UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA MOLECULAR

Ana Paula Carneiro Rodrigues

INVESTIGATION OF BEHAVIORAL ALTERATIONS OF PRENATAL NICOTINE EXPOSURE IN MICE

Investigação das alterações comportamentais da exposição pré-natal de nicotina em camundongos

Belo Horizonte
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Investigação das alterações comportamentais da exposição pré-natal de nicotina em camundongos

Dissertação apresentada ao Programa de Pós-graduação em Medicina Molecular da Faculdade de Medicina da UFMG como requisito parcial para obtenção de título de mestre.

Orientador: Professor Marco Aurélio Romano Silva (MD-PhD)

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FOLHA DE APROVAÇÃO

INVESTIGAÇÃO DAS ALTERAÇÕES COMPORTAMENTAIS DA EXPOSIÇÃO PRÉ-NATAL DE NICOTINA EM CAMUNDONGOS

ANA PAULA CARNEIRO RODRIGUES

Dissertação de Mestrado defendida e aprovada, no dia trinta de julho de dois mil vinte e um, pela Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação Medicina Molecular da Universidade Federal de Minas Gerais constituída pelos seguintes professores doutores:

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RESUMO

A exposição pré-natal ao tabaco (EPT) e terapias de reposição de nicotina (TRN) durante a gravidez foram clinicamente associadas a sintomas e transtornos psiquiátricos, como depressão, ansiedade e agressividade, e a uma maior incidência de crianças com Transtorno de Déficit de Atenção/Hiperatividade (TDAH). Para estudar mais e compreender melhor o TDAH e suas alterações no desenvolvimento neurológico, os modelos animais fornecem uma ferramenta útil. Infelizmente, ainda não existe um modelo clinicamente perfeito, e cada um tem seus prós e contras. O modelo de exposição pré-natal à nicotina (EPN) é um deles. Devido às infinitas possibilidades de protocolo, existem resultados contraditórios entre as publicações. Neste trabalho testamos diferentes doses de nicotina e investigamos extensivamente as alterações comportamentais apresentadas por camundongos machos EPN. Aqui, mostramos que o tratamento com nicotina de 0.1 e 0.15 mg/mL não forneceu um modelo de TDAH confiável, pois tivemos resultados mistos ou negativos. No entanto, o tratamento com nicotina 0.2 mg/mL fornece um modelo com hiperlocomoção, ansiedade social e impulsividade. Também encontramos um pequeno efeito na memória de longo prazo, e nenhum sinal de comportamentos semelhantes aos de ansiedade ou depressivos. Em conclusão, este modelo apresenta validade de face, preditiva e de construto e pode ser uma ferramenta poderosa para melhor compreender os mecanismos subjacentes às manifestações clínicas do TDAH, especialmente considerando a relação entre exposição à nicotina durante a gravidez e maior risco de desenvolvimento de TDAH.

Palavras-chave: Exposição pré-natal à nicotina, TDAH, comportamento, hiperatividade, cognição, impulsividade, memória, ansiedade, depressão.

ABSTRACT

Prenatal exposure of tobacco (PET) and nicotine replacement therapies (NRT) during pregnancy has been clinically associated with psychiatric disorders and symptoms, such as depression, anxiety and aggressivity, and to a higher incidence of children with Attention-Deficit/Hyperactivity Disorder (ADHD). To further study and better understand ADHD and its neurodevelopmental alterations, animal models provide a useful tool. Unfortunately, there is still no clinically perfect model, and each has its pros and cons. The prenatal nicotine exposure (PNE) model is one of them. Due to infinite protocol possibilities, there are contradicting results between publications. In this work, we tested different nicotine doses and extensively investigated behavioral alterations presented by the PNE male mice. Here we show that the 0.1 and 0.15 mg/mL nicotine treatment did not provide a reliable ADHD model, as we had mixed or negative results. However, we found that the 0.2 mg/mL nicotine treatment provides a model with hyperlocomotion, social anxiety and impulsivity. We also found a small effect in the long-term memory and no signs of anxiety-like or depressive-like behaviors. In conclusion, this model presents face, predictive and construct validity and can be a powerful tool to better understand the mechanisms underlying ADHD clinical manifestations, especially considering the relation between nicotine exposure during pregnancy and higher risk of ADHD development.

Keywords: Prenatal nicotine exposure, ADHD, behavior, hyperactivity, cognition, impulsivity, memory, anxiety, depression.

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LIST OF ACRONYMS AND INITIALS

%SAB	Percentage of Spontaneous Alternation Behavior
3-CSSNT	Three-Chamber Sociability and Social Novelty Test
5-CSRTT	5-Choice Serial Reaction Time Task Test
ADHD	Attention-Deficit/Hyperactivity Disorder
BM	Barnes Maze
CAR	Cliff Avoidance Reaction
Ctrl	Control
DAT-KO	Dopamine Transporter Knockout
DI	Discrimination index
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
E	Empty Cage
EPM	Elevated Plus Maze
FST	Forced Swim Test
LDT	Light/Dark Test
MBT	Marble Burying
MD	Mean Difference
MedD	Median Difference
MPH	Methylphenidate
6-OHDA	6-Hydroxydopamine
nAChR	Nicotinic Acetylcholine Receptor
NRT	Nicotine Replacement Therapy
OFT	Open Field Test
ORT	Object Recognition Test
PET	Prenatal Exposure of Tobacco
PNE	Prenatal Nicotine Exposure
S1	Stranger 1
S2	Stranger 2
SHR	Spontaneously Hypertensive Rat
SPT	Sucrose Preference Test
YM	Y-maze
WHO	World Health Organization

SUMMARY

1.0 INTRODUCTION

1.1 Smoking during pregnancy and its effects

Although the World Health Organization (WHO) reported that the general prevalence rates of tobacco use are declining, especially among women, the rates are still high in certain countries (WHO, 2019). In Europe, the prevalence rates are stagnated with a high probability of minimal decline until 2025 (WHO, 2019). Recent studies report that in some European countries, tobacco use is still prevalent in 20% of pregnancies, and also that in some regions, the rates are stagnated or even increased (Lange et al., 2018). Even though cigarettes have thousands of chemical components, studies suggest that the negative effects observed in some babies born from mothers who smoked may be caused by nicotine (Abbott et al., 2012; Zhu et al., 2014), as it is known to be the major psychoactive component (Dani and Harris, 2005). It is known that nicotine can cross the placenta and accumulate in the fetus tissue (Luck, 1985; Polli et al., 2019). As the mechanisms by which it could interfere with the development and act as a teratogen is still unknown, the evidence is suggestive of a causal relationship. Some studies hypothesized that its long-term effects could be either because of overstimulation and/or desensitization of nicotinic acetylcholine receptors (nAChRs) and possible epigenetic alterations (Slotkin et al., 1987; Navarro et al., 1989; Nguyen et al., 2000; Zhu et al., 2014). The nicotine interactions with the receptors are likely both in the mother and the fetus, as the nAChRs are expressed in the early stages of development and are widely present in the central nervous system, as well as in the peripheral and enteric nervous systems (Zoli et al., 1995; Ernst et al., 2001; Garza et al., 2009; Abbott et al., 2012).

