg, i.p.) em camundongos *Swiss* fêmeas;

- Avaliar se o tratamento agudo ou crônico com o fármaco fluoxetina bloqueia o efeito do tipo depressivo induzido por uma única injeção de PILO (75 mg/Kg, i.p.) em camundongos *Swiss* fêmeas;

- Quantificar a curto e longo prazo os níveis sorológicos de corticosterona após a administração de uma única injeção de PILO (75 mg/Kg, i.p.) em camundongos *Swiss* fêmeas;

- Avaliar a neurodegeneração hipocampal após a administração de uma única injeção de PILO (75 mg/Kg, i.p.) em camundongos *Swiss* fêmeas.

ARTIGO 1

Pharmacological validation of the novel model of enduring anxiety induced by pilocarpine in mice

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Author contribution

FMA Souza, FS Duarte, TCM de Lima, M Duzzioni: Conceptualization; FMA Souza, MPS Vieira, GF Souza, RGD Torres, JGS Neto, M Duzzioni: Methodology; FMA Souza, MPS Vieira, GF Souza, RGD Torres, NKG Tavareiros, JGS Neto, MA Amarós, AHR Cofré, DLG Gitaí, OW Castro, M Duzzioni: Investigation; FMA Souza, NKG Taveiros, JA Junkes, M Duzzioni: Formal analysis; FS Duarte, TCM de Lima, M Duzzioni: Supervision and fund acquisition; FMA Souza, AHR Cofré, JA Junkes, DLG Gitaí, OW Castro, FS Duarte, TCM de Lima, M Duzzioni: Writing - review and editing.

Declaration of Competing Interest

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Abstract

Rationale: Animal models are useful tools to study the neurobiological basis of anxiety. However, reproducing human anxiety in preclinical trials is a major challenge. Recent studies have suggested a novel murine model of enduring anxiety following the administration of a single subconvulsive dose of pilocarpine. *Objectives:* Pharmacological validation of the novel murine model for enduring anxiety induced by pilocarpine (PILO). *Methods:* Female Swiss mice were treated with a single dose of PILO (75 mg/Kg, i.p.), followed by 24 h or 30 consecutive days with diazepam (DZP; 1.5 mg/Kg, i.p.) or fluoxetine (FLU; 10 mg/Kg, i.p.) treatment. Thirty minutes after the last treatment, animals were submitted to the elevated plus-maze (EPM) and open-field (OF) tests to assess anxiety- and locomotion-related behaviors. *Results:* Animals treated with PILO showed short- and long-term anxiogenic-like behavior (*e.g.*, decreased percentage of entries and time spent on open arms) in the EPM test. The acute or chronic treatment with DZP blocked PILO-induced anxiogenic-like behavior. On the other hand, only chronic treatment with FLU (for 30 d) was able to block the PILO-induced anxiogenic-like behavior. Results cannot be attributed to locomotor alterations. *Conclusions:* To our knowledge, we are the first to report that standard anxiolytic drugs block the anxiogenic-like effect produced by PILO, which contributes to the pharmacological validation of the murine model.

Keywords: Murine animal anxiety; pilocarpine; diazepam; fluoxetine; anxiety; pharmacological validity

1 Introduction

Anxiety is an adaptive and evolutionary emotion, which under normal conditions, protects the individual from potentially dangerous situations. However, when anxiety levels are exacerbated, its protective function becomes detrimental to emotional, psychological, cognitive and behavioural characterizing a pathological disorder that requires immediate treatment (American Psychiatric Association, 2013; Crocq, 2015). The consequences of anxiety have a strong impact on the quality of life (World Health Organization, 2017), especially for women, who are twice as likely to develop this mental disorder (Jalnapurkar, 2018; Kinrys and Wygant, 2005; National Institute of Mental Health, 2017).

Animal models of anxiety are useful tools for the study of the neurobiological basis of anxiety, and for the pharmacological screening of therapeutic compounds to treat it (Belzung and Lemoine, 2011; McGonigle, 2014). However, there are some limitations associated with the validation of these models, which include the lack of long-term behavioural assessment and sex bias that excludes females (Griebel and Holmes, 2013; Palanza, 2001). Furthermore, the pharmacological validity must be investigated in the establishment of a new animal model for the study of mental disorders (Belzung and Lemoine, 2011; Willner, 1984; van der Staay, 2006).

Studies have suggested a novel murine model of enduring anxiety following the administration of a single subconvulsive dose of pilocarpine (PILO; non-selective agonist of muscarinic receptors; Duarte et al., 2010; 2013; Hoeller et al., 2016, 2013; Ramos Costa et al., 2019). Briefly, they demonstrated that male *Wistar* rats exhibited short- and long-term anxiogenic-like behaviour (Duarte et al., 2013, 2010; Hoeller et al., 2013), neurogenesis and downregulation of the hippocampal expression of microRNAs (Ramos Costa et al., 2019). Our research group has shown that *Swiss* mice of both sexes exhibit anxiogenic-like behaviour after PILO-treatment, with females being more

responsive than males to long-term effects (data no showed). To the best of our knowledge, there are no studies demonstrating the pharmacological validity of a single administration of PILO for modelling enduring anxiety in female mice. Therefore, we aim to pharmacologically validate the novel model of enduring anxiety induced by PILO in mice.

2 Materials and Methods

2.1 Animals

Female *Swiss* mice (60 days old, weighing 30 - 35 g) were obtained from the animal facility of the Federal University of Alagoas (BIOCEN/UFAL). The animals were housed in collective cages (up to 10 animals/cage; 41 x 34 x 16 cm), maintained at a temperature of 22 ± 2 °C, and 12 h light-dark cycle (with lights on at 7:00 a.m.). Food and water were available *ad libitum*, except during the experiments. The experimental protocols were approved by the Ethics Committee on Animal Use of the Federal University of Alagoas (Protocol number #52/2018) in accordance with the Brazilian National Council for Animal Experimentation Control (Conselho Nacional de Controle da Experimentação com Animais, CONCEA). Before the beginning of the experiments, the animals underwent a one-week acclimatization. All experiments were performed between 12:00 p.m. and 6:00 p.m.

2.2 Drug treatments and experimental design

The following drugs were used: pilocarpine hydrochloride (PILO; Ao Pharmacêutico®, Alagoas, Brazil), methyl scopolamine bromide (Sigma-Aldrich Co., St. Louis, USA), diazepam (DZP; Santista Lab. Farmacêutico S.A), fluoxetine (FLU; Ao Pharmacêutico®, Alagoas, Brazil) and saline solution (SAL; NaCl 0.9%, used as a

control solution). All substances were diluted in SAL and prepared on the same day of administration at a volume of 10 ml/Kg.

To minimize the peripheral adverse effects of PILO, all animals were treated with methyl scopolamine bromide [1 mg/Kg; subcutaneous injection (s.c.); Clifford et al., 1987)]. After 30 minutes, a dose of PILO [75 mg/Kg; intraperitoneal injection (i.p.)] or SAL (NaCl, 0.9%; i.p.) was administered. The behavioural analysis of seizures were performed for 90 minutes, in accordance with the Racine scale (Racine 1972). For acute protocol, after 24 h of treatment with SAL or PILO, animals receveid SAL (i.p.), DZP (1.5 mg/Kg; i.p.) or FLU (10 mg/Kg; i.p.), and 30 minutes later, subjected to the elevated plus-maze (EPM) and open-field (OF) tests (Fig.1A).

For chronic protocol, a second group of animals were treated for 30 days (30 d) with the same drugs and doses, and subject to the same behavioural tests as the previous 24 h group (Fig. 2A). At the end of behavioural tests, animals were euthanized with a lethal dose of thiopental (Thiopentax®; 150 mg/Kg, i.p.). All experiments were recorded by video camera (Intelbras®, Santa Catarina, Brazil; model VD 3108) positioned above the behavioural apparatus

2.3 Racine scale

Behavioural seizures were classified according to the Racine scale (Racine, 1972), with the following stages (S): S0, immobility; S1, facial automation; S2, head and neck myoclonus; S3, forelimb clonus; S4, raising of the forelimbs with clonic convulsions; S5, raising of the forelimbs with clonic convulsions and loss of posture.

2.4 Estrous cycle identification

The estrous cycle stage was verified by vaginal cytology (Byers et al., 2012; McLean et al., 2012) 24 h or 30 d after the behavioral tests. Briefly, 50 µL of SAL was

introduced to the vaginal canal with a 200 µL pipette tip. Immidiately, the fluid was aspirated using the same tip and transferred to a slide to air-dry for 10 minutes, fixed with methanol and stanning with Panotico Kit (Insta Prov Panotico, NewProv). The estrous cycle stage is determined by the presence or absence of leukocytes, cornified epithelial cells, and nucleated epithelial cells, which were visualized by microscopy (Bioval, L-1000B). The female mice were in the diestrus cycle phase during the behavioral tests (data not shown).

2.5 Behavioural tests

2.5.1 Elevated plus-maze

The elevated plus-maze (EPM; Insight®, São Paulo, Brazil) is made of plexiglas and consists of two open (30 x 5 cm) and closed arms (30 x 5 x 25 cm), raised from the ground level (38.5 cm). The open arms are surrounded by lateral bars (0.25 cm in height) to prevent the animals from falling. Individually, the mice were placed on the central platform, with its face towards a closed arm. For 5 minutes, the following spatiotemporal parameters were analyzed: (1) frequency of the time spent (seconds) in open arms (OAT), and of entries into either open arms (OAE) and closed arms (ECA). The number of ECA was used as an index of general activity (Rodgers et al., 1997). To increase test sensitivity, ethological parameters, such as protected stretch-attend posture (pSAP), unprotected head-dipping (uHD), rearing (REA) and grooming (GRO) were also recorded (Cole; Rodgers, 1994). Following each experiment, the EPM apparatus was carefully cleaned with a 10 % ethanol solution.

2.5.2 Open-field

The OF (Insight®, São Paulo, Brazil) is made of plexiglas and consists of a circular acrylic arena (30 cm high, 30 cm diameter), with its floor divided into eight squares. Mice were individually placed in the center of the arena to record the number of lines crossed (CRO) with the four paws and rearing (REA), for a period of 5 minutes (Prut and Belzung, 2003). Following each experiment, the OF apparatus was carefully cleaned with a 10 % ethanol solution.

2.6 Statistics analysis

All results are expressed as mean ± standard mean error (S.E.M). We used the two-way ANOVA test with the pretreatment (PT; PILO or SAL) and treatment (T; SAL, DZP or FLU) and interaction of this (PT x T) as factors. The Student Newman–Keuls *post-hoc* test was used to analyze the differences between the experimental groups. We assume a value of $P \leq 0.05$ to determine the statistical differences. These data were plotted using GraphPad Prism (Version 8.0).

3. Results

3.1 PILO-injection induced low stages of Racine scale

After treatment with a single dose of PILO (75 mg/Kg, i.p.), mice were analyzed according to the Racine scale for 90 minutes. In the 24 h-group, PILO-treated mice presented stages at S0 (n = 2; 7.69 %) and S2 (n = 24; 92.31 %). In the 30 d-group, stages at S0 (n = 4; 12.5 %), S1 (n = 3; 9.37 %), and S2 (n = 25; 78.12 %) were observed. None of the animals presented stages at S3. The observed behavioural seizures were characterized as isolated, and upon their reintroduction to housing cages, PILO-pretreated mice were indistinguishable from the control mice. The animals were devoid of self-sustained seizures and the seizures presented lasted less than 1 minute. In

addition, previous analyzes by our research group demonstrated the absence of hippocampal neurodegeneration at the dose of PILO 75 mg/kg (unpublished data).

3.2 Acute treatment with DZP, but not FLU, blocked short-term anxiogenic-like effect induced by PILO

To assess whether PILO-induced short-term anxiogenic-like behaviors can be blocked by anti-anxiety agents, mice were treated with PILO, and 24 h later, with either DZP or FLU, and subjected to the EPM test (Fig.1A).

Two-way ANOVA showed that PILO-injection affects the % OAT [PT: $F(1, 38) = 35.97$; $P<0.0001$], such as the treatments [T: $F(2,38) = 40.51$; $P<0.0001$] and the interaction between these factors [PTxT: $F(2,38) = 3.24$; $P=0.0499$]. Similarly, pretreatment with PILO changed the % OAE [PT: $F(1, 38) = 21.64$; $P<0.0001$], and the treatments [T: $F(2, 38) = 32.36$; $P<0.0001$] without the interaction between these factors [PTxT: $F(2, 38) = 0.93$; $P=0.4041$]. The Newman–Keuls *post-hoc* test showed that 24 h treatment with SAL after PILO-injection decreased the % OAT ($P<0.05$; Fig.1B) and the % OAE ($P<0.05$; Fig.1C) compared to the control group (SAL+SAL), indicating an anxiogenic-like behavior. Administration of DZP after 24 h of PILO-pretreatment increased the % OAT ($P<0.01$; Fig.1B) and the % OAE ($P<0.05$; Fig. 1C), by blocking PILO-induced anxiogenic-like behavior. However, acute treatment with FLU after PILO-injection potentiated its anxiogenic-like behaviour by decreased the % OAT ($P<0.01$) and the % OAE ($P<0.01$). Treatment with DZP after SAL-injection increased the % OAT and the % OAE, as compared to PILO+SAL ($P<0.001$) and control (SAL+SAL; $P<0.01$; $P<0.05$), indicating an anxiolytic-like behaviour (Fig.1B and Fig.1C).

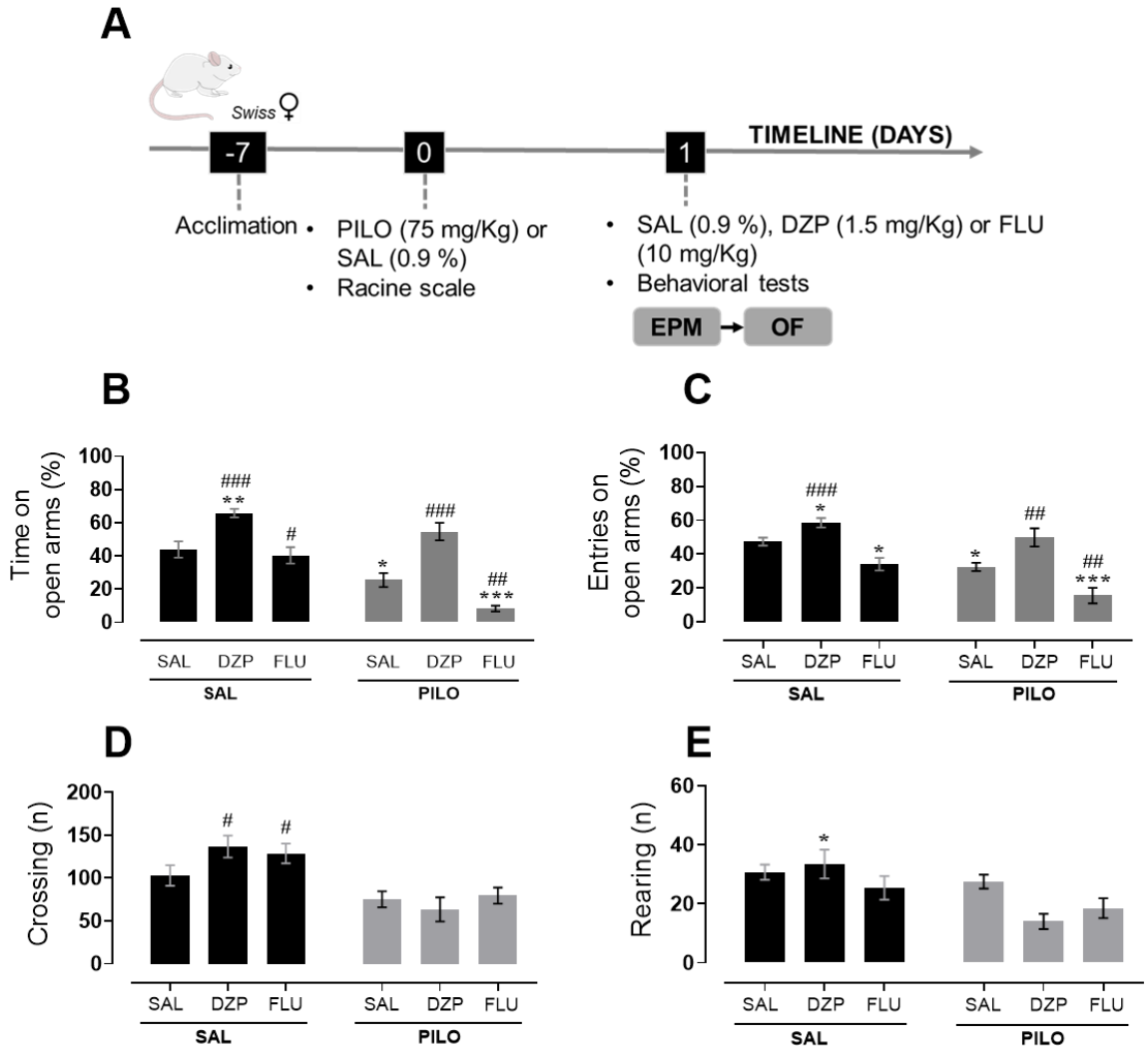

Figure 1. Short-term anxiogenic-like effect induced by pilocarpine (PILO; 75 mg/Kg, i.p.) was blocked by acute treatment with diazepam (DZP; 1.5 mg/Kg, i.p.), but not by fluoxetine (FLU; 10 mg/Kg, i.p.) in female mice submitted to the elevated plus-maze test (EPM). Diagram of the experimental design (A). Spatial and temporal parameters: percentage of time (B) and entries (C) to open arms on EPM, number of crossings (D) and rearing (E) on open-field (OF). Each value represents the mean ± S.E.M of 6-9 animals per group. **P<0.05*, ***P<0.01* or ****P<0.001* compared to control (SAL+SAL) and $^{\#}$*P<0.05*, $^{\#\#}$*P<0.01* or $^{\#\#\#}$*P<0.001* compared to PILO+SAL. Two-away ANOVA followed by Student Newman–Keuls test. SAL; saline.