Prenatal exposure of tobacco (PET) has been clinically associated with psychiatric disorders and symptoms, such as depression, anxiety and aggressivity, mainly in children and adolescents, and also predisposition to smoking as adults (Biederman and Faraone, 2002; Gatzke-Kopp and Beauchaine, 2007; Biederman et al., 2009; Cornelius and Goldschmidt, 2012; Moylan et al., 2015). There are also reports associating smoking or nicotine replacement therapies (NRT) during gestation to a higher incidence of children with Attention-Deficit/Hyperactivity Disorder (ADHD). According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), ADHD is classified as a heterogeneous and chronic neurodevelopmental disorder. Its main symptoms include inattention, hyperactivity

and impulsivity, usually showing signs during childhood, and may cause behavioral, academic and social problems (Barkley, 2002; APA, 2013; Kaiser et al. 2015).

1.2 ADHD rodent models

To further study and better understand ADHD and its neurodevelopmental alterations, animal models provide a useful tool. A perfect ADHD animal model would have similar clinical symptoms (face validity), positive response when currently available therapies applied (predictive validity) and a good translational value (construct validity), especially regarding the development of the disorder in humans. Unfortunately, there is still no clinically perfect model, and each has its pros and cons. The most studied animal models are spontaneously hypertensive rats (SHR), neonatal 6-hydroxydopamine (6-OHDA) lesion, dopamine transporter knockout (DAT-KO) and prenatal nicotine exposure (PNE).

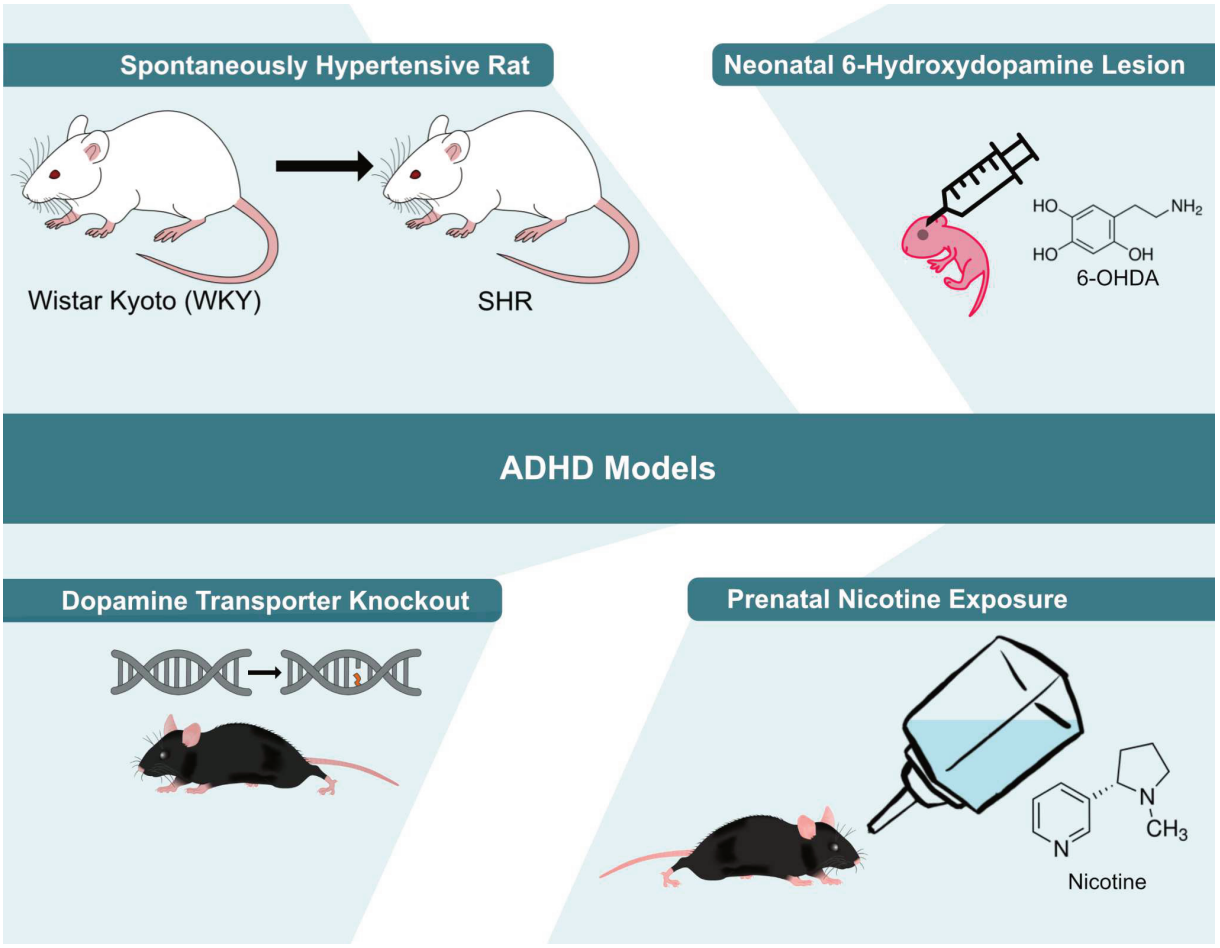

Figure 1. Different ADHD animal models. Representation of the most studied animal models of ADHD and its development.

The SHR attracted attention for its hyperactivity (Wultz et al., 1990; Sagvolden et al., 1992), but further research showed also inattention and impulsivity (Evenden and Meyerson, 1999; De Bruin et al., 2003; Jentsch, 2005; Bizot et al., 2007; Fox et al., 2008), which made it possible to be used as an ADHD model even though it does not have a translational value. The neonatal 6-OHDA lesion is one of the oldest models available and still used (Shaywitz et al., 1976). They present hyperactivity (Erinoff et al., 1979; Miller et al., 1981; Archer et al., 1988) and inattention (Oke and Adams, 1978; Archer et al., 1988; Bouchatta et al., 2018) but impulsivity has mixed results (Arime et al., 2011; Bouchatta et al., 2018). Unfortunately, it also lacks translational value. The DAT-KO mouse show hyperactivity (Giros et al., 1996), impulsivity and impaired learning and memory (Gainetdinov et al., 1999; Gainetdinov and Caron 2000; Trinh et al., 2003; Li et al., 2010; Yamashita et al., 2013). The role of DAT in ADHD is still not completely elucidated, but there is enough evidence to assume its major involvement (Volkow et al., 2002; Madras et al., 2005; Volkow et al., 2007, 2009; Sakrikar et al., 2012) and it has one of the best construct validity of all models available (Leo et al., 2013).

The PNE model has several different administration routes, including drinking solution, subcutaneous or intravenous injections, osmotic minipump and tobacco smoke. In this work, we will only discuss the drinking solution route, but as each route has advantages and disadvantages, the main information is summarized in Figure 2 (for review, see Polli et al., 2019).

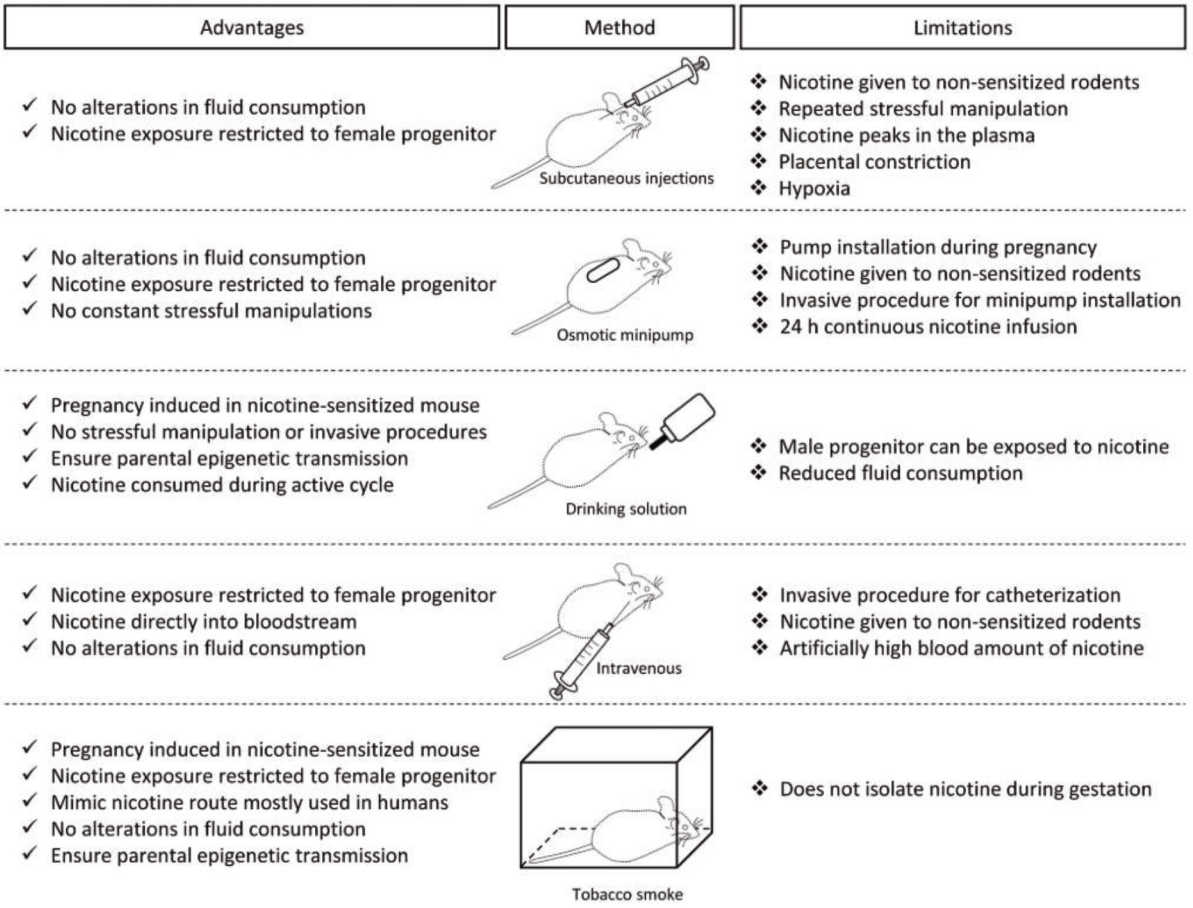

Figure 2. Different administration routes of PNE animal models. Resume of the advantages and disadvantages of different routes of nicotine administration in PNE models. Adapted from Polli et al., 2019.

1.3 PNE model: oral administration

In this route, the nicotine solution is given to a pregnant or soon-to-be pregnant mouse, as a replacement for their drinking water (Figure 3), usually containing saccharine to increase palatability and reduce the risk of dehydration (Paz et al., 2007; Zhu et al., 2012; Zhu et al., 2017; Zhang L. et al., 2018; Polli et al., 2020). The oral treatment has shown distribution in both central and peripheral tissues (Rowell et al., 1983) and cotinine plasma levels (nicotine's main metabolite and biomarker) were similar to the levels found in habitual cigarette smokers (Pauly et al., 2004). Studies also did not find changes in maternal care, suggesting a low stress-inducing model (Pauly et al., 2004; Heath et al., 2010; Alkam et al., 2013). This model is also consistent with the natural fluctuation of nicotine/cotinine levels

during the day, with higher levels during the active phase and lower levels during the inactive phase (Polli et al., 2019).