For ethological parameters analyzed in the EPM test (Table 1), two-way ANOVA showed that the pretreatment, treatment or the interaction of these factors changed the number of REA [PT: F(1, 39) = 24.52; *P<0.0001*], T: F(2, 39) = 12.91; *P<0.0001* and PTxT: F(2, 39) = 4.09; *P=0.0244*], such as uHD [PT: F(1, 38) = 24.37; *P<0.0001*; T: F(2, 38) = 34.37; *P<0.0001* and PTxT: F(2, 38) = 11.77; *P=0.0001*]. In

the REA, *post-hoc* analysis revelead that the FLU-injection 24 h after PILO significantly decreased this parameter compared to PILO+SAL (*P<0.001*) or SAL+SAL (*P<0.001*), while the treatment of DZP after SAL-administration increased it compared to PILO+SAL (*P<0.01*) and SAL+SAL (*P<0.05*). The treatment with DZP 24 h after SAL-injection also increased the number of uHD compared to SAL+SAL (*P<0.001*) and PILO+SAL (*P<0.001*). The pSAP was affected by treatments [T: F(2, 38) = 3.29; *P=0.0481*], but neither by the pretreatment [PT: F(1, 38) = 0.01; *P=0.9796*] or interaction of these factors [PTxT: F(2, 38)= 0.07; *P=0.9336*]. In the GRO, two-way ANOVA showed that only the pretreatment altered this parameter [PT: F(1, 38) = 5.90; *P=0.0200*] without influence of treatments [T: F(2, 38)= 0.03; *P=0.9687*] or interaction of these factors [PTxT:F (2, 38)= 0.08; *P=0.9190*]. Finally, as revealed by two-way ANOVA, the number of ECA was not altered by any factors [PT: F(1, 38) = 0.05; *P=0.8268*, T: F(2, 38) = 1.16; *P=0.3250* and PTxT: F(2, 38) = 1.942; *P=0.1574*]. *Post-hoc* tests did not detect any difference among the groups for the numbers of GRO, ECA and pSAP (*P>0.05*) (Table 1).

In the OF, two-way ANOVA showed that the number of CRO and REA was affected only by the pre-treatment [CRO; PT: F(1, 38) = 26.26; *P<0.0001*, T: F(2, 38) = 0.80; *P=0.4544* and PTxT: F(2, 38) = 1.87; *P=0.1685*]; and REA [PT: F(1, 38) = 10.62 *P=0.0024*], [T: F(2, 38) = 1.953; *P=0.1558*] and [PTxT: F(2, 38) = 2.780; *P=0.0747*]}. The Newman–Keuls *post-hoc* showed an increase for CRO in SAL+DZP (*P<0.05*) and SAL+FLU (*P<0.05*) groups compared to PILO+SAL (Fig.1D), such as an increase of REA in SAL+DZP (*P<0.05*), as compared to SAL+SAL (*P<0.05*; Fig.1E).

Elevated plus-maze (EPM)					
Treatment	ECA	REA	pSAP	uHD	GRO
SAL+SAL	11.83±1.25	13.33±2.42	0.17±0.17	6.33±2.32	1.00±0.26
PILO+SAL	15.00±1.30	12.11±1.71	0.01±0.01	3.87±1.01	0.50±0.19
SAL+DZP	16.22±1.39	18.78±1.04$^{*\#\#}$	0.10±0.10	30.57±3.48$^{***\#\#\#}$	1.00±0.24
PILO+DZP	12,86±5.40	10.86±1.24	0.01±0.01	8.71±2.37	0.43±0.20
SAL+FLU	16.38±1.27	12.00±1.57	1.37±0.70	4.00±1.43	0.87±0.23
PILO+FLU	15.67±2.06	2.17±0.99$^{***\#\#\#}$	1.67±1.67	0.50±0.22	0.50±0.34

Table 1. Effects on the behaviour of female mice of acute treatment with diazepam (DZP; 1.5 mg/Kg) or fluoxetine (FLU; 10 mg/Kg) 24 h after PILO-injection evaluated in the EPM. Each value represents the mean ± S.E.M. $*P<0.01$ or $***P<0.001$ compared with SAL+SAL and $^{\#\#}P<0.01$ or $^{\#\#\#}P<0.001$ compared with PILO+SAL. Two-way ANOVA followed by Student Newman–Keuls test of 6 - 9 animals per group. ECA: entries into enclosed arms; uHD: unprotected head-dipping; pSAP: protected stretched attend posture; REA: rearing; and GRO: grooming. PILO: pilocarpine. SAL: Saline.

3.3 Chronic treatment with DZP and FLU blocked long-term anxiogenic-like effects induced by PILO

To assess whether chronic treatment with DZP or FLU can alleviate the PILO-induced long-term anxiogenic-like behaviors in the EPM test, mice were treated for 30 days with these anti-anxiety drugs starting 24 hours later PILO-injection (Fig.2A).

The ANOVA two-way showed that the % OAT was influenced by the pre-treatment [PT: $F(1, 34) = 4.450$; $P=0.0423$], treatment [T: $F(2, 34) = 10.74$; $P=0.0002$], and the interaction of these factors [PTxT: $F(2, 34) = 4.20$; $P=0.0234$]. *Post-hoc* analysis revelead that the PILO-injection decreased the % OAT compared to the SAL+SAL group ($P<0.01$; Fig.2B), indicating PILO-induced long-term anxiogenic-like behavior. Interestingly, the chronic treatment with DZP or FLU in animals pretreated with PILO increased the % OAT in the EPM (Fig.2B), indicating that the PILO-induced long-lasting anxiogenic-like behaviour was blocked with these anti-anxiety drugs. In the % OAE, ANOVA two-way detected a influence for the treatment [T: $F(2, 34) = 4.37$;

P=0.0205] and interaction [PT x T: F(2, 34) = 4.93; *P=0.0132*] factors, but not for pre-treatment [PT:F (1, 34) = 0.09188; *P=0.7636*]. *Post-hoc* analysis revelead an increase in the % OAE of the mice pretreated with PILO and treated with DZP or FLU, when compared to the PILO+SAL group (*P<0.01*; Fig.2C).

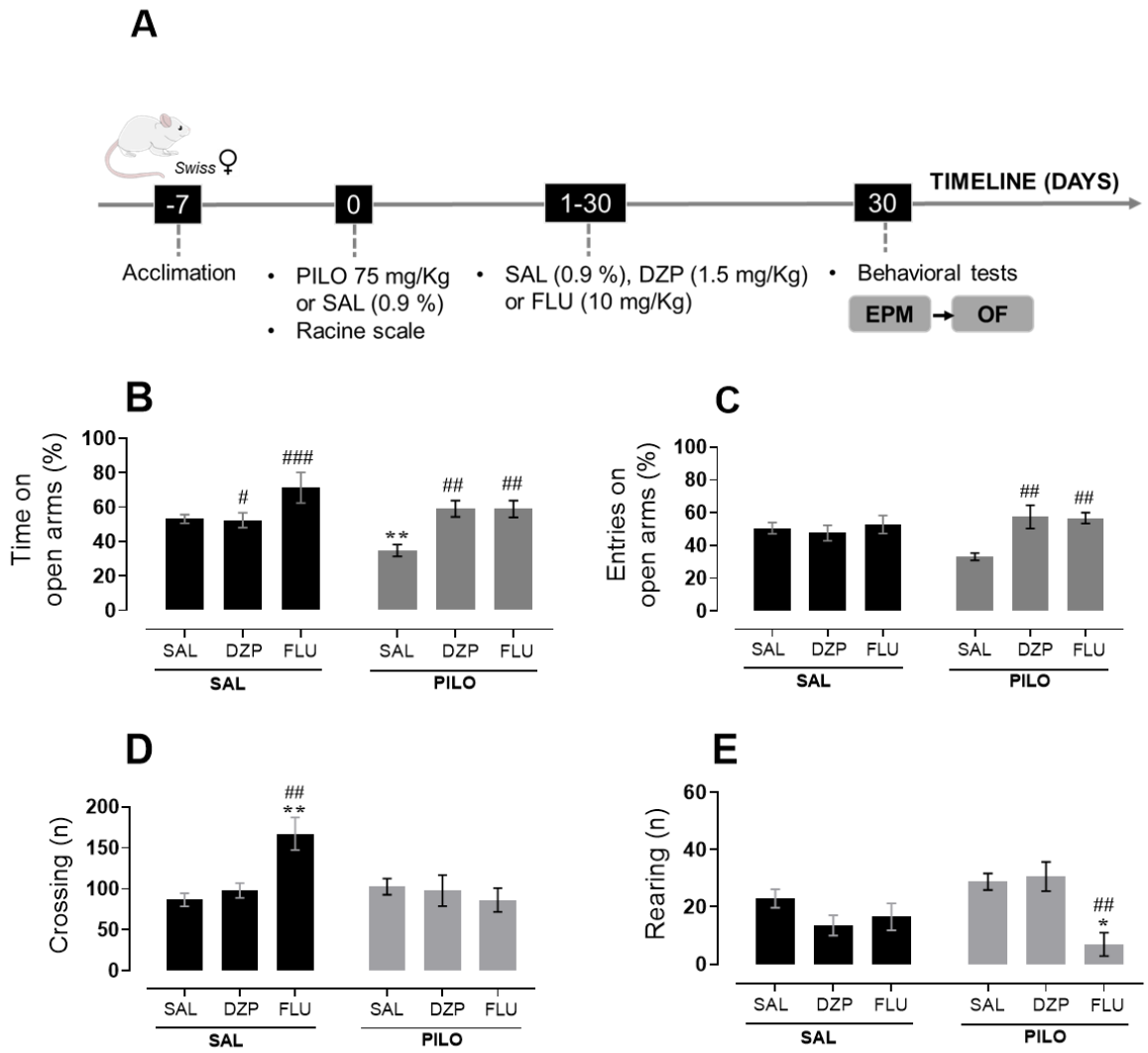

Figure 2. Long-term anxiogenic-like effect induced by pilocarpine (PILO; 75 mg/Kg, i.p.) was blocked by chronic treatment with diazepam (DZP; 1.5 mg/Kg, i.p.) and fluoxetine (FLU; 10 mg/Kg, i.p.) in female mice submitted to the elevated plus-maze test (EPM). Diagram of the experimental design (A). Spatial and temporal parameters: percentage of time (B) and entries (C) to open arms on EPM, number of crossings (D) and rearing (E) on open-field (OF). Each value represents the mean ± S.E.M of 4-8 animals per group. **P<0.05*, ***P<0.01* or ****P<0.001* compared to control (SAL+SAL) and $^{\#}P<0.05$, $^{\#\#}P<0.01$ or $^{\#\#\#}P<0.001$ compared to PILO+SAL. Two-away ANOVA followed by Student Newman–Keuls test. SAL; saline

In the ethological parameters (Table 2), two-way ANOVA showed that the number of REA and ECA was affected by the interaction of factors {REA [PTxT: F(2, 34)= 6.673; *P=0.0036*] and ECA [PTxT: F(2, 34) = 8.51; *P=0.0010*]}, but neither by pretreatment {REA [PT: F(1, 34) = 2.54; *P=0.1204*] and ECA [PT: F(1, 34) = 3.32; *P=0.0774*]} or treatment {REA [T:F (2, 34) = 3.03; *P=0.0617*] and ECA [T:F (2, 34) = 2.31; *P=0.1146*]}. *Post-hoc* analysis revelead that only chronic treatment with FLU after PILO-injection significantly decreased the number of REA and ECA compared to PILO+SAL (*P<0.001*; Table 2). Additionally, two-way ANOVA showed the influence of pretreatment in the number of uHD [PT: F(1, 34) = 4.24; *P=0.0471*] and GRO [PT: F(1, 36) = 8.48; *P=0.0061*], but not of the treatment {uHD [T: F(2, 34) = 1.49; *P=0.2386*] and GRO [T:F (1, 36) = 8.48; *P=0.0061*]} and interaction of the factors {uHD [PTxT: F(2, 34) = 1.54; *P=0.2295*] and GRO [PTxT: F(2, 36) = 0.1644; *P=0.8490*]. However, *post-hoc* analysis did not find significant differences among the groups for these two parameters. Finally, two-way ANOVA showed that pSAP was influenced by the treatment [T: F(2, 34) = 5.36; *P=0.0095*] and the interaction of factors [PTxT: F(2, 34) = 8.38; *P=0.0011*], but not by pretreatment [PT: F(1, 34) = 0.1407; *P=0.7100*]. According to *post-hoc* analysis, the chronic treatment with DZP and FLU after PILO-administration decreased the pSAP compared to PILO+SAL. Simirlay, the treatment with FLU after SAL-injection decreased this parameter compared to the PILO+FLU (*P<0.05*).

Elevated plus-maze (EPM)					
Treatment	ECA	REA	pSAP	uHD	GRO
SAL+SAL	11.50±1.16	13.0±2.66	0.75±0.16	6.87±1.74	0.87±0.23
PILO+SAL	16.12±1.00	18.87±1.77	2.12±0.44	6.25±1.66	1.75±0.16
SAL+DZP	15.20±1.71	15.20±1.98	2.00±0.89	17.40±1.47	1.14±0.26
PILO+DZP	10.62±1.96	10.62±2.03$^{\#}$	0.37±0.18$^{\#}$	10.63±2.36	1.75±0.41
SAL+FLU	14.00±1.58	15.50±1.76	0.25±0.25$^{\#}$	23.00±19.74	1.00±0.41
PILO+FLU	7.00±1.1.33$^{\#\#\#}$	5.14±2.72$^{\#\#\#}$	0.14±0.14$^{\#\#}$	4.29±2.66	2.00±0.49

Table 2. Effects on female mice of chronic treatment with diazepam (DZP; 1.5 mg/Kg) or fluoxetine (FLU; 10 mg/Kg) 30 days after PILO-administration evaluated in the EPM. Each value represents the mean ± S.E.M of 4 - 8 animals per group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ or $^{\#\#\#}P<0.001$ compared with PILO+SAL. Two-way ANOVA followed by Student Newman–Keuls test. ECA: entries into enclosed arms; uHD: unprotected head-dipping; pSAP: protected stretched attend posture; REA: rearing; and GRO: grooming. PILO: pilocarpine. SAL: Saline.