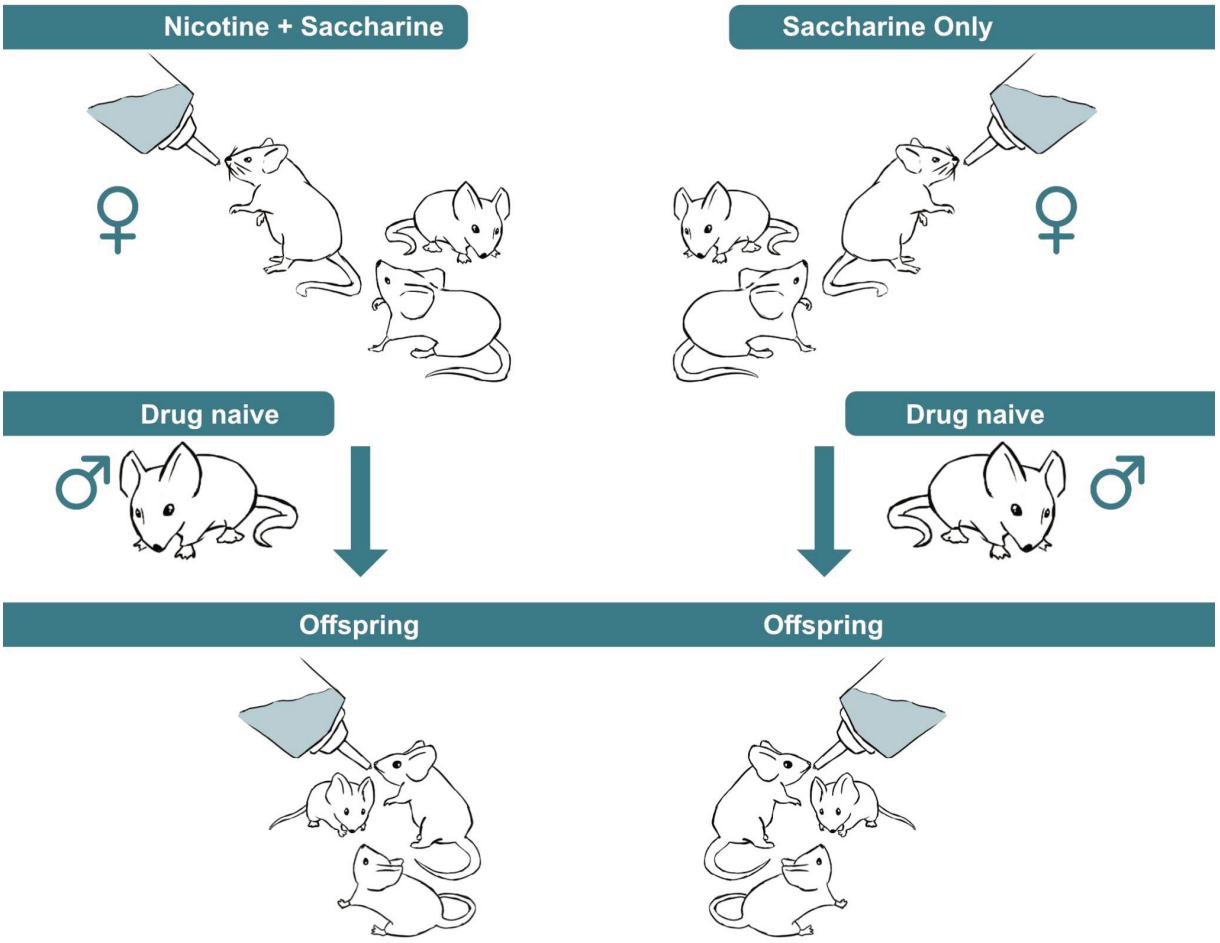

Figure 3. Protocol for oral PNE model. Scheme showing the basic steps of the protocol for oral administration of nicotine. Firstly, female mice are offered nicotine + saccharine solutions or only saccharine solutions (controls) for a defined period (usually 21 days) before breeding. The females are then housed together with males for mating. After 19-21 days (of the mating) the females will give birth to the pups. The nicotine solution is removed and only water is offered from now on. After 21-28 days (from the day of birth) the offspring are weaned and housed in 4-5 same-sex mice per cage. The usual age for mice behavioral testing is 6-12 weeks old.

As the experimental animals are pups of these treated females, the results observed will change drastically depending on the protocol followed, even within the oral route group. Some of the main points are: the species (rats or mice), maternal or paternal treatment, the nicotine dose, the start point and duration of the treatment, if there is crossfostering or not, the age and sex of the offspring tested, conditions of the behavioral test, among others. So considering all possible protocols used in

publications with this model, some contradicting results are expected. Usually they present hyperactivity (Pauly et al., 2004; Paz et al., 2007; Zhu et al., 2012; Schneider et al., 2012; Balsevich et al., 2014; Buck et al., 2019), but some authors found no differences (Heath et al., 2010; Schneider et al., 2011; Zhang L et al., 2018). They also present deficits in learning and attention (Alkam et al., 2013; Zhu et al., 2017; Zhang L. et al., 2018; McCarthy et al., 2018; Polli et al., 2020), and increased impulsive behaviors (Schneider et al., 2011; Zhu et al., 2017; Zhang L. et al., 2018). However, for emotional assessments, reports are showing anxiogenic-like (Alkam et al., 2013; Balsevich et al., 2014; Buck et al., 2019; Polli et al., 2020) or anxiolytic-like behaviors results (Zhang L et al., 2019). The model has also shown improved cognitive deficits when treated with methylphenidate (MPH), the most used medication in human patients (Zhu et al., 2017; Alkam et al., 2017).

In conclusion, this model presents face, predictive and construct validity. Its main problem is the inconsistency between studies, especially due to different protocols applied that make it impossible to compare the findings directly. The resolution of this problem could help better understand the mechanisms underlying ADHD clinical manifestations, considering the relationship between nicotine exposure during pregnancy and a higher risk of ADHD development.

2.0 OBJECTIVES

2.1 Overall Objectives

Validate the behavioral aspects of prenatal nicotine exposure in mice, as a mouse model of ADHD, for further investigation of its complex neurobiological mechanisms.

2.1.1 Specific Objectives

2.1.1 Test most commonly used nicotine doses in existing PNE protocols.
2.2.2 Investigate behavioral alterations evoked by the different PNE protocols.
2.2.3 Develop an easier and more reliable protocol for PNE models.

3.0 ARTICLE

INVESTIGATION OF BEHAVIORAL ALTERATIONS OF PRENATAL NICOTINE EXPOSURE IN MICE: A mouse model of ADHD

3.1 ABSTRACT

Prenatal exposure of tobacco (PET) and nicotine replacement therapies (NRTs) during pregnancy has been clinically associated with psychiatric disorders and symptoms, as depression, anxiety and aggressivity, and to a higher incidence of children with Attention-Deficit/Hyperactivity Disorder (ADHD). To further study and better understand ADHD and its neurodevelopmental alterations, animal models provide a useful tool. Unfortunately, there is still no clinically perfect model, and each has its pros and cons. The prenatal nicotine exposure (PNE) model is one of them. Due to infinite protocol possibilities, there are contradicting results between publications. In this work, we tested different nicotine doses and extensively investigated behavioral alterations presented by the PNE male mice. Here we show that the 0.1 and 0.15 mg/mL nicotine treatment did not provide a reliable ADHD model, as we had mixed or negative results. However, we found that the 0.2 mg/mL nicotine treatment provides a model with hyperlocomotion, social anxiety and impulsivity. We also found a small effect in the long-term memory and no signs of anxiety-like or depressive-like behaviors. In conclusion, this model presents face, predictive and construct validity and can be a powerful tool to better understand the mechanisms underlying ADHD clinical manifestations, especially considering the relation between nicotine exposure during pregnancy and higher risk of ADHD development.

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3.2 INTRODUCTION

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In conclusion, this model presents face, predictive and construct validity. Its main problem is the inconsistency between studies, especially due to different protocols applied that make it impossible to compare the findings directly. The resolution of this problem could help better understand the mechanisms underlying ADHD clinical manifestations, considering the relation between nicotine exposure during pregnancy and a higher risk of ADHD development.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Female and male adult C57BL/6J mice (8-12 weeks) were used to obtain the PNE mice. Male adolescent and adult C57BL/6J mice (6-12weeks) were used to investigate behavioral alterations evoked by the different PNE protocols. All mice were housed in a temperature-controlled facility with an inverted 12h light/dark schedule (lights on at 7 p.m./lights off at 7 a.m.) and provided with food and water or PNE solutions *ad libitum*. All procedures were approved by the Animal Use Ethics Committee from the Federal University of Minas Gerais (protocol 313/2019).