For spontaneous locomotor activity evaluated in the OF, two-way ANOVA revealed influence in the number of CRO and REA by interacting of the factors {CRO [PTxT: F(2, 45) = 7.167; *P=0.0020*] and REA [PTxT: F(2, 46)= 4.957; *P=0.0112*]} and treatment {CRO [T: F(2, 45) = 3,296; *P=0.0462*] and REA [T: F(2, 46) = 7,124; *P=0.0020*], but not by the pretreatment {CRO [PT: F(1, 45) = 3.57; *P=0.0654*] and REA [PT: F(1, 46) = 1.82; *P=0.1835*]}. *Post-hoc* analysis showed an increase in the number of CRO after chronic treatment with FLU in animals previously treated with SAL compared to SAL+SAL (*P<0.01*) and PILO+SAL (*P<0.01*), as seen in Fig.2D. In relation to REA, *post-hoc* analysis revealed that only chronic treatment with FLU after PILO-administration decreased this parameter compared to SAL+SAL (*P<0.05*) and PILO+SAL (*P<0.01*).

4. Discussion

Previous data from our research group have demonstrated a short- and long-term anxiogenic-like effect in female Swiss mice after a single injection of PILO 75 mg/kg

(unpublished data). Here, we demonstrated that PILO-injection induced a short- and long-term anxiogenic-like effect in female mice evaluated in the EPM test. Furthermore, acute and chronic treatment with DZP blocked these PILO-induced behavioral changes. However, acute and chronic treatment with FLU showed opposite effects in the EPM test. Acute FLU treatment potentiated the behavioral effects induced by PILO, while chronic treatment blocked them. Taken together, these results contribute to pharmacological validation of this novel murine anxiety model.

PILO have been widely used as a model for temporal lobe epilepsy (for review see Scorza et al., 2009). The activation of the muscarinic receptors following the convulsive PILO-administration, can trigger electrical seizures and changes in hippocampal thetha rhythm (Turski et al., 1983; 1984). The cholinergic signaling in the hippocampus has been reported to be regulating behaviors related to anxiety and depression (Mineur et al., 2013). Interestingly, Duarte et al. (2013) demonstrated an increase in the hippocampal theta rhythm in male rats with an anxious-like phenotype after PILO-injection at a subconvulsant dose. In addition, a non-convulsive dose of PILO promotes neurogenesis and downregulation of miRNAs in the hippocampus (Ramos Costa et al., 2019). These studies indicate that low doses of PILO can cause enduring neuronal changes that contribute to long-lasting behavioral effects. However, further studies should be conducted to understand how these neuronal changes, especially in the hippocampus, mediate PILO-induced behavioral responses.

Animal models of anxiety are used to assess innate and conflicting responses after exposure to potentially threatening situation (Calhoon and Tye, 2015). And the behavioral and physiological responses of animals are accessed through specific tests (Harro, 2018; Palanza, 2001). Here, we used the EPM test to investigate the short- and long-term anxiogenic-like effects induced by a single dose of PILO. The EPM test has

been widely used for the screening of anti-anxiety compounds, such as, benzodiazepine agonists (Varty et al., 2002; Walf and Frye, 2007), 5-HT1A agonists and selective serotonin (5-HT) reuptake inhibitors (Varty et al., 2002), and natural and synthetic substances (Calhoon and Tye, 2015; Walf and Frye, 2007). Our data showed that female mice treated with a single dose of PILO exhibit a short- and long-term anxiogenic-like behavior in the EPM, corroborating with studies developed by Duarte et al., 2013, 2010; Höeller et al., 2013, 2016).

In our research for pharmacological validation of the novel model of enduring anxiety induced by PILO administration in mice, animals were treated acutely and chronically with standard anxiolytic drugs – DZP (Blanchard et al., 1990; Crawley, 1985; Griebel et al., 2000) and FLU (Griebel et al., 1999; Nowakowska et al., 2000). DZP is one of the most common benzodiazepines used for anxiety (Altamura et al., 2013; Letizia Trincavelli et al., 2012). In mice, the anxiolytic effect of DZP has been shown in different strains and behavioral analysis apparatus, such as EPM (Griebel et al., 2000; Kurt et al., 2000). Furthermore, classical anxiolytics such as benzodiazepines have been shown to change the septohippocampal theta rhythm, a neurocircuitry crucial for the perception of threatening environmental signals (Gray and Mcnaughton, 2000). Benzodiazepines also reduce the release of acetylcholine in the cortex (Moore et al., 1995). Höeller et al. (2013), blocked the anxiogenic-like behavior induced by a subconvulsant dose of PILO with benzodiazepine drug, in rats. Similarly, we demonstrate that the PILO-induced anxiety-like phenotype is blocked by DZP contributing to the pharmacological validation of this model.

FLU exhibits a delayed onset of anxiolytic therapeutic response, with some clinical reports of symptom deterioration in the first weeks of treatment (Perez-Caballero et al., 2014). In animals, the anxiolytic-like effect of chronic treatment with

FLU can be observed in different behavioral tests, such as EPM (Kurt et al., 2000), light-dark box (Farhan and Haleem, 2016; Nowakowska et al., 2000), novelty-induced hypophagia (Farhan and Haleem, 2016) and OF (Farhan and Haleem, 2016). Our results demonstrated that acute treatment with FLU exacerbated PILO-induced anxiety-like effect, while chronic treatment blocked them. In addition to contributing to the pharmacological validation of this PILO-induced anxiety model, our results demonstrate that the PILO model is sensitive to the acute and chronic effects of FLU, that highlights the model's relevance to the screening of new anti-anxiety agents, as well as their mechanisms of action.

After PILO-injection, animals did not present a Racine scale score greater than S2 or features of sustained seizures. Although we did not perform EEG recordings, Turski et al. (1984) showed that fully-developed EEG seizures did not appear up to 24 h after administration of a low (100 mg/Kg) or intermediate (200 mg/Kg) dose of PILO. Duarte et al. (2010) showed this same response up to 90 days after treatment with a subconvulsant dose of PILO. Furthermore, female mice are more resistant to developing *status epilepticus,* thus requiring higher doses (> 400 mg/Kg) of PILO as compared to male mice (Römermann et al., 2015). Therefore, we assume that the behavioral changes observed in this work are not induced by an epileptic seizure.

Interestingly in the OF, the chronically treated group with FLU (SAL+FLU) showed an increase in the number of crossings, however these change are associated with an increase of locomotor activity. The alteration of this parameter can be explained by an increase in the exploratory activity of the animals, as a result of a decrease in the valence of the aversive stimulus promoted by the FLU. In line with this, increased number of crossings in familiar housing cages and novel environment (open-field) have been shown after repeated FLU-injection (Farhan and Haleem, 2016). Additionally, sex

must be considered, as female rodents naturally demonstrate greater exploratory activity compared to males (Hughes, 1968).

A limiting factor in the experimental design of preclinical anxiety research is sex bias, in which males are used up to 14 times more than females (Griebel; Holmes, 2013). Relevant to our study, anxiogenic-like effects of PILO were performed only on male rodents (Duarte et al., 2010, 2013; Hoeller et al., 2016, 2013; Ramos Costa et al., 2019). Thus, our study is the first to report that female mice are not only responsive to PILO, but also to DZP and FLU treatments. Thus, we validated this pharmacologically relevant murine model to be used regardless of the mice' sex.

In summary, our results demonstrate that the administration of PILO induced an anxious-like phenotype in female mice; and acute and chronic treatment with standard anti-anxiety drugs blocked this behavioral effect, contributing to the pharmacological validation of this novel murine model of enduring anxiety.

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ARTIGO 2

Behavioural and pharmacological validation of the novel model of depression induced by pilocarpine in mice

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Declaration of Competing Interest

All authors state to have no conflict of interest.

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Abstract

Rationale: Animal models are important tools to study the pathophysiology of depression and finding new antidepressant agents. *Objectives:* Behavioral and pharmacological validation of the novel murine model for depression induced by pilocarpine (PILO). *Methods:* Female Swiss mice were treated with a single dose of PILO (75 mg/Kg, i.p.), followed by 24 h or 30 consecutive days with fluoxetine (FLU; 10 mg/Kg, i.p.) or diazepam (DZP; 1,5 mg/Kg, i.p.). Thirty minutes after the last treatment, animals were submitted to open-field (OF) and forced swim (FST) tests to investigate locomotion and depressive-like behaviors. Blood samples and whole brain were collected to determination of the levels of corticosterone in serum and hippocampal neurodegeration, respectively. *Results:* Animals treated with PILO showed a short- and long-term depressive-like behaviour (e.g., increased immobility time) in the FST, without affecting locomotor activity and hippocampal neurodegeneration. Only chronic treatment with FLU blocked PILO-induced depressive-like behavior (e.g., decreased immobility time) in the FST. Futhermore, an increase in serum corticosterone levels was observed 24h after PILO-injection. *Conclusions:* To the best of our knowledge, this is the first report that PILO induces a depressive-like behavior in female mice, followed by an increase in serum corticosterone levels without producing hippocampal neurodegeneration. And this depressive-like phenotype was blocked after chronic treatment with an antidepressant agent. Taken together, these results contribute to the validation of a new animal model of depression.

1 Introduction

Depression is a mood disorder that causes worldwide disability and produces major economic burden. According to reports from the World Heatlh Organization (2017) more than 300 million people (4.4 % of the world population) present some type of depressive disorder, with women being twice affected. Depressive individuals are 30 % unhelthier, and 85 % of them present one or more associated-chronic-health condition which results in an estimated loss of 10 years life (Blue Cross Blue Shield Association, 2018). Therefore, anxiety is the main comorbidity associated with depressive disorders, where more than 60 % of people present overlapping symptoms (Lamers et al., 2011).

Animal models are crucial for understanding the neurobiological and/or molecular mechanisms underlying psychiatric disorders (Mello et al., 2003; Yan et al., 2010). Recently, our research group showed the validation of a novel model of enduring anxiety induced by pilocarpine (PILO, a non-selective muscarinic receptor agonist) in female mice (date not showed), extending previous data with male rats (Duarte et al., 2013, 2010; Hoeller et al., 2016, 2013). Taking into account the critical relationship between depression and anxiety disorders, we decided to investigate the PILO-induced effects on depression-related behaviors in female mice.

An important and persistent limitation of current animal models of psychiatric disorders, and the one we address in this study, is the gender bias. In general, female animals are neglected, which leads to partial results that cannot be easily projected to preclinical and clinical research (Beery and Zucker, 2011; Palanza, 2001). In fact, the few studies that include female animals report behavioral differences in depression tests, such as forced swim (Alonso et al., 1991; Brotto et al., 2000), and learned helplessness and sucrose preference (Reviewed by Kokras and Dalla, 2014). Due to these behavioral differences and the limitations of current animal models, it is crucial to validate novel

animal models for depression. Here, we tested the hypothesis that PILO induces a depressive-like behavior in female mice, which can be blocked by an antidepressant (fluoxetine), as measured by an increase in serum corticosterone levels without producing hippocampal neurodegeneration.

2. Material and Methods

2.1 Animals

Adults female *Swiss* mice (weighing 30 - 35 g) were obtained from the animal facility of the Federal University of Alagoas (BIOCEN/UFAL). Mice were housed in collective cages (up to 10 animals/cage; 41 x 34 x 16 cm), maintained at a 22 ± 2 °C, and 12h light-dark cycle (lights on at 7:00 a.m.). Food and water were available *ad libitum*, excepted during the experiments. All procedures were approved by the Ethics Committee on Animal Use of the Federal University of Alagoas (Protocols numbers #52/2018; #35/2020) in accordance with the Brazilian National Council for Animal Experimentation Control (*Conselho Nacional de Controle da Experimentação com Animais*, CONCEA). All experiments were performed between 12:00 a.m. and 6:00 p.m..

2.2 Drugs treatments and experimental design

The following drugs were used: pilocarpine hydrochloride (PILO; Ao Pharmacêutico®, Alagoas, Brazil), methyl scopolamine bromide (Sigma-Aldrich Co., St. Louis, USA), diazepam (DZP; Santista Lab. Farmacêutico S.A), fluoxetine (FLU; Ao Pharmacêutico®, Alagoas, Brazil) and saline solution (SAL; NaCl 0.9%, used as a control solution). All drugs were dissolved in SAL and prepared on the same day of administration, at a volume of 10 ml/Kg.

To prevent peripheral adverse effects of PILO, animals were treated with methyl scopolamine bromide [1 mg/Kg; subcutaneous injection (s.c.)] (Clifford et al., 1987).

After 30 minutes, a dose of PILO [75 mg/Kg; intraperitoneal injection (i.p.)] or SAL (i.p.) was administered. The behavioural analysis of seizures were performed for 90 minutes, in accordance with the Racine scale (Racine 1972). After 24 hours of treatment with SAL or PILO, animals receveid SAL (i.p.), DZP (1.5 mg/Kg; i.p.) or FLU (10 mg/Kg; i.p.), and 30 minutes later, were subjected to elevated open-field (OF) and forced swim (FST; Fig.1A) tests. This group, treated for 24 h, will be refered to as the *acute* treated group.

A second group of animals was treated for 30 days with the same drugs and doses, and subject to the same behavioural tests as the previous acute treated group (Fig.2A). From here on, this group is refered to as the *chronically* treated group. At the end of behavioural tests, animals were euthanized with a lethal dose of thiopental [Thiopentax®; 150 mg/Kg, (i.p.)]. Samples of whole-brain and blood were collected. All behavioral experiments were recorded by video camera (Intelbras®, Santa Catarina, Brazil; model VD 3108) positioned above the apparatus of tests.

2.3 Racines scale

Behavioural seizures were classified according to the Racine scale (Racine, 1972), with the following stages (S): S0, immobility; S1, facial automation; S2, head and neck myoclonus; S3, forelimb clonus; S4, raising of the forelimbs with clonic convulsions; S5, raising of the forelimbs with clonic convulsions and loss of posture.

2.4 Estrous cycle identification

The estrous cycle stage was verified by vaginal cytology (Byers et al. 2012; McLean et al., 2012). Briefly, 50 µL of SAL was introduced to the vaginal canal with a 200 µL pipette tip. Inmidetly, the fluid was aspirated using the same tip, transferred to a slide to air-dry for 10 minutes, fixed with methanol and stained with Panoptic Kit

(Instant Prov Panotico, NewProv). The estrous cycle stage is determined by the presence or absence of leukocytes, cornified epithelial cells, and nucleated epithelial cells, which were visualized by microscopy (Bioval, L-1000B). The vaginal fluid was collected 24 h or 30 d after the PILO-injection, after behavioral tests. The female mice were in the diestrus cycle phase during the behavioural tests (data not shown).

2.5 Behavioural tests

2.5.1 Forced swim test

To test depression-related behavior in mice we subjected the animals to FST (Yankelevitch-Yahav et al., 2015). The test is performed in a cylindrical tank made of Plexiglass (30 cm height x 20 cm diameters) filled with 15 cm of water at 26 °C. Prior to the experiments, mice were trained by being gently placed in the water for 6 minutes. This was repeated 24 h later and following parameters where recorded: (1) latency of immobility and (2) immobility, understood as the absence of body movement and passivly hanging. When the sessions were concluded, the mice were immediately removed from the tank, dried with a paper towel, heated with the aid of a lamp (40W), and returned to its homecage. Before subjecting another mice to the test, water was renewed and its temperature stabilized.

2.5.2 Open-field

The OF (Insight®, São Paulo, Brazil) is made of Plexiglas, and consists of a circular acrylic arena (30 cm high, 30 cm diameter), with its floor divided into eight squares. Mice were individually placed in the center of the arena for 5 minutes to record the following parameters: the (1) number of lines crossed (CRO) with the four paws, and (2) rearing (REA) understood as behavior of stand on the hind legs (Prut and

Belzung, 2003). Following each experiment, the OF apparatus was carefully cleaned with a 10 % ethanol solution.

2.6. Corticosterone Assay

Blood samples were stored at room temperature for a period of 60 minutes to allow them to clot appropriately before being centrifuged at 2000 RCF for 15 min at 24 °C (Centrifuge Heal Force®). The serums were collected and stored in microtubes at -80 °C until analyzed. Levels of corticosterone in serum was determined in duplicate, using a commercially available kit (Corticosterone Kit, Cayman, no.501320).