3.3.2 Prenatal nicotine exposure model

For the first dose test (0.1 mg/mL), individually-housed female mice received an aqueous solution with nicotine (Nicotine tartrate, Sigma) and 2% sugar or sugar only (control) ad libitum for 21 days. The nicotine was given at small doses first and changed every 3 days, starting at 0.025 mg/mL, then 0.05 mg/mL, 0.075 mg/mL, and finally 0.1 mg/mL. Their weight and solution intake were measured daily until reproduction. After 19 days, we mixed scented male bedding (urine, feces and natural scent) to their home cage bedding to synchronize the estrous cycle (Whitten effect). On day 21st, females were housed with drug-naive males to reproduce (one female for each male) for 7 days, while still receiving the nicotine or control solution. After birth, the nicotine doses were 50% reduced every 2 days until they were drinking only water. The pups were weaned at 21-28 days old and were housed with 4-5 same-sex mice per cage.

For the “best dose” test (0.1 mg/mL, 0.15 mg/mL or 0.2 mg/mL), the same previous protocol was used, with a few improvements: (1) The female mice were housed with 4-5 same-sex mice per cage; (2) The nicotine doses were still introduced slowly, but started at the dose of 0.05 mg/mL, and every 3 days we added 0.05 mg/mL until they reached the maximum dosage for the group (either 0.1 mg/mL, 0.15 mg/mL or 0.2 mg/mL). The 21 days started counting after they reached the final dose; (3) This time, only the weight was evaluated; (4) The reproduction design changed to two females for each male. For the last experiment, using only the higher dose, we repeated the previous improved protocol.

3.3.3 Behavioral testing

For all tests, the animals were habituated at the testing room for, at least, 30 minutes. All apparatuses were cleaned with 70% alcohol before testing. The first tests (open field, elevated plus maze and object recognition test) were performed during the animals’ light cycle (inactive) and the room was illuminated the same as their home cage room. The following tests were performed during the animals’ dark cycle (active), so we used red dim lighting for most tests, except the anxiety-motivated ones (elevated plus maze, Barnes maze and light/dark box).

3.3.3.1 Open field

Widely used to evaluate the locomotor function and anxiety-like behaviors in mice, the animals were positioned in the center of the apparatus and were free to explore for 10 minutes, 2 hours or 5 hours. Distance traveled, speed, time spent in the center and periphery zones were evaluated in open field boxes (40cm long, 40cm wide, and 30cm deep) at different ages.

3.3.3.2 Object recognition

This test is based on the innate exploratory and novelty seeking behavior of mice. In open field boxes, the objects were placed in two of the four corners of the box, 5cm far from the nearby walls. The test was divided into two stages, 10 minutes sessions each: Training, with both objects identical, and Test, with one of the previously used objects (old) and one new (novel). Two different protocols were used in this paper: one test after 2 hours (short-term memory) and the other after 24 hours (long-term memory). The animals were placed in the center of the box, facing the opposite wall from the objects. Time interacting with the objects, discrimination index, distance traveled and speed were evaluated. Interaction with the object was defined as facing the object inside a 2cm wide zone around it. Touching or climbing was not considered an interaction. The discrimination index (DI) allows us to see if the animals could discriminate between the objects, by exploring more the new than the old one. We defined the index according to the following formula: DI = (Novel Time − Old Time)/(Novel Time + Old Time). A positive index indicates more time exploring the new object and a negative index indicates more time exploring the old object.

3.3.3.3 Elevated plus maze

This test was used to evaluate anxiety-like behaviors in mice, making them choose between a high and well-illuminated place or a hidden and less exposed place. The apparatus consisted of four arms (50cm long) elevated at 55cm from the floor, two open without walls and two closed with 30cm tall walls. The animals were placed at the center and each session lasted 5 minutes. Time spent in the open arms, time spent in the closed arms, distance traveled and speed were evaluated.

3.3.3.4 Y-maze

The Y-maze is based on the innate novelty-seeking behavior of mice and the apparatus consists of three arms in a Y shape (40cm long, 15cm wide, and 30cm tall). The animals were placed at the end of the starting arm, facing forward, and each session lasted 5 minutes. We considered spontaneous alternation if the animal made consecutive entries into the arms without going back to the previous one. The percentage of spontaneous alternation, total entries in the arms, distance traveled and speed were evaluated. The formula of the percentage of spontaneous alternation was defined as: %SAB = [(number of alternations)/(total number of arm entries − 2)] × 100%.

3.3.3.5 Barnes maze

The Barnes Maze is a complex behavioral assessment of visuospatial learning and memory in rodents. The apparatus is a circular metal platform (90cm diameter and elevated 1m from the floor) with 19 shallow holes (fake escape holes) and 1 hole leading to the escape chamber (true escape hole). As visual cues, 4 images of different geometric figures were positioned around the apparatus in the main cardinal points. To avoid positioning bias, we used an opaque hollow rectangular prism made of acrylic to place the animals in the apparatus. This way they were randomly positioned before the test started. This test was divided into 3 parts: habituation, training and test. The habituation was only 1 trial on the first day and serves to introduce the apparatus and the overall routine. The mice were placed in the middle of the platform (inside the prism), and after 10 seconds, the prism was lifted, freeing the animal to explore for 2 minutes. If it didn’t find the correct exit until the end of the trial, we used a glass cylinder to help the animal locate it and enter it by itself. If after 1 minute the animal still didn’t enter the escape chamber, we gently placed it inside the chamber through the hole. Mice stayed in the chamber for 1 minute and then returned to their home cage.

The habituation was followed by the training stage, lasting 3 days with 3 trials each day. These trials were conducted as the habituation stage, but with a longer exploration time (3 minutes) and a shorter glass cylinder time (30 seconds). The trials were consecutive, with a 3 minutes inter-trial interval. The last stage consists of the test, to evaluate if they learned the task. In this stage, the escape chamber is

replaced by a shallow fake exit, identical to the other 19 ones. It is only one trial of 1 minute and 30 seconds. Distance traveled, speed, primary and total errors, primary and total latency, strategy and learning curve was evaluated. Errors are the mistakes the animals made by exploring the fake escape holes. Primary measures are the events recorded until finding the true escape. We considered three types of strategy: direct (animals made 2 or fewer errors), serial (animals explore the holes in a sequence, making more than 2 errors) or random (animals explore the apparatus randomly).

3.3.3.6 Three-chamber sociability and social novelty

This test is a complex behavioral assessment of sociability and social novelty-seeking in mice. The apparatus is a three-chamber box made of transparent acrylic (19cm long and 45cm wide, each chamber) with a metal base. The two main chambers (interaction chambers) are separated by a middle neutral chamber. The connections between the chambers are closed with a removable lid. Inside the interaction chambers, there is one small circular metal cage for the social animals (Strangers 1 and 2). The chosen Strangers were of the same lineage, age, sex and weight as the experimental and control mice. Also, they were habituated to both metal cages and apparatus before testing to avoid aversive cues. The test consists of three consecutive stages: Habituation, sociability and social novelty.