2.7. Neurodegeneration pattern analysis

2.7.1. Brain perfusion and slide mounting

Mice treated with PILO or SAL received a lethal dose of Tiopental (150 mg/Kg i.p.) and later, subjected to transcardical perfusion of SAL (50 ml) followed by paraformaldehyde (PFA 4%; 100 ml, PH 7.5). The whole-brain was immediately dissected and stored in a PFA 4 % at – 20 °C. Prior to mount brain slides, the brains were transferred to 20 % sucrose solution, freezed and sectioned (30 μm thickness) using a cryostat (Leica CM 1860). The resulting coronal sections (Paxinos and Franklin, 2001) were fixed and mounted on gelatinized slides. Each mice genereated 8 to 10 samples.

2.7.2. Fluoro-Jade C (FJ-C) staining

Mice brain slides were washed in ethanol 100 % (3 min), ethanol 70 % (1 min), and destile water (1 min), in order to submerge them in potassium permanganate with gentle agitation (20 RPM; 15 min; SL-184/4DT). Slides were then washed in destile

water (1 min) three times and stained with FJ-C solution in gentle agitation (20 RPM; 30 min: SL-184/4DT). FJ-C excess was washed in destile water (1 min) three times.

2.7.3. Image processing

Stained brain sections were visualized with a fluorescence microscope (EVOS M5000 Imaging System) using a GFP filter (Invitrogen Alexa Fluor, 546; SYBR Green) at 10x and 20x magnification. Hippocampus (dorsal, medial and ventral) images were captured and analyzed according to the fluoroscein signal (FJ-C+) or absence (FJ-C-). Images were processed in Adobe Photoshop Software (2015).

2.8. Statistical analysis

All results are expressed as mean ± standard mean error (S.E.M). We used the Two-way ANOVA test with the pretreatment (PT; PILO or SAL) and treatment (T; SAL, DZP or FLU) and interaction of this (PT x T) as factors. The Student Newman–Keuls *post-hoc* test was used to analyze the differences between the experimental groups. The Unpaired t-test was used to analyze the serum levels of corticosterone. We assume a value of $P \leq 0.05$ to determine the statistical differences. These data were plotted using GraphPad Prism (Version 8.0).

3. Results

3.1. PILO-injection induced low stages of Racine scale

To assess the possible development of behavioural seizures post PILO-injection, mice were monitored for 90 minutes. The *acute* group presented the following Racine stages, at S0 (n = 2; 7.69 %) and S2 (n = 24; 92.31 %). While the *chronic* group presented the following Racine stages, at S0 (n = 4; 12.5 %), S1 (n = 3; 9.37 %), and S2 (n = 25; 78.12 %;). The observed behavioural seizures were characterized as isolated, and upon their reintroduction to housing cages, PILO-treated mice were indistinguishable from the control mice.

3.2. Neither DZP nor FLU blocks short-term depressive-like PILO-induced effects

To investigate whether *acute*-treatment with DZP or FLU blocks PILO-induced short-term depressive-like behaviour, mice were subject to FST (Fig.1A). Two-way ANOVA shows that the interaction of factors [PTxF: $F(2, 48) = 9.183$; $P=0.0004$] and treatments [T: $F(2, 48) = 3.67$; $P=0.0329$], but not the pretreatment [PT: $F(1, 48) = 3.28$; $P=0.0763$] influenced the latency to immobility. The Newman-Keuls *post-hoc* analysis demonstrated a significant decrease in the latency to immobility in SAL+DZP ($P<0.05$) and SAL+FLU ($P<0.05$) groups compared to SAL+SAL (Fig.1B), such as an increased of this parameter in PILO+FLU compared to PILO+SAL ($P<0.05$). During the last 4 minutes of the test, the two-way ANOVA revealed that the interaction of the factors [PTxT: $F(2, 48) = 8.87$; $P=0.0005$], and treatment [T: $F(2, 48) = 13.92$; $P<0.0001$], but not pretreatment [PTxT: $F(1, 48) = 0.54$; $P=0.4654$] affects immobility. The *post-hoc analysis* shows an increase of immobility time during the last 4 minutes when animals were treated with PILO+SAL ($P<0.01$), PILO+DZP ($P<0.05$) or PILO+FLU ($P<0.01$), compared to SAL+SAL (Fig.1C). Similarly, mice treated with SAL+DZP ($P<0.001$) or SAL+FLU ($P<0.001$) showed an increase of immobility

compared to SAL+SAL (*P<0.001*, Fig.1C). The SAL+DZP group also increased of immobility compared to PILO+SAL (*P<0.05*, Fig.1C). Finally, the two-way ANOVA analysis indicated that the pretreatment [PT: F(1,48) = 4.08; *P<0.0491*], treatment [T: F(2,48) = 14.69; *P<0.0001*] and the interaction of these factors [PT x T: F(2,48) = 10.04; *P<0.0002*] affected the total time of immobility (Fig.1D). The *post-hoc* analysis demonstrated an increase of the total time of immobility, after PILO+SAL (*P<0.05*) or PILO+DZP (*P<0.01*), when compared to SAL+SAL (Fig.1D). Similar results were obtained when mice were treatment with SAL+DZP (*P<0.001*) or SAL+FLU (*P<0.001*)compared to SAL+SAL or PILO+SAL [SAL+DZP; (*P<0.01)* and SAL+FLU; (*P<0.05*)] (Fig. 1D).

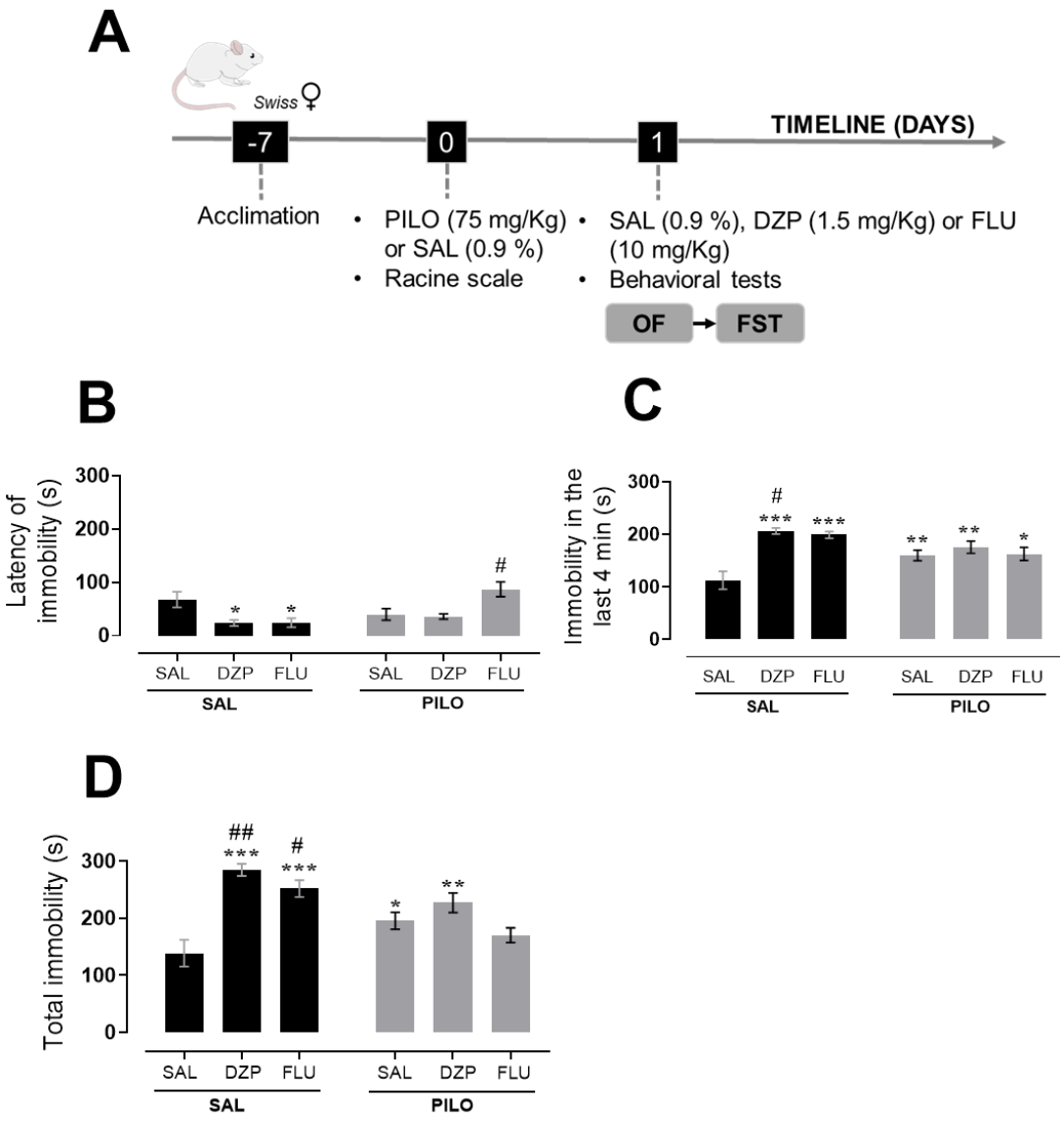

Figure 1. Acute treatment with saline (SAL; NaCl 0.9 %), diazepam (DZP; 1.5 mg/Kg) or fluoxetine (FLU; 10 mg/Kg) 24h after injection of pilocarpine (PILO; 75 mg/Kg). (A) Experimental design, (B) Latency of immobility; (C) Immobility during the last 4 minutes and (D) total time of immobility in forced swim test (FST). Each value represents the mean ± S.E.M of 9-10 mices in each group. **P<0.05*, ***P<0.01* or ****P<0.001* when compared to SAL+SAL; $^{\#}$*P<0.05* or $^{\#\#}$*P<0.01* when compared to PILO+SAL. Two-way ANOVA followed by Student Newman–Keuls *post hoc* test.

For the OF, the two-way ANOVA analysis detected the influence of pre-treatment [PT: F(1, 38) = 26.26; *P<0.0001*], but not the treatment [T: F(2, 38)= 0.80; *P=0.4544*] or the interaction of these factors [PTxT: F(2, 38) = 1.87; *P=0.1685*] in the number of crossings (Table 1). The *post-hoc* analysis detected an increase in the number of crossings in animals pretreated with SAL and 24h after with DZP (*P<0.05*) or FLU

(*P<0.05*) compared to PILO+SAL group. When analyzing rearing, two-way ANOVA showed the influence of pre-treatment [PT: F(1, 38) = 10.62 *P=0.0024*], without influence of the treatment [T: F(2, 38) = 1.953; P=0.1558] or the interaction of these factors [PTxT: F(2, 38) = 2.780; *P=0.0747*]. Furthermore, the *post-hoc* analysis shows an increased in the number of rearing by SAL+DZP (*P<0.05*) compared to SAL+SAL group (Table 1).

Open-field				
	24h		30 days	
Treatment	CRO	REA	CRO	REA
SAL+SAL	103.00±11.99	30.67±2.59	86.67±8.07	22.90±3.23
PILO+SAL	75.25±9.23	27.50±2.38	102.67±9.86	28.75±2.87
SAL+DZP	136.78±12.71$^{\#}$	33.44±4.85*	98.00±9.06	13.50±3.53
PILO+DZP	63.43±14.01	14.00±2.59	97.86±18.91	30.57±5.10
SAL+FLU	128.62±11.47$^{\#}$	25.37±3.99	167.33±19.75$^{**\#\#}$	16.50±4.67
PILO+FLU	79.50±9.40	18.50±3.33	86.45±14.56	6.09±4.07$^{*\#\#}$

Table 1. Effects on the behaviour during the open-field after PILO acute- or chronic-treatment with saline (SAL; NaCl 0.9 %), diazepam (DZP; 1.5 mg/Kg) or fluoxetine (FLU; 10 mg/Kg). Each value represents the mean ± S.E.M of 6-11 mices in each group. **P<0.05* or ***P<0.01* when compared with SAL+SAL and *#P<0.05* or *##P<0.01* compared with PILO+SAL. Two-away ANOVA followed by Student Newman–Keuls test. Behavioral parameters; CRO (crossings) and REA (rearings).

3.3. FLU, but not DZP, blocks long-term PILO-induced depressive-like effects

To investigate whether *chronic*-treatment with DZP or FLU blocks PILO-induced depressive-like behavior, we performed FST (Fig.2A). The two-way ANOVA shows that the latency of immobility was affected by pretreatment [PTxT: F(1, 40) = 6.535; *P=0.0145*] and treatment [T: F(2, 40) = 14.54; P<0.0001)], but not by the interaction of these factors [PT x T: F(2,41)=1.769; *P=0.183*)]. The *post-hoc* analysis shows that the SAL+FLU group increase the latency of immobility compare to SAL+SAL (*P<0.001*) or PILO+SAL group (*P<0.001*, Fig.2B). In addition, two-way

ANOVA shows that pretreatment and treatments but not interaction these factors affected the imobility during the last 4 minutes [PT: F(1, 42) = 16.62; *P=0.0002*, T: F(2, 42) = 45.61; *P<0.0001* and PTxT: F(2, 42) = 0.84; *P=0.4403*] and total immobility [PT: F(1, 42) = 12.38; *P=0.0011*, T: F(2, 42) = 30.10; *P<0.0001* and PTxT: F(2, 42) = 0.2207; *P=0.8029*]. *Post-hoc* analysis shows that the PILO+SAL group increases immobility during the last 4 minutes and total immobility when comparing with SAL+SAL group (P<0.05, Fig.2C and Fig.2D). This behavior was blocked when PILO-pretreated mice were injected with FLU (*P<0.001*; Fig.2C, *P<0.01*; Fig 2D). In contrast to this result, treatment with DZP was ineffective in blocking PILO-induced behavioural effects, and potentiated an increase of immobility time during the last 4 minutes and total immobility when comparing with PILO+SAL (*P<0.05*) or SAL+SAL (*P<0.001*;) (Fig.2C, Fig.2D). The group treated with SAL+DZP increases of immobility time during the last 4 minutes and total immobility when comparing with SAL+SAL (*P<0.01;* Fig.2C, *P<0.01*; Fig.2D). In addition, the *post-hoc* analysis showed that SAL+FLU group decrease the immobility time during the last 4 min and total immobility when compared to SAL+SAL (*P<0.001*; Fig. 2C, *P<0.05*; Fig.2D) and PILO+SAL (*P<0.001*; Fig. 2C and Fig.2D).

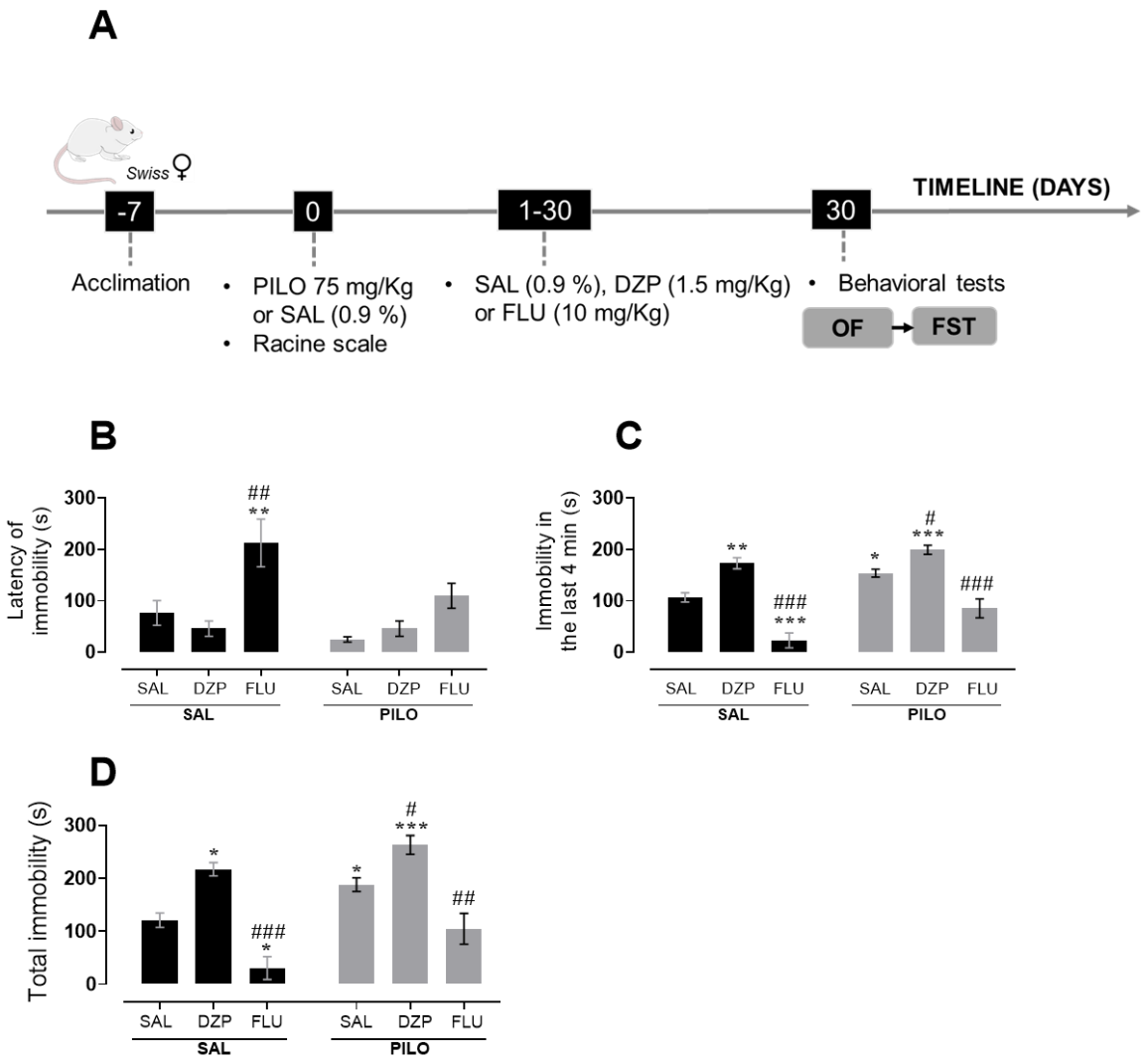

Figure 2. Chronic treatment with saline (SAL; NaCl 0.9 %), diazepam (DZP; 1.5 mg/Kg) or fluoxetine (FLU; 10 mg/Kg) starting 24 h after injection of pilocarpine (PILO; 75 mg/Kg). (A) Experimental design; (B) Latency of immobility; (C) Immobility during the last 4 minutes and (D) total time of immobility in forced swim test (FST) . Each value represents the mean ± S.E.M of 6-11 mices in each group. **P<0.05*, ***P<0.01* or ****P<0.001* when compared with SAL+SAL; $^{\#}$*P<0.05* or $^{\#\#}$*P<0.01* when compared with PILO+SAL. Two-way ANOVA followed by Student Newman–Keuls *post hoc* test.

For the open-field results, the two-way ANOVA shows that interaction of the factors [PTxT: F (2, 45) = 7.167; *P=0.0020*] and treatment [T: F(2, 45) = 3,296; *P=0.0462*], but not pre-treatment [PT:F (1, 45) = 3.57; *P=0.0654*] affect the number of crossings. *Post-hoc* analysis shows that SAL+FLU-administered mice increased their crossings compared to SAL+SAL (*P<0.01*) and PILO+SAL (*P<0.001*) (Table 1). Furthermore, two-way ANOVA analysis shows that the interaction of factors [PTxT:F

(2, 46) = 4.957; P=0.0112] and treatment [T:F (2, 46) = 7.124; P=0.0020], but not pre-treatment [F (1, 46) = 1.823; P=0.1835] affected rearing. The *post-hoc* analysis shows that only chronic treatment with FLU after PILO-injection decreased rearing compared to SAL+SAL (P<0.05) or PILO+SAL (*P<0.01*) (Table 1).

3.4. Short-term PILO-treatment, but not long-term, increase corticosterone levels in serum

Corticosterone levels were measured from blood samples collected 30 minutes after the FST (Fig.3A). Unpaired t-test analysis reveals that serum corticosterone levels were increased after acute-treatment with PILO (T= 2.54; *P < 0.05*, Fig.3B) compared to control group. In contrast, chronic-treatment with PILO-treatment did not change corticosterone levels, eventhough an increasing tendency can be appreciated (T= 2.033; *P = 0.076*, Fig.3C).

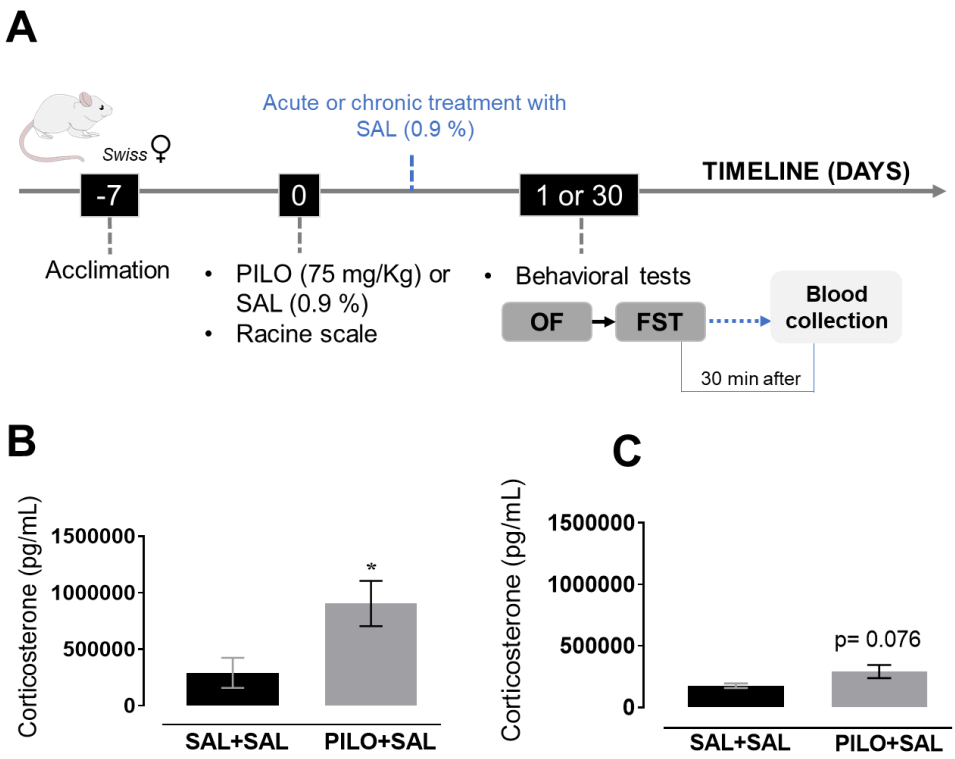

Figure 3. Acute and chronic effects of PILO-injection (75 mg/Kg) on serum corticosterone levels. (A) Experimental design of blood samples collected 24h or 30 d after PILO-injection. (B) Short- and (C)

long-term effects of PILO-injection. Each value represents the mean ± S.E.M of 4-5 mice in each group. *$P<0.05$ when compared with SAL+SAL. Unpaired t-test. FST; forced swim test, OF; open-field, SAL; saline.

3.5. Short- and long-term PILO treatment did not induce a short- and long-term FJ-C+ hippocampal

Both acute and chronic PILO treatment did not present FJ-C+ hippocampal (Fig.4). As a control positive, high-dose of PILO induces neurodegenetion in the dentate gyrus (Fig.4A and 4A').

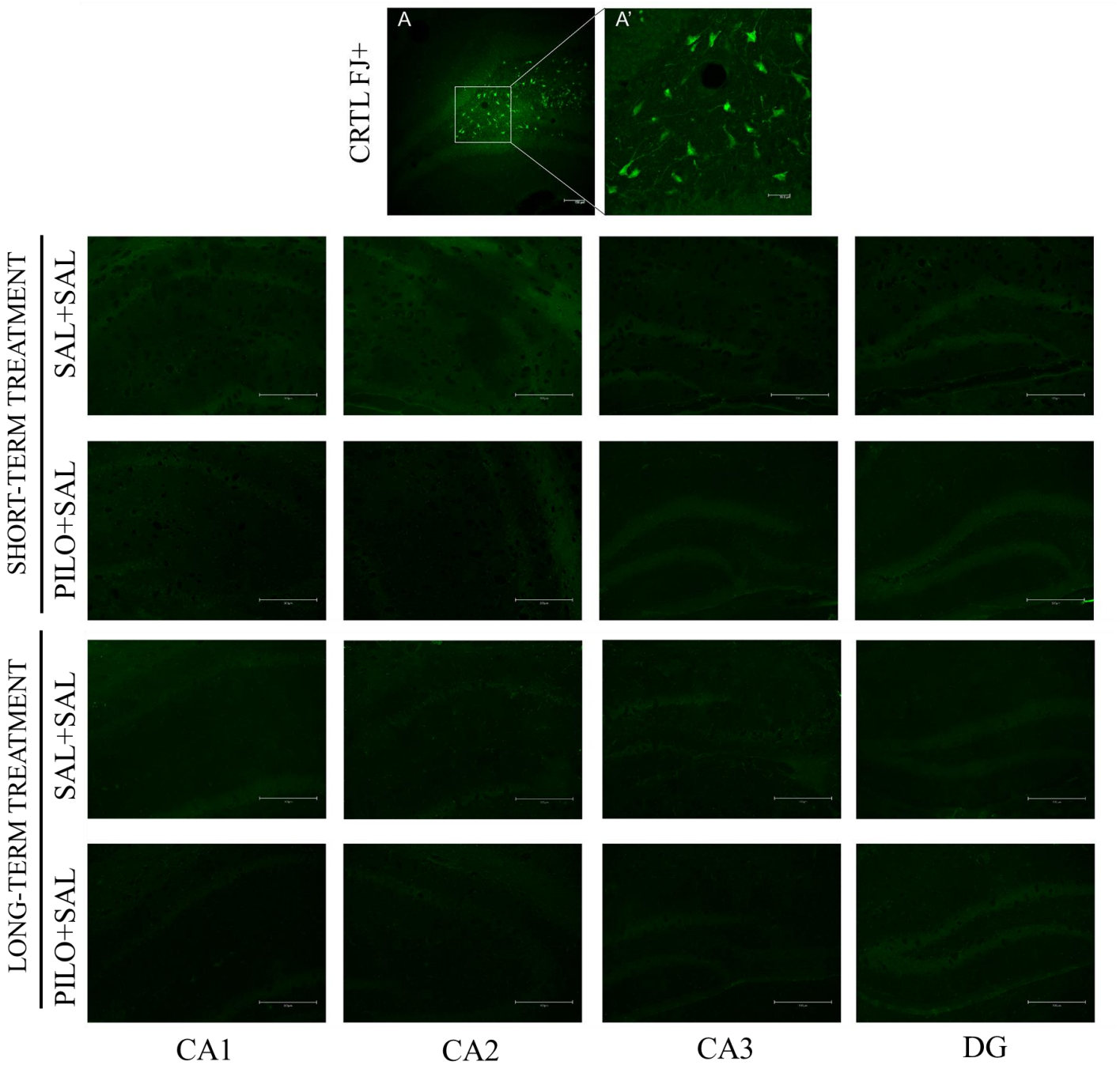

Figure 4. Fluoro-Jade staining of brain sections after acute and chronic treatment with PILO-injection (75 mg/Kg). (A) Positive control (CRTL FJ+), high-dose of PILO induces neurodegenetion in the dentate gyrus (DG). Note the absence of FJ-C+ staining in PILO+SAL groups compared to SAL+SAL. Each group has 8 mices.

4 Discussion

In the present study, we showed that PILO induced short- and long-term depressive-like behavior in female *Swiss* mice as evaluatted in the FST, with no hippocampal neurodegeneration. PILO-injection also increased short-term level of corticosterone in serum. Moreover, the chronic, but not acute, treatment with FLU blocked the PILO-induced depressive-like phenotype.

PILO is an alkaloid extracted from *Pilocarpus microphyllus* leaves and a non-selective muscarinic receptor agonist (Pinheiro, 1997). Several studies have implicated the cholinergic system in depressive disorder. In fact, an increase of chollinergic transmission after administration of acetylcholinesterase (AChE) inhibitors (Rowntree et al., 1950) or activation of cholinergic receptors (Gershon and Shaw, 1961) induces symptomatological features of depression in humans. Depressive-like behaviour has been reported after high-dose of PILO-injection, followed by status epilepticus, in animals (Mazarati et al., 2008; Zanirati et al., 2018). On the other hands, Ramos Costa et al. (2019) demonstrated that the administration of a non-convulsant dose of PILO reduced immobility time in FST in rats, indicating antidepressant-like behavior. In contrast to these results, our data showed that PILO-injection induced a depressive-like phenotype in the same behavioral test but in female mice, suggesting that this species and sex are more responsive to the depressive-like profile induced by cholinergic activation via single dose of PILO.

Depressive disorders are heterogeneous conditions making them a challenge for reproduction in preclinical trials (Yan et al., 2010). In this sense, some criteria are needed to validate a new animal model of psychiatric disorders including predictive or pharmacological validity (Belzung and Lemoine, 2011). To analyze the pharmacological validity of PILO-injection as a novel model of depression, we evaluated the effects of acute and chronic treatment with FLU, a commonly used

antidepressant-drugs (see more in Pinna, 2015) and that in animal models its chronic use has demonstrated an antidepressant-like effect in several behavioral tests such as splash test (Machado et al., 2012), FST (Hasey and Hanin, 1991; Tyagi and Walia, 2017), sucrose preference (Grippo et al., 2006) and tail suspesion test (Mineur et al., 2013). Here, we observe that only chronic treatment with FLU blocked the PILO-induced depressive-like phenotype, corroborating with the work of Mineur et al. (2013), who showed that depressive-like behavior induced by physostigmine-administration, a AChE inhibitor, was blocked after chronic FLU-injection in mice. Furthermore, FLU increases hippocampal AChE activity (Mineur et al., 2013) and reverses depressive-like behavior induced by hippocampal AChE knockdown (Mineur et al., 2013). Therefore, our results suggest that the novel model of depression induced by PILO in mice is responsive to a antidepressant-drug commonly used in clinical medicine.

As a negative control in the FST, we chose DZP – a benzodiazepine extensively used in the treatment of anxiety disorders and devoid of antidepressant activity that promotes the facilitation of GABAergic neuronal transmission through positive allosteric modulation of the $GABA_A$ receptor (Altamura et al., 2013; Forman et al., 2009.). In an expected way, DZP did not block the short- and long-term depressive-effects induced by PILO. On the contrary, DZP potentiated the PILO-induced depressive-like phenotype. In agreement with our results, a similar effect of DZP treatment has already been found in the FST (Martl and Armario, 1993; Nishimura et al., 1989; Tyagi and Walia, 2017). And this can be explained by a sedative or ataxia effects (Nishimura et al., 1989), as well as a reduction of serotonergic transmission (Ahmed and Elhwuegi, 2014).

The hypothalamic-pituitary-adrenal (HPA) axis dysregulation is the most common neuroendocrine alteration found in depressive disorders (reviewed by Leistner

and Menke, 2020; Troubat et al., 2021). Abnormalities in the HPA axis result in increased glucocorticoid levels and of inflammatory mediators (reviewed by Pariante and Lightman, 2008), as well as the presence of neuronal damage in the hippocamppus (Swaab et al., 2005). The regulation of the HPA axis can be performed by hippocampal cholinergic activation from the forebrain (Paul et al., 2015). Inhibition of AChE with the consequent upregulation of acetylcholine induces an increase of the adrenocorticotropic hormone (ACTH) and cortisol levels (Janowsky et al., 1986). Futhermore, acetylcholine levels are elevated in depressive pacientes (Saricicek et al., 2012), and AChE inhibitors induce symptoms of depression (Janowsky et al., 1974), while muscarinic antagonists releave them (Furey and Drevets, 2006), suggesting an important cholinergic involvement in the neuropsychopathology of depressive disorders. Hoeller et al. (2016) showed an increase in plasma corticosterone levels 24 h and 30 d after administration of a non-convulsive dose of PILO in rats. In this work, PILO-injection resulted in a similar response profile in Swiss female mice, indicating HPA axis dysregulation as one of the possible mechanisms for the depressive phenotype observed after the injection of this drug.