In the habituation stage, the mouse is placed in the middle chamber, with the separating lids still in place, and is allowed to explore it for 5 minutes. After that period, we placed the first Stranger in one of the interaction chambers and removed the separating lids. The animal is then free to explore all chambers for 10 minutes. The trial mouse was then restricted back in the neutral chamber while we placed the second Stranger in the remaining cage. The lids were removed again and it was free to explore all chambers for another 10 minutes. Interaction time and the number of entries in the chambers with Stranger 1 and the empty cage were evaluated for sociability measures. interaction time and the number of entries in the chambers with Stranger 1 and Stranger 2 were evaluated for social novelty measures.

3.3.3.7 Light/dark box

The light/dark box test was used to evaluate anxiety-like behaviors in mice, making them choose between a highly illuminated and open place or a dark and hidden place. The apparatus is a two-chamber box (42cm long, 25cm large and 21cm tall). One chamber is smaller, approximately one-third, and in black opaque acrylic. The bigger one, approximately two-thirds, is made of transparent acrylic. The “light chamber” was illuminated with light between 200-400 lux as the “dark chamber” was not illuminated, presenting 5 or less lux. The animals were positioned inside the dark chamber and explored freely for 5 minutes. Light chamber entries were considered when the animal had all four paws outside the dark chamber. If the animal still had one paw inside the dark chamber and head facing the light chamber, it was considered a risk assessment. The number of risk assessments and entries in the light chamber, latency until first risk assessment and entry in the light chamber, time spent in each chamber and distance traveled was evaluated.

3.3.3.8 Forced swim

The forced swim test was used to evaluate depressive-like behaviors in mice. The apparatus consisted of a 50cm tall and 20cm diameter transparent glass cylinder (2L becker). It was filled with approximately 1.4 L clean tap water (25ºC). Mice were placed on top of the water and allowed to swim freely for 6 minutes. The animals were then placed inside a clean and warm cage to dry. The cage was placed on top of a heated pad below 37ºC. After drying, they returned to their home cage. Before each animal, the cylinders were clean with 70% alcohol and thoroughly rinsed with water several times. Immobility was considered as none or minimal paw movements for floating purposes. Swimming was considered as any other paw activity. Time swimming, time immobile and longest swimming episode were evaluated.

3.3.3.9 Marble-burying

This test was used to evaluate repetitive movement behaviors in mice. We used an acrylic mouse cage filled with 2cm of proper clean bedding. We positioned 15 glass marbles (1.5cm diameter), cleaned with 70% alcohol, using a 3 by 5 grid

with approximately 2.5cm spacing between each other. Mice were placed in one corner of the cage, facing the wall, and were free to explore for 30 minutes. The number of buried marbles is this test's main result.

3.3.3.10 Cliff avoidance reaction

The cliff avoidance reaction test is commonly used in ADHD rodent models to evaluate impulsive-like behaviors in mice. The apparatus used was a 50cm tall and 20cm diameter transparent glass cylinder (2L becker) upside down, so that the base turned into a small platform. The cylinder was placed inside an open field box to prevent escapes when the animals fell. Mice were positioned on the center of the platform and were free to explore for 15 minutes. When a fall occurred, they were replaced gently on the platform until the end of the trial. The number of falls and latency until the first fall were evaluated.

3.3.4 Statistical analysis

Datasets normality was tested using the D'Agostino & Pearson normality test ($\alpha > 0.05$). All tests were two-tailed and had an $\alpha = 0.05$. For comparisons of a single variable between unrelated datasets, we used the unpaired Student's *t* test or Mann-Whitney test. For comparisons of a single variable between related datasets, we used paired Student's *t* test or Wilcoxon matched-pairs signed-rank test. For comparisons of four groups with only a single variable, we used one-way ANOVA followed by Holm-Sidak's multiple-comparisons tests or Kruskal-Wallis's test followed by Dunn's multiple-comparisons tests. For comparisons of double variables, datasets were analyzed using two-way ANOVA with individual trials as repeated measures and groups (treated vs. control) as independent variables. Sidak's multiple-comparisons test was used for *post hoc* testing with correction for multiple comparisons. All statistical analysis was performed using Prism version 8 (GraphPad Software).

3.4 RESULTS

3.4.1 Nicotine solution does not cause weight loss, reduces water intake or natality

To obtain the prenatal nicotine exposure (PNE) model, we treated female mice with an aqueous solution containing nicotine and sugar or just sugar, for controls. Since nicotine solutions have an unattractive flavor for mice, besides adding sugar, we also introduced nicotine gradually, starting at 0.025 mg/mL. We then increased the dose every three days until reaching the final 0.1 mg/mL dose (**Figure 1A**). After 19 days, we mixed scented male bedding to their home cage to synchronize the estrous cycle, known as the “Whitten effect” (McCartney and Marshal, 2013; Hickman et al., 2017; Brennan, 2018). On the 21st day, the females were housed with drug-naive males to reproduce (one female for each male) while still receiving the nicotine or control solution (**Figure 1A**). Male mice also drank the nicotine solution while they stayed with the females for breeding (7 days). After birth, the nicotine dose was 50% reduced every 2 days until they were drinking only water. The pups were weaned at 21-28 days old and were housed with 4-5 same-sex mice per cage (**Figure 1A**).

To investigate if the nicotine added to their drinking water would cause aversion, and consequent weight loss, we weighed the females and their bottles (full and after drinking) daily during treatment (**Figure 1B** and **C**). We considered the “final day” the day we housed the females and males together (**Figure 1B** and **C,** graphs at the right). Both groups presented an increase in their weight, as expected as they grew up, but there were no differences between the groups (Interaction effect: $F_{(20, 260)} = 1.844$, $p = 0.0169$; Days effect: $F_{(20, 260)} = 33.29$, $p < 0.0001$; Treatment effect: $F_{(1, 13)} = 0.6650$, $p = 0.4295$; Animal effect: $F_{(13, 260)} = 131.9$, $p < 0.0001$, two-way repeated-measures ANOVA; Final Day: Mean Difference (MD) = -0.4000, 95% CI = -2.161 to 1.361, $t_{(13)} = 0.4907$, $p = 0.6318$, Student’s *t* test; **Figure 1B**). The nicotine added also had no effects on their solution intake (Interaction effect: $F_{(20, 260)} = 1.176$, $p = 0.2752$; Days effect: $F_{(20, 260)} = 2.288$, $p = 0.0017$; Treatment effect: $F_{(1, 13)} = 0.03249$, $p = 0.8597$; Animal effect: $F_{(13, 260)} = 8.216$, $p < 0.0001$, two-way repeated-measures ANOVA; Final Day 0.1 vs. Ctrl, MD = -0.5000, 95% CI = -2.043 to

1.043, $t_{(13)}$ = 0.7001, p = 0.4962, Student's t test; **Figure 1C**) or on their natality (Ctrl: 6.600 ± 0.4000; 0.1: 5.300 ± 1.193; Mean ± SEM. 0.1 vs. Ctrl, MD = 0.000, U = 21.50, p = 0.7043, Mann-Whitney test; data not shown).