In addition, during estress are observed elevated levels of glucocorticoids and its association with hippocampal damage, such as inhibition of neurogenesis, neurotoxicity and loss of neurons (as reviewed by Mello et al., 2003). According to Bremner et al. (2000), patients with major depression have a smaller left hippocampal volume (up to 19%) compared to non-depressed subjects. Our results did not show necrotic hippocampal neurodegeneration after PILO-injection, suggesting another possible mechanism as a target for the dysregulation of the HPA axis and PILO-induced enduring depressive-like behaviors. On the other hands, the dose of PILO administered to animals in this work did not promote neurobiological alterations characteristic of

status epilepticus, a condition that induces hippocampal neuronal death (Rashid et al., 2021; Tan et al., 2018). Therefore, we assume that the behavioral seizures scores less than 3 on Racine' scale observed in the mice were caused after injecting a subconvulsive PILO dose.

The prevalence of neuropsychiatric disorders differs between the sexes with women experiencing about twice as many depression-related symptoms as men (American Psychiatric Association, 2014; World Health Organization, 2017). Despite that and animal models of depression, in studies of neuropsypathologies, femeals are generally neglected in experimental designs (Griebel and Holmes, 2013; Kokras and Dalla, 2014). One reason is hormonal fluctuation during the estrous cycle, which can impact behavioral outcomes (Beery and Zucker, 2011; Kokras and Dalla, 2014; Palanza, 2001; Planchez et al., 2019). Unfortunately, many of this studies carried out with females did not identify the estrous cycle, which may difficult their reproduction by other research laboratories (Bolea-Alamanac et al., 2018). Here, female mice were in diestrus cycle as submitted to the behavioral tests. In this phase of estrous cycle, animals show a reduction in progesterone and its neurosteroid metabolite allopregnanolone levels resulting in an increase of neuronal excitability especially into the hippocampus (Wu et al., 2013). However, this hormonal difference in estrous cycle does not seem to interfere with the immobility time in rats (Alonso et al., 1991) and mice (Yohn et al., 2018) submitted to the FST. Further studies are necessery to investigate the effect of the PILO-induced depressive-like behavior on other stages of the animals' estrous cycle and assess a phase-dependent susceptibility for its behavior.

In summary, our results demonstrate for the first time that single dose of PILO induces a depressive-like behavior in female mice. This PILO-induced depressive-like phenotype to be sensitive to manipulation with antidepressant drugs.

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4 CONCLUSÕES

Nossos resultados mostraram que efeito do tipo ansiogênico e depressivo induzido por uma única injeção de PILO (75 mg/Kg), a curto e longo prazo em camundongos *Swiss* fêmeas, foi bloqueado por fármacos ansiolítico e antidepressivo. Este achado foi visto nos animais desprovidos de alteração locomotora espontânea e marcação positiva para neurodegeneração hipocampal. Com isso, pela primeira vez, nossos dados demonstraram a validade farmacológica de um novo modelo animal (a partir de uma dose única de PILO) no estudo da ansiedade duradoura e depressão em roedores fêmeas.

Nossos resultados também demonstraram que a administração de PILO produziu um aumento dos níveis sorológicos de corticosterona a curto prazo. Esses dados sugerem uma possível ativação do eixo HPA na circuitaria neural envolvida nos transtornos mentais e que deve, posteriormente, ser melhor estudada.

Novos estudos devem ser realizados afim de se conhecer sobre as vias colinérgicas ativadas, bem como, outras alterações neuronais mediadas pelo tratamento único de PILO. Como os transtornos de ansiedade e depressão são mais prevalentes em mulheres, a validação de um novo modelo animal em roedores fêmeas pode responder lacunas substanciais na identificação da predisposição do sexo ao desenvolvimento destes transtornos. As contribuições podem se estender ao futuro, com o descobrimento de novas alternativas farmacológicas e com isso, a melhoria da condição de vida dos acometidos.

ANEXOS

ANEXO 1. Aprovações da Comissão de Ética no Uso de Animais (CEUA/UFAL).

UNIVERSIDADE FEDERAL DE ALAGOAS
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que a proposta intitulada *"Validação farmacológica do modelo animal de ansiedade e depressão induzido por dose intermediária de pilocarpina em camundongos Swiss"*, registrada com o **nº 52/2018**, sob a responsabilidade do pesquisador **Prof. Dr. Marcelo Duzzioni**, que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), **para fins de pesquisa científica**, 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 da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais da Universidade Federal de Alagoas (CEUA/UFAL), em reunião de 27 de julho de 2018.

Vigência da autorização	01.08.2018 a 01.08.2021
Espécie/linhagem/raça	Camundongo heterogênico / Swiss
Nº de animais	280
Peso/idade	35 g / 3 meses
Sexo	Fêmeas
Origem/Local de manutenção	Biotério Central da Ufal / Biotério do LNFI-ICBS-UFAL
Colaboradores	Fernanda M. A. de Souza, Dannyele C. S. Nicácio, José G. S. Neto, Gabriela T. S. Cavalcante, Mirella P. S. Vieira, Ozileudiane B. S. Silva e Gabriela F. De Souza

Maceió, 31 de julho de 2018.

Elvan Nascimento dos Santos Filho
Coordenador da CEUA
SIAPE 1756479

UNIVERSIDADE FEDERAL DE ALAGOAS
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CEUA

CERTIFICADO

Certificamos que a proposta intitulada "Análise dos efeitos a curto e em longo prazo da administração única de uma dose subconvulsivante de pilocarpina na ativação neuronal e neurogênese hipocampal de camundongo Swiss", registrada com o **nº 35/2020**, sob a responsabilidade do pesquisador **Prof. Dr. Marcelo Duzzioni**, que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), **para fins de pesquisa científica,** 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 da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais da Universidade Federal de Alagoas (CEUA/UFAL), em reunião de 11 de março de 2021.

Vigência da autorização	25.03.2021 a 25.03.2023
Espécie/linhagem/raça	Mus musculus - camundongo heterogênico - linhagem Swiss.
N° de animais	96
Peso/idade	35 g / 60 dias
Sexo	Fêmeas
Origem/Local de manutenção	Biocen - Ufal /Biotério Setorial do Laboratório de Neurofarmacologia e Fisiologia Integrativa (LNFI) – ICBS/UFAL
Colaboradores	José Gomes dos Santos Neto; Fernanda Maria Araújo de Souza; Walleska Bismaida Zacarias Galvão Barros Correia; Gabriela Ferreira de Souza; Mirella Priscilla dos Santos Vieira; Rayssa Gabriely Duarte Torres;

Maceió, 24 de março de 2021.

Elvan Nascimento dos Santos Filho
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ANEXO 2. Manuscrito “Behavioral validation of the novel model of enduring anxiety induced by pilocarpine in mice” submetido na Psychopharmacology, 2022.

BEHAVIORAL VALIDATION OF THE NOVEL MODEL OF ENDURING ANXIETY INDUCED BY PILOCARPINE IN MICE

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Abstract

Rationale: A couple of recent studies have suggested a novel model of enduring anxiety following the administration of a single subconvulsive dose of pilocarpine in rodents. Despite the consistent results, this animal model of anxiety needs to be better validated, as well as the use of different animal species and sexes, and behavioral tests. *Objectives:* To evaluate the short- and long-lasting behavioral effects of a single dose of pilocarpine administration in female and male Swiss mice subjected to a behavioral test battery. *Methods:* The animals were treated with pilocarpine (75 or 150 mg/Kg, i.p.) and, after 24 hours or 30 days, they were exposed to anxiety (elevated plus-maze and light/dark box tests) and locomotion (open-field and rota-rod tests) tests. *Results:* The administration of pilocarpine (75 mg/Kg) induced anxiogenic-like effects in male and female mice in the elevated plus-maze test 24 hours after treatment. This same anxiety-like phenotype was observed in the light/dark box test 30 days after treatment, but only in female mice. Treatment with pilocarpine (150 mg/kg) did not change the anxiety-related behavioral parameters evaluated in these apparatus. Moreover, no changes in locomotion were found after administration of both pilocarpine doses. *Conclusions:* Our findings corroborate and extend previous data in the literature that showed the administration of a single subconvulsive dose of pilocarpine as a model of enduring anxiety.

Keywords anxiety, pilocarpine, mice

1. Introduction

Anxiety can be considered an adaptive and evolutionary emotion provided that it protects the individual from potentially threatening situations and promotes survival. However, when anxiety is pathological and categorized as an anxiety disorder, it becomes detrimental to emotional, psychological, cognitive and behavioral states and requires immediate treatment (American Psychiatric Association 2014; Crocq 2015). Globally, more than 260 million people suffer from anxiety disorders and prevalence is higher in females than in males (World Health Organization 2017). In fact, anxiety disorders are among the most prevalent and disabling psychiatric disorders worldwide (Fineberg et al. 2013; Seth et al. 2016).

Unfortunately, many patients suffering from anxiety disorders are refractory to current pharmacological treatments. In other cases, the unwanted effects of these drugs cause abrupt treatment abandonment, further aggravating progression and prevalence of the pathology (Tiller 2012; Griebel and Holmes 2013a). To understand the neurobiological basis of anxiety and to screen for new compounds with anxiolytic activity, several animal models have been developed (Stenzel-Poore et al. 1994; Chandra et al. 2005; Delawary et al. 2010; Belzung and Lemoine 2011; Griebel and Holmes 2013b; Xiang et al. 2018). Despite the progress made in this area, the validity of these animal models of anxiety is limited, hindering the development of new anxiolytic compounds (Murrough et al. 2015; Stewart et al. 2015).

Recently, a couple of studies have reported short- and long-lasting anxiety-like phenotype in male rats after a single injection of a subconvulsive dose of pilocarpine, a non-selective agonist of muscarinic receptors (Duarte et al. 2010, 2013; Hoeller et al. 2016). In fact, intraperitoneal administration of pilocarpine (150 mg/Kg) induces an anxiogenic-like effect in the elevated plus-maze test in rats when evaluated 24 hours

and up to 3 months after treatment (Duarte et al. 2010, 2013; Hoeller et al. 2016). Moreover, pilocarpine increases hippocampal theta rhythm (Duarte et al. 2013), corticosterone release (Hoeller et al. 2016) and induces hippocampal neurogenesis through post-transcriptional mechanisms (Ramos Costa et al., 2019), responses related to stress, fear and anxiety. These results suggest the single administration of pilocarpine as a novel model of enduring anxiety.

However, validation of a new animal model to study psychopathology ideally includes criteria such as the use of different animal species of both sexes and behavioral tests (McKinney and Bunney 1979; Willner 1984; Belzung and Lemoine 2011). Thus, the present study aimed at evaluating the novel model of enduring anxiety induced by the single administration of subconvulsive pilocarpine doses in female and male mice subjected to a behavioral test battery.

2. Experimental procedures

2.1 Animals

Male and female Swiss mice (2-3 months old, weighing 30-35 g) were obtained from the animal facility of the Federal University of Alagoas. The animals were kept in a temperature- (22 ± 2°C) and light- (12h light-dark cycle; lights on at 7:00 a.m.) controlled room and housed in groups of 6-10 per cage. The experimental protocols were approved by the Ethics Committee on Animal Use of the Federal University of Alagoas (Protocol number #17/2014) in accordance with the Brazilian National Council for Animal Experimentation Control (*Conselho Nacional de Controle da Experimentação com Animais*, CONCEA). The animals were maintained with free access to food and water, except during the experiments. All experiments were performed between 12:00 p.m. and 6:00 p.m.

2.2 Drugs, treatment, and experimental schedule

Pilocarpine hydrochloride (Ao Pharmacêutico®, Alagoas, Brazil) and methyl scopolamine bromide (Sigma-Aldrich Co., St. Louis, USA) were dissolved in a saline solution (NaCl 0.9%, also used as a control solution) immediately before the experiments and injected at a constant volume of 10 ml/Kg. The mice were treated with methyl scopolamine bromide (1 m/Kg, s.c.) to prevent the peripheral effects of pilocarpine (Clifford et al. 1987). After 30 minutes, the animals received saline solution (i.p.) or pilocarpine (75 or 150 mg/Kg, i.p.), and behavioral analysis of seizures was performed for 90 minutes according to the Racine scale (Racine 1972). After 24 hours (24h) or 30 days (30d) of treatment with saline solution or pilocarpine, the animals were exposed to a behavioral test battery used in locomotion/anxiety assessment (Figures 1A-4A). The pilocarpine doses used in the current study were based on Duarte et al. (Duarte et al. 2010, 2013). All experiments were recorded with a video camera (Intelbras®, Santa Catarina, Brazil; model VD 3108) positioned above the device.

2.3 Estrous cycle identification

The estrous cycle stage was verified by vaginal cytology according to Byers et al. (2012) and McLean et al. (2012). Briefly, 24h or 30d after the behavioral tests, sterile saline at room temperature was pulled into an attached pipette tip (200 µL) and the end of the tip was placed in the opening of the vaginal canal. Approximately 50 µL of saline were gently pushed out into the opening of the vaginal canal, and then the fluid was spontaneously aspirated into the tip after slowly releasing the plunger. The cells were transferred to a dry glass slide and stained (Insta Prov Panotico, NewProv). The slide was air-dried for approximately 10 minutes for fixation and later analyzed using a binocular biological microscope in 10x magnification (Bioval, L-1000B). The estrous cycle stage determined the presence or absence of leukocytes, cornified epithelial cells,

and nucleated epithelial cells. The female mice were in the diestrus cycle phase during the behavioral tests (data not shown).

2.4 Racine scale

Behavioral analysis of seizures was performed according to the Racine scale (Racine 1972), with the following stages: 0 (S0), immobility; 1 (S1), facial automation; 2 (S2), head and neck myoclonus; 3 (S3), forelimb clonus; 4 (S4), raising of the forelimbs with clonic convulsions; 5 (S5), raising of the forelimbs with clonic convulsions and loss of posture.

According to Phelan et al. (2015), during the transition period of the pilocarpine model of epilepsy, multiple bouts of stage 4–5 convulsive behaviors suggest full progression to *status epilepticus*. Thus, only animals with Racine stages 1-3 were subjected to the behavioral test battery after single intraperitoneal administration of pilocarpine (low and intermediate doses; Tursk et al., 1984).

2.5 Behavioral tests

2.5.1 Elevated plus-maze

The elevated plus-maze device (Insight®, São Paulo, Brazil) was made of Plexiglas and consisted of two open (30 x 5 x 25 cm) and two closed (30 x 5 x 25 cm) arms opposite cross-shaped and raised 38.5 cm from the ground level. The open arms are surrounded by lateral bars (0.25 cm in height) to prevent the animals from falling. Each animal was individually placed into the central platform with its head directed towards one of the closed arms. For a 5-minute period, the frequency of entries into either open or enclosed arms, as well as the time spent in each arm type, were recorded (in seconds). The number of entries into the closed arms was used as an index of general activity (Rodgers et al. 1997). Ethological parameters such as protected Stretch-Attend Posture (pSAP), and unprotected Head-Dipping (uHD) were also recorded (Cole and

Rodgers 1994; Rodgers and Dalvi 1997). All tests were taped by using a video camera. After each test, the device was carefully cleaned with a 10% ethanol solution.

2.5.2 Light/dark box

The light/dark box device (Insight®, São Paulo, Brazil) consisted of a Plexiglas box divided into two different compartments: unprotected (light, 20 x 26.5 x 26 cm, with the floor divided into nine squares) and protected (dark, 20 x 26.5 x 17.5 cm). The two compartments were connected by a small door (5 x 5 cm). The light box was illuminated with a 40 W bulb at a height of 60 cm above the device. Each animal was placed in the unprotected compartment facing the door and allowed to freely explore the entire device for a 5-minute period. The following parameters were evaluated: time spent and the number of squares crossed with the four paws in the unprotected compartment and latency and the number of entries into the protected compartment (Crawley 1981; Costall et al. 1989). All tests were taped by using a video camera. After each test, the device was carefully cleaned with a 10% ethanol solution.

2.5.3 Open-field test

The open-field test (Insight®, São Paulo, Brazil) was made of Plexiglas and consisted of a circular acrylic arena (30 cm high, 30 cm diameter), with the floor divided into eight squares. The open-field test was performed immediately after the elevated plus-maze test. Each animal was placed in the center of the arena and the number of lines crossed with the four paws and the number of rearings was recorded for a 5-minute period (Prut and Belzung 2003). All tests were taped by using a video camera. After each test, the device was carefully cleaned with a 10% ethanol solution.

2.5.4 Rota-rod

The automated rota-rod device (Insight®, São Paulo, Brazil) consisted of a rotating rod (3.5 cm diameter, 30 cm length) covered with a wire mesh. Each animal

was preselected through a learning period assessed 24h before treatment with pilocarpine on its capacity to remain on the rotating rod (16 rpm) for 60 seconds. The animals that fell during this period were discarded (adapted from Dunham and Miya 1957). After 24h or 30d of pilocarpine administration, the animals were again subjected to the rota-rod test for 90 seconds and the number of falls was recorded. After each test, the device was carefully cleaned with a 10% ethanol solution.

2.6 Statistical analyses

All results are expressed as mean ± SEM. The data were analyzed by unpaired two-tailed Student's *t* test or one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test for multiple comparisons when appropriate. Differences were considered significant at $p \leq 0.05$. All statistical analyses were performed with GraphPad Prism software (Version 5.01).

3. Results

3.1 Single low and intermediate pilocarpine dose and Racine stage scores

All the male and female mice treated with pilocarpine at a dose of 75 mg/kg scored stage 1-3 in the Racine scale [male: S0 (31%); S1 (3%); S2 (38%); and S3 (28%); female: S0 (37%); S1 (25%); S2 (13%); and S3 (25%)]. On the other hand, some mice of both sexes that received pilocarpine at a dose of 150 mg/kg scored stage 4-5 in the Racine scale [male: S0 (0%); S1 (2%); S2 (39%); S3 (22%); S4 (6%); and S5 (9%); female: S0 (3%); S1 (9%); S2 (41%); S3 (22%); S4 (3%); and S5 (3%)] or died [19% female; 18% male].

3.2 Pilocarpine produces a short-term anxiogenic-like effect in the elevated plus-maze test in male and female mice

To assess pilocarpine's short- and long-term effects on anxiety- and locomotion-related behaviors, the animals were subjected to the elevated plus-maze and open-field

tests (Figures 1A and 2A). As shown in Figures 1B and 1E, administration of pilocarpine at a dose of 75 mg/Kg significantly reduced the time spent ($F_{2,20} = 5.48$; $p < 0.05$) and frequency of entries ($F_{2,19} = 4.08$; $p < 0.05$) into the open arms of the elevated plus-maze test in female and male mice, respectively, 24h after treatment, as compared to the saline group. This same pilocarpine dose increased the number of protected Stretch-Attend Postures ($F_{2,19} = 6.86$; $p < 0.01$; Figure 1G) in the maze when compared to the saline group, but only in male mice. On the other hand, in female mice, pilocarpine at a dose of 150 mg/Kg reduced the number of protected Stretch-Attend Postures ($F_{2,19} = 3.61$; $p < 0.05$; Figure 1F), as compared to the pilocarpine 75 mg/Kg group. However, one-way ANOVA did not detect significant differences in the other behavioral parameters evaluated in the maze in both female [frequency of entries into open arms ($F_{2,20} = 2.50$; $p > 0.05$; Figure 1D) and male [time spent in open arms ($F_{2,19} = 1.30$; $p > 0.05$; Figure 1C)] mice 24h after pilocarpine treatment.

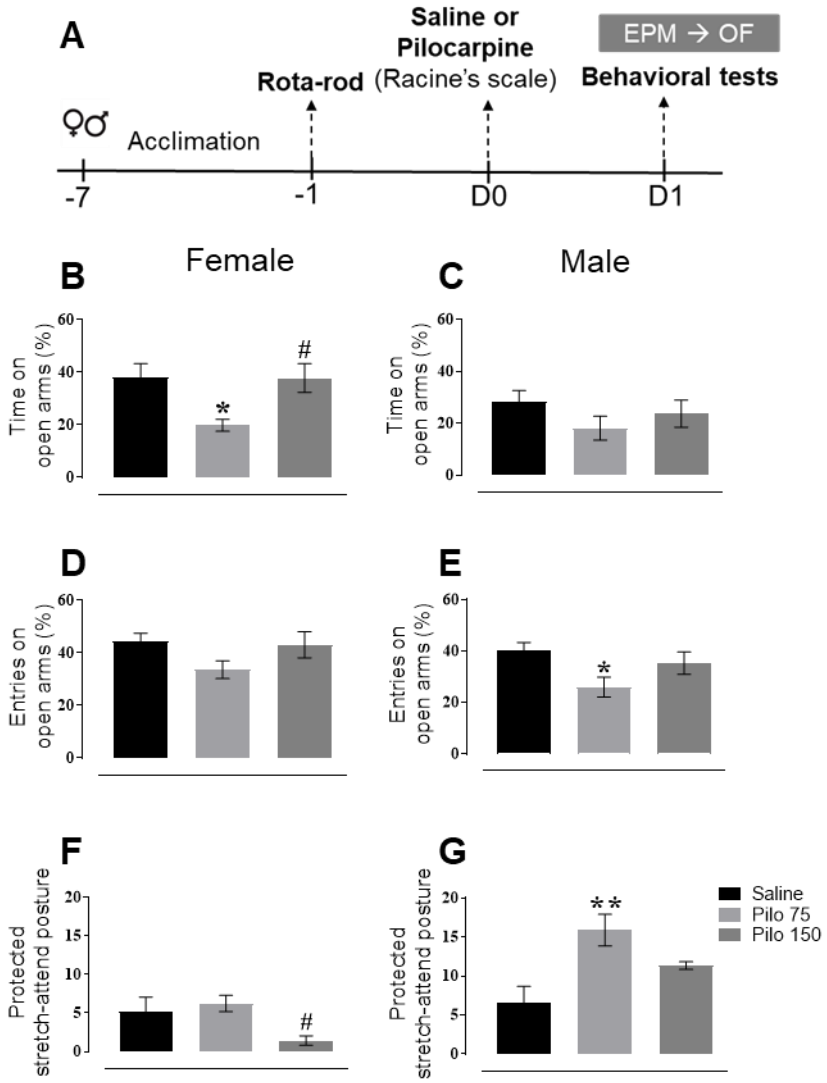

Figure 1. (A) Diagram of the experimental design. **(B - G)** Short-term effects of pilocarpine (75 or 150 mg/Kg, i.p.) on anxiety-related behavior in female and male mice subjected to the elevated plus-maze test. The animals were evaluated 24h after treatment. Results expressed as mean ± SEM of 6 to 8 animals per group. *$p < 0.05$ or **$p < 0.01$ as compared to the saline solution group. $^{\#}p < 0.05$ as compared to the Pilo 75 group. One-way ANOVA followed the Newman-Keuls post hoc test. EPM: Elevated Plus-maze test and OF: Open-field test.

Thirty days after pilocarpine (75 or 150 mg/kg) administration, the behavioral parameters evaluated in the elevated plus-maze test were not altered in mice of both sexes [time spent in open arms: female ($F_{2,21} = 0.59$; $p > 0.05$; Figure 2B) and male ($t = 0.47$; $p > 0.05$; Figure 2C); frequency of entries into open arms: female ($F_{2,21} = 1.21$; $p >$

0.05; Figure 2D) and male: (t = 0.50; p > 0.05; Figure 2E); and number of protected Stretch-Attend Postures: female ($F_{2,21}$ = 0.10; p > 0.05; Figure 2F) and male (t = 0.83; p > 0.05; Figure 2G)].

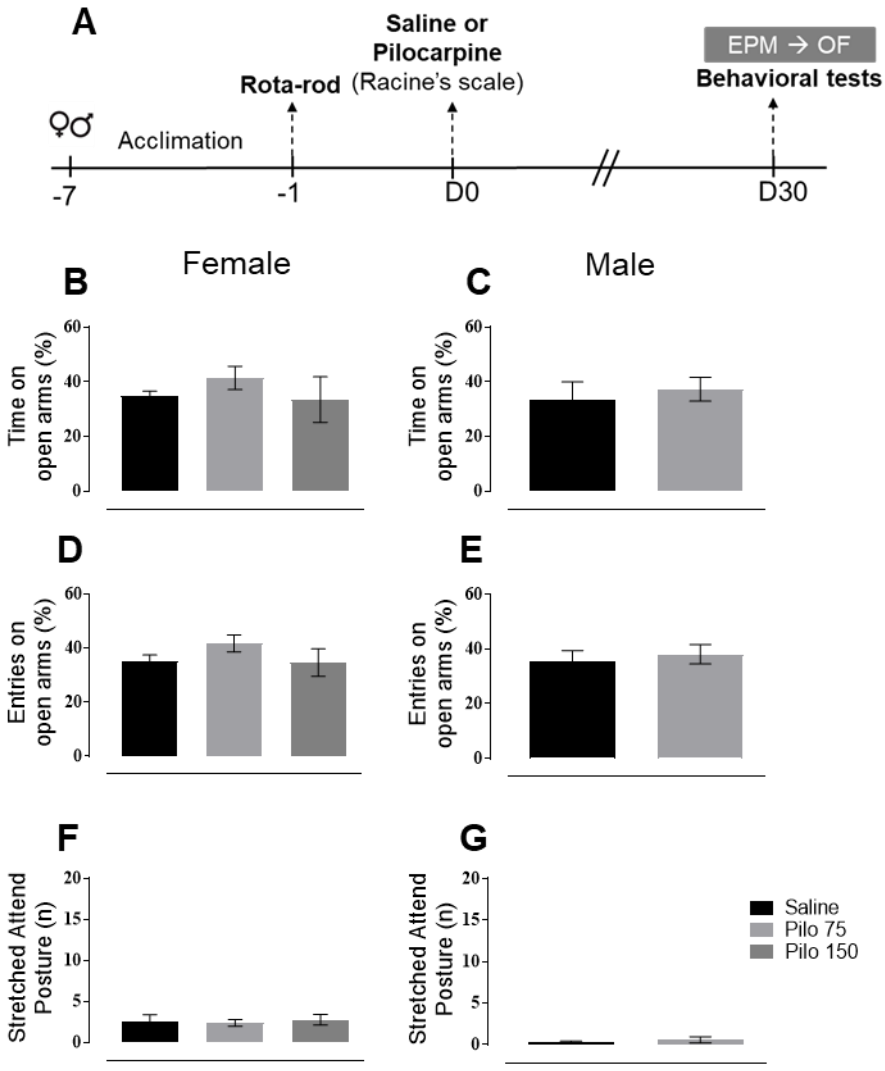

Figure 2. (A) Diagram of the experimental design. **(B - G)** Long-term effects of pilocarpine (75 or 150 mg/Kg, i.p.) on anxiety-related behavior in female and male mice subjected to the elevated plus-maze test. The animals were evaluated 30d after treatment. Results expressed as mean ± SEM of 7 to 8 animals per group. p = N.S., no significant difference. One-way ANOVA or Student's t test. EPM: Elevated Plus-maze test and OF: Open-field test.

As shown in Table 1, administration of pilocarpine (75 or 150 mg/kg) did not change the number of entries into the closed arms [female: ($F_{2,20}$ = 2.30; p > 0.05);

male: ($F_{2,19}$ = 0.42; p > 0.05)], rearings [female: ($F_{2,20}$ = 1.82; p > 0.05); male: ($F_{2,19}$ = 0.36; p > 0.05)], and unprotected Head-Dipping [female: ($F_{2,20}$ = 2.25; p > 0.05); male: ($F_{2,19}$ = 1.47; p > 0.05)], as evaluated in the elevated plus-maze test 24h after treatment. No effect on these same behavioral parameters evaluated in the maze [number of entries into the closed arms: female ($F_{2,21}$ = 1.62; p > 0.05) and male (t = 0.92; p > 0.05); rearings: female ($F_{2,21}$ = 2.87; p > 0.05) and male (t = 0.53; p > 0.05); and unprotected Head-Dipping: female ($F_{2,21}$ = 2.67; p > 0.05)] and male (t = 1.81; p > 0.05)] was found 30d after pilocarpine administration, according to Table 1. Unfortunately, some of the male mice treated with the highest pilocarpine dose developed seizures with Racine stages 4-5 (n=3), or died (n=3). Thus, the number of animals remaining in this group was very low (n=2), precluding any statistical analysis.

In relation to spontaneous locomotor activity evaluated in the open-field test, no significant difference was found after 24h treatment with pilocarpine (75 or 150 mg/Kg) in the number of crossings [female ($F_{2,20}$ = 0.60; p > 0.05) and male ($F_{2,19}$ = 0.08; p > 0.05)] and rearings [female ($F_{2,20}$ = 1.03; p > 0.05) and male ($F_{2,19}$ = 0.75; p > 0.05)], as illustrated in Table 1. Similar results were observed in the open-field test 30d after pilocarpine administration (75 or 150 mg/Kg) in the number of crossings [female ($F_{2,21}$ = 2.95; p > 0.05) and male (t = 0.28; p > 0.05)] and rearings [female ($F_{2,21}$ = 1.76; p > 0.05) and male (t = 0.27; p > 0.05)].

		Elevated Plus Maze (EPM)			Open Field (OF)	
Groups	Treatment	ECA	REA	uHD	CRO	REA
24h Female	Saline	11,13 ± 0,79	6,37 ± 0,92	10,63 ± 2,49	90,63 ± 9,68	29.88 ± 2.94
	Pilo 75	13,25 ± 1,11	10,38 ± 1,93	6,75 ± 1,35	83,14 ± 5,08	26.86 ± 3.35
	Pilo 150	13,71 ± 0,75	8,57 ± 1,52	13,29 ± 2,56	72,38 ± 16,92	22.00 ±5.14
Male	Saline	11,63 ± 0,94	11,50 ± 1,67	12,75 ± 4,73	97,75 ± 12,49	26,25 ± 3,42
	Pilo 75	13,00 ± 1,12	11,00 ± 1,19	3,87 ± 1,53	91,50 ± 7,81	19,75 ± 3,83
	Pilo 150	12,17 ± 1,33	9,67 ± 1,65	6,50 ± 4,97	92,83 ± 16,70	20,83 ± 5,56
30d Female	Saline	11,88 ± 1,08	11,63 ± 1,41	7,37 ± 1,02	103,4 ± 6,33	18,00 ± 2,74
	Pilo 75	12,88 ± 0,81	12,50 ± 1,68	11,50 ± 1,22	133,4 ± 14,96	28,38 ± 4,23
	Pilo 150	10,50 ± 0,91	7,75 ± 1,36	6,75 ± 2,23	146,4 ± 15,16	24,63 ± 4,65
Male	Saline	11.13 ± 1.14	10,00 ± 2,19	6,50 ± 1,07	141,4 ± 7,36	28,75 ± 2,86
	Pilo 75	12.29 ± 0.28	8.57 ± 1.43	9.86 ± 1.56	136,1 ± 17,83	30,57 ± 6,55

Table 1. Short- and long-term effects of pilocarpine (75 or 150 mg/Kg, i.p.) on anxiety- and locomotion-related behaviors in female and male mice subjected to the elevated plus-maze and open-field tests. Results expressed as mean ± SEM, p = no significant difference of 6 to 8 animals per group. One-way ANOVA or Student's t test. ECA: Entries into Closed Arms. REA: Rearing. uHD: unprotected Head-Dipping. CRO: Number of Crossings.

In the rota-rod test, pilocarpine (75 or 150 mg/kg) did not change the number of falls when evaluated 24h [female ($F_{2,21}$ = 1.00; p > 0.05) and male ($F_{2,22}$ = 1.07; p > 0.05)] or 30d [female ($F_{2,20}$ = 1.48; p > 0.05) and male ($F_{2,22}$ = 0.73; p > 0.05)] after administration (data not shown).

3.3 Pilocarpine produces a long-term anxiogenic-like effect in the light/dark box test only in female mice

To characterize the anxiogenic-like phenotype induced by pilocarpine in the elevated plus-maze test in another anxiety device, mice of both sexes were subjected to the light/dark box (Figures 3A and 4A). One-way ANOVA did not detect differences in the time spent in the light box [female ($F_{2,21}$ = 0.97; p > 0.05; Figure 3B) and male ($F_{2,22}$ = 2.18; p > 0.05; Figure 3C), both in latency time to enter the dark box [male ($F_{2,22}$ = 1.37; p > 0.05; Figure 3E)] and in number of entries into the dark box [female ($F_{2,21}$ = 0.29; p > 0.05; Figure 3F) and male ($F_{2,22}$ = 0.78; p > 0.05; Figure 3G)], as evaluated

24h after treatment. However, a difference between pilocarpine doses was observed in latency time to enter the dark box ($F_{2,21}$ = 4.74; p > 0.05; Figure 3D), but only in female mice.