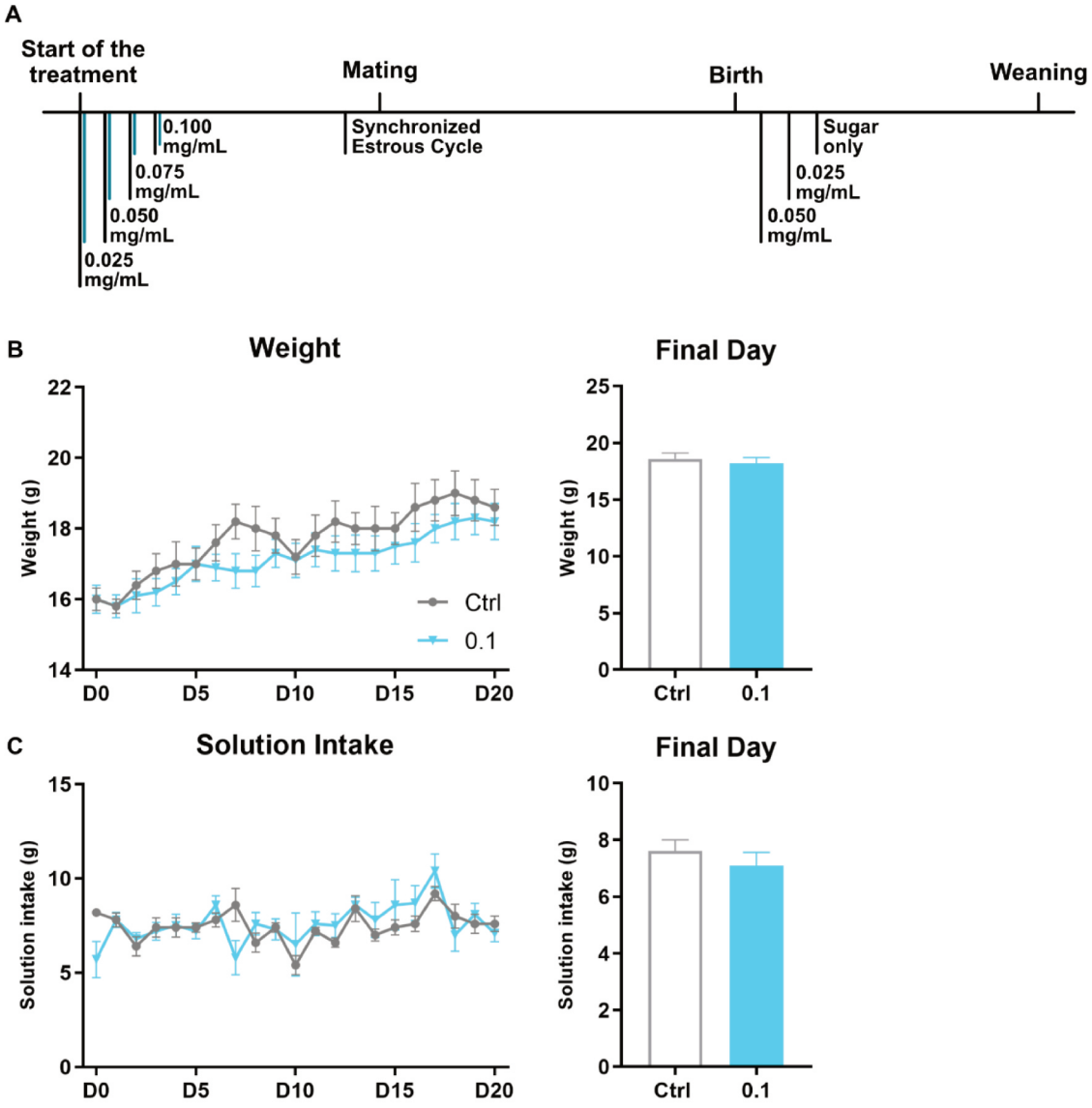

Figure 1. Nicotine solution at 0.1 mg/mL does not cause weight loss or reduces water intake. **(A)** Experimental design of the treatment with 0.1 mg/mL nicotine + 2% sugar or just 2% sugar (controls) in their drinking water. The female mice received nicotine gradually, starting at 0.025 mg/mL. Every three days, the dose went higher, until it reached the final dose of 0.1 mg/mL. After 19 days of treatment, we synchronized the estrous cycle using scented male bedding. On the 21st day, we started the mating process. Mouse pregnancy lasts around 19-21 days and they received the nicotine solution during the whole period. After they gave birth, we removed the nicotine gradually, reducing the dose by 50% every three days, until they were drinking only water. Pups were weaned when around 21-28 days old. **(B)** Mice were weighed every day until they started mating. There was no difference in their weight besides the expected weight gain as they grew up. We considered the final day (graph at the right) as the last day before mating. **(C)** We also weighed their bottles every day, when they were just filled with the solution and right before changing it the next day. The difference is what they drank, here we named it "solution

intake". There was no difference between the groups. We considered the final day (graph at the right) as the last day before mating. Data represented as mean ± S.E.M. (n = 10 0.1 group/ 5 Ctrl group).

After the first results using the 0.1 mg/mL dose (detailed in the next topic), we decided to test higher doses, trying to find the "best dose". We then choose to test 0.15 mg/mL and 0.2 mg/mL doses. At the time, 0.2 mg/mL was the higher dose we could find and decided to not go further higher as we were trying to test existing and used doses and not to test the animals' limits. We also made a few improvements to the previous protocol, especially in the social aspect of the treatment (see METHODS for more details). Since we observed that the females did not have any problems drinking the nicotine solutions, we decided to house them together (4-5 same-dose mice per cage) and only monitor their weight. We still introduced the nicotine slowly and the 21 days started counting after they reached the final dose (**Figure 2A**). The reproduction style also changed to two females for each male, as it decreases female's stress and increases pups' survival (Braden et al., 2017).