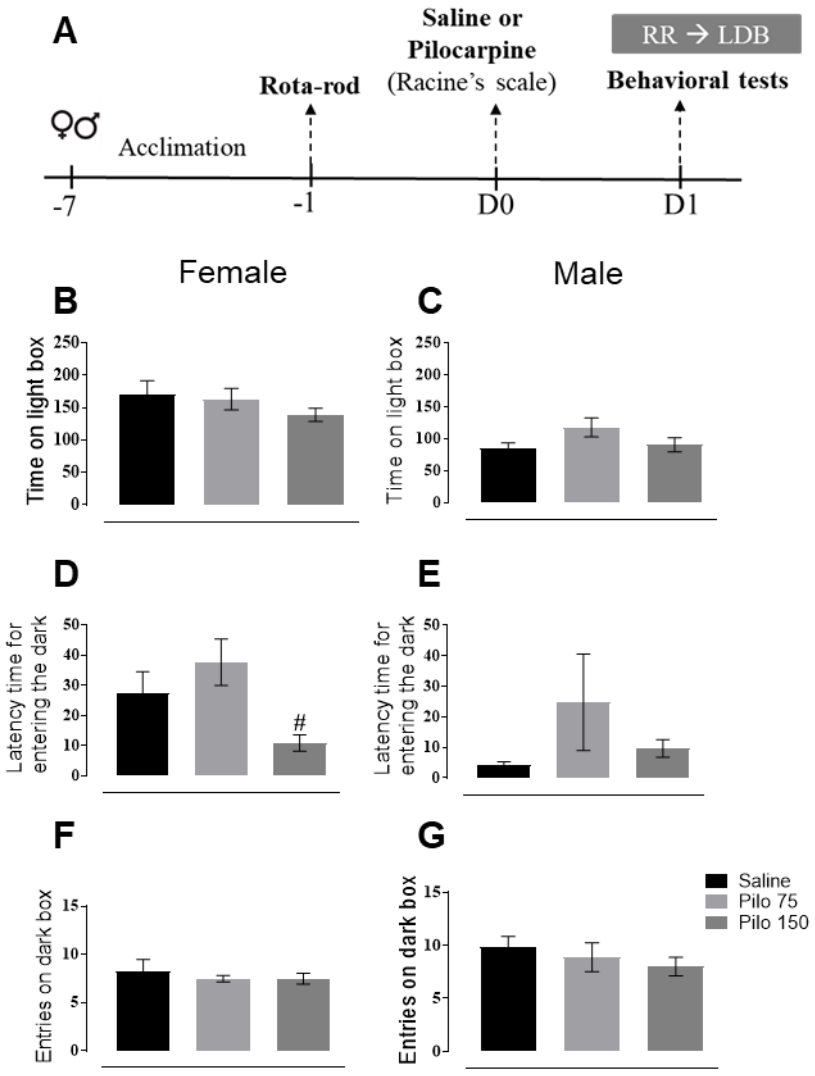

Figure 3. (A) Diagram of the experimental design. **(B-G)** Short-term effects of pilocarpine (75 or 150 mg/Kg, i.p.) on anxiety-related behavior in female and male mice subjected to the light/dark box test. The animals were evaluated 24h after treatment. Results expressed as mean ± SEM of 8 to 9 animals per group. $^{\#}p < 0.05$ as compared to the Pilo 75 group. One-way ANOVA followed the Newman-Keuls post hoc test. RR: Rota-Rod and LDB: Light/Dark Box.

One-way ANOVA did not detect differences in the time spent in the light box [male ($F_{2,22}$ = 0.04; p > 0.05; Figure 4C), in latency time to enter the dark box [female

($F_{2,20}$ = 3.15; p > 0.05; Figure 4D) and male ($F_{2,22}$ = 0.32; p > 0.05; Figure 4E)], and in number of entries into the dark box [female ($F_{2,20}$ = 0.14; p > 0.05; Figure 4F)], as evaluated 30d after treatment. In female mice, the pilocarpine 75 mg/Kg group reduced the time spent in the light box when compared to the control group, while the highest pilocarpine dose increased such time, when compared to the lowest pilocarpine dose ($F_{2,20}$ = 9.68; p < 0.01; Figure 4B). On the other hand, in male mice, the pilocarpine 150 mg/Kg group increased the number of entries into the dark box, when compared to the saline solution group ($F_{2,22}$ = 4.72; p < 0.05; Figure 4G).

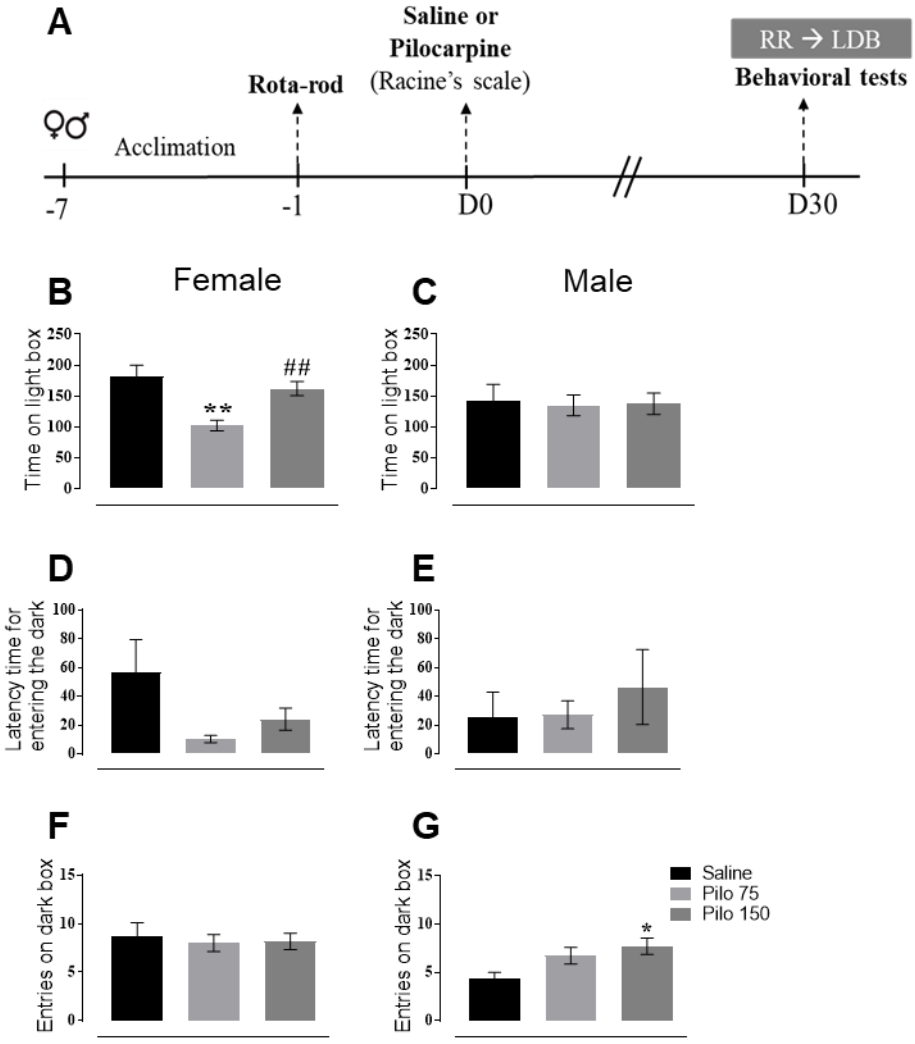

Figure 4. (A) Diagram of the experimental design. **(B-G)** Long-term effects of pilocarpine (75 or 150 mg/Kg, i.p.) on anxiety-related behavior in female and male mice subjected to the light/dark box test. The animals were evaluated 30d after treatment.

Results expressed as mean ± SEM of 6 to 9 animals per group. *p < 0.05 or **p < 0.01 as compared to the saline solution group and $^{\#\#}p < 0.01$ as compared to the Pilo 75 group (one-way ANOVA followed the Newman-Keuls post hoc test). RR: Rota-Rod and LDB: Light/Dark Box.

4 Discussion

Our results showed that the single administration of pilocarpine in a dose-dependent manner induced short- and long-lasting anxiogenic-like effects in mice. In fact, 24h after treatment with pilocarpine (75 mg/kg, subconvulsive dose), male and female mice presented an anxiogenic-like effect in the elevated plus-maze test; while 30d after treatment with the same pilocarpine dose, the similar behavioral response was shown in the light/dark box test, but only in female mice. Treatment with a higher pilocarpine dose (150 mg/kg) did not change these behavioral parameters. Moreover, no changes in locomotion-related parameters were observed during evaluation in the elevated plus-maze, rota-rod and open-field tests 24h or 30d after treatment with pilocarpine (75 or 150 mg/kg) in mice of both sexes. Thus, our results corroborate and extend previous literature data showing a short- and long-lasting anxiogenic-like phenotype in male Wistar rats (Duarte et al. 2010, 2013; Hoeller et al. 2016).

Pilocarpine-induced *status epilepticus* has been widely used to understand the pathogenesis of temporal lobe epilepsy and to evaluate potential antiepileptogenic drugs in rodents (Leite et al. 2002; Curia et al. 2008; Scorza et al. 2009). It is also known that temporal lobe epilepsy is commonly associated with behavioral and cognitive alterations in humans and that the pilocarpine model reproduces several of the comorbid psychopathologies. The mice that developed epilepsy after pilocarpine-induced *status epilepticus* exhibited anxiety-like behavior and impairment in the learning and memory processes (Gröticke et al. 2007; Müller et al. 2009). On the other hand, in the C57BL/6 mice that received convulsive pilocarpine doses but did not develop *status epilepticus*,

no anxiety-related behavior was observed in the elevated plus-maze, light/dark box and open-field tests (Müller et al. 2009). However, in this study, a single intraperitoneal injection of a low pilocarpine dose increased anxiety-like behavior in Swiss mice, indicating that *status epilepticus* induced by muscarinic receptors activation is not required to promote the anxious phenotype or that the mouse strains can influence behavioral response in some anxiety tests.

Several studies have shown that, in a dose-dependent manner, pilocarpine injection promotes behavioral, electroencephalographic (EEG), and neurochemical changes (for a review, see Scorza et al. 2009). The Racine scale has been frequently used to assess seizure intensity in pilocarpine-induced *status epilepticus*. Turski et al. (1984) showed EEG seizures starting after injection of large pilocarpine doses (300, 325 and 350 mg/Kg) but that, in animals that received pilocarpine in a low (100 mg/Kg) and intermediary (200 mg/Kg) dose, fully-developed EEG seizures did not appear up to 24h after pilocarpine administration. Moreover, injection of a low pilocarpine dose (100 mg/kg) does not promote neurodegeneration, unlike higher pilocarpine doses that induce *status epilepticus* and neuronal loss in various areas of the limbic system (Turski et al. 1984; Cavalheiro et al. 1996). However, Phelan et al. (2015) revealed a complex relationship between cortical EEG recordings and convulsive behaviors described by the Racine scale. Although we did not perform EEG recordings and neuropathological analysis, we assume that the behavioral changes observed in the animals that did not present *status epilepticus* and had a Racine scale value ≤ 3 were caused after injecting a subconvulsive pilocarpine dose.

During the last decade, several studies have shown that a single systemic injection of pilocarpine (150 mg/Kg, a subconvulsive dose) promotes long-lasting anxiety-like behaviors in adult Wistar rats. Also, rats spend more time in the protected

area (closed arms) of the elevated plus-maze test, when evaluated 24h or 1 month (Duarte et al. 2010, 2013; Hoeller et al. 2013, 2016), and up to 3 months (Duarte et al. 2010) after pilocarpine treatment, an indication of anxiety-related behavior. In the present study, we demonstrated that mice of both sexes present an anxious-like phenotype in the elevated plus-maze test 24h after a single administration of pilocarpine in a subconvulsive dose. In addition, 1 month after pilocarpine treatment, the anxiety-related behavioral responses were only found in female mice evaluated in the light/dark box. Therefore, our results corroborate and extend previous data from the literature that suggested a novel cholinergic-based model of enduring anxiety using a single systemic injection of subconvulsive pilocarpine.

Mouse models of anxiety have been widely used to demonstrate the anxiolytic behavioral effect of compounds derived from natural (Galdino et al. 2012; Narasingam et al. 2017; Diniz et al. 2019; Alonso-Castro et al. 2020; Tian et al. 2020; Oliveira et al. 2020; Zhang et al. 2020) or synthetic (Bektas et al. 2020; Moreira et al. 2020; Shanmugasundaram et al. 2020) products, as well as to investigate the molecular mechanisms underlying the behavioral results obtained with these bioactive compounds (Galdino et al. 2012; Narasingam et al. 2017; Diniz et al. 2019; Alonso-Castro et al. 2020; Bektas et al. 2020; Moreira et al. 2020; Shanmugasundaram et al. 2020; Tian et al. 2020; Zhang et al. 2020). Additionally, the mouse genome can be manipulated to study gene function in normal and diseased states (Dow and Lowe 2012). However, the best rodent anxiety model (mouse *vs.* rat) depends on which species best resembles human conditions (Bryda 2013). Our data show for the first time that a low pilocarpine dose promotes long-lasting anxiety-like behaviors in mice, contributing to the use of this animal species to study the neurobiology of anxiety disorders. Therefore, further

studies are needed to establish the rodent anxiety model that best represents the long-lasting anxiety-like effects of pilocarpine.

Moreover, the short- and long-term anxious phenotype found after the systemic injection of pilocarpine was observed in two distinct behavioral tests: elevated plus-maze and light/dark box, respectively. Several factors can interfere with animal behavior on anxiety tests (Harro 2018), exploring different anxiety-provoking situations. Although elevated plus-maze and light/dark box are approach-avoidance tests, the pharmacology and underlying neurobiology are not necessarily identical (Cryan and Sweeney 2011). Thus, it is necessary to use two or more anxiety tests to assess selective anxiolytic compounds, for example (Crawley 1981; Bourin and Hascoët 2003; Fraser et al. 2010; Cryan and Sweeney 2011). However, other studies from our laboratory (unpublished data) have shown short- and long-lasting anxiety-like behaviors derived from pilocarpine on the elevated plus-maze test, indicating that a single anxiety test can detect the behavioral effects of pilocarpine on anxiety.

It is important to point out that only female mice showed a long-lasting anxiety-like phenotype, indicating that male and female mice respond differently to the internal stimulus (pilocarpine). The model of enduring anxiety induced by pilocarpine originally reported in male rats can describe a model of state anxiety in male mice, whereas in female mice it can depict a model of trait anxiety. Even though female mice in the diestrus phase are more anxious than males (Galeeva and Tuohimaa 2001), we cannot attribute the behavioral differences found between genders in our experiments due to hormonal issues only. Therefore, our results are in line with data showing that women are more prone to develop anxiety disorders when compared to men (World Health Organization 2017), supporting our findings regarding distinct sex-related behavior.

Finally, we evaluated the effects of pilocarpine treatment on locomotion. The open-field test is widely used to analyze exploratory and anxiety-related behaviors (Gould et al. 2009). In the open-field test, a 5-minute test length is typical for assessing novel environment exploration. However, an increase in locomotion can be considered a stimulating effect, while a reduction in locomotion and vertical activity can be related to sedation (Prut and Belzung 2003). Thus, our results in the open-field, rota-rod, and elevated plus-maze (entries into closed arms) tests ruled out motor influence on the pilocarpine-induced anxiogenic-like effect in mice.

In conclusion, our results showed that subconvulsive pilocarpine, in a dose-dependent manner, induced a short- and long-term anxiogenic-like effect in mice. Unlike those previously observed in male rats (Duarte et al. 2010, 2013; Hoeller et al. 2016), our results were found only in female mice; and these effects cannot be associated with motor impairment. Therefore, our results enhance the validity of the novel cholinergic-based model of enduring anxiety using a single systemic injection of subconvulsive pilocarpine in rodents.

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