This nicotine treatment also did not show any effects on their weight, even at the higher dose of 0.2 mg/mL (Interaction effect: $F_{(42,\ 294)} = 1.927$, $p = 0.0010$; Days effect: $F_{(14,\ 294)} = 16.44$, $p < 0.0001$; Treatment effect: $F_{(3,\ 21)} = 0.5405$, $p = 0.6598$; Animal effect: $F_{(21,\ 294)} = 63.93$, $p < 0.0001$, two-way repeated-measures ANOVA; Final Day: 0.1 vs. Ctrl, MedD = 1.000, $U = 7.500$, $p = 0.1667$; 0.15 vs. Ctrl, MD = -0.3143, 95% CI = -1.526 to 0.8976; 0.2 vs. Ctrl, MedD = 0.000, $U = 15$, $p = 0.6667$, Student's *t* test or Mann-Whitney test; **Figure 2B**). Nicotine also did not significantly interfere with the natality (Ctrl: 3.200 ± 1.960; 0.1: 4.000 ± 1.291; 0.15: 2.714 ± 1.358; 0.2: 2.857 ± 1.519; Mean ± SEM. Treatment, Kruskal-Wallis (KW) = 0.2742, p = 0.9648, Kruskal-Wallis test; data not shown).

For the last reproduction using the higher dose only, we repeated the previous protocol to test the replicability of the model (see METHODS for more details; **Figure 2C**). Again, there were no differences in their weight caused by the treatment (0.2 vs. Ctrl, Interaction effect: $F_{(11,\ 352)} = 0.3017$, $p = 0.9853$; Days effect: $F_{(11,\ 352)} = 48.92$, $p < 0.0001$; Treatment effect: $F_{(1,\ 32)} = 1.904$, $p = 0.1772$; Animal effect: $F_{(32,\ 352)} = 40.87$, $p < 0.0001$, two-way repeated-measures ANOVA; Final Day 0.2 vs. Ctrl, MD = -0.4583, 95% CI = -1.218 to 0.3018, $t_{(32)} = 1.228$, $p = 0.2283$, Student's *t* test; **Figure 2D**). Nicotine also did not significantly interfere with the natality (Ctrl: 5.000 ± 0.9545; 0.2

5.917 ± 0.5175; Mean ± SEM. 0.1 vs. Ctrl, MD = 0.500, U = 98.50, p = 0.4160, Mann-Whitney test; data not shown).

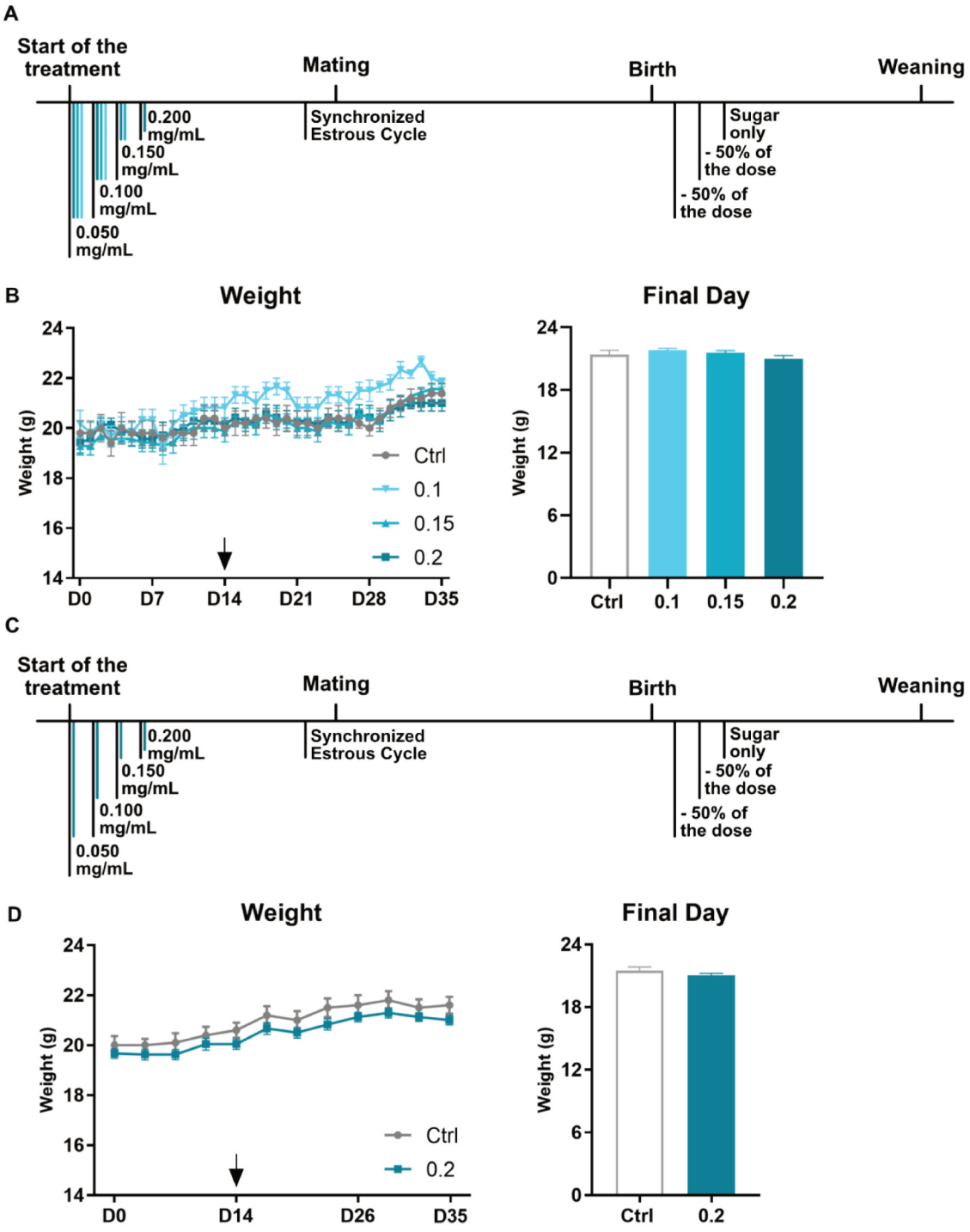

Figure 2. Nicotine solution at 0.1, 0.15 or 0.2 mg/mL does not cause weight loss. **(A)** Experimental design of the treatment with 0.1 mg/mL, 0.15 mg/mL or 0.2 mg/mL nicotine + 2% sugar solutions or just 2% sugar (controls) in their drinking water. The female mice received nicotine gradually, starting at 0.05 mg/mL. Every three days, the dose went higher, until it reached the final dose for each group. On the 14th day, all groups had reached the final dose, and we started counting 21 days from this mark. After 19 days of treatment, we synchronized the estrous cycle using scented male bedding. On the 21st day, we started the mating process. Mouse pregnancy lasts

around 19-21 days and they received the nicotine solution during the whole period. After they gave birth, we removed the nicotine gradually, reducing the dose by 50% every three days, until they were drinking only water. Pups were weaned when around 21-28 days old. **(B)** Mice were weighed every day until they started mating. There was no difference in their weight besides the expected weight gain as they grew up. We considered the final day (graph at the right) as the last day before mating. The arrow marks the 14th day. **(C)** Experimental design of the treatment with 0.2 mg/mL nicotine + 2% sugar solution or just 2% sugar (controls) in their drinking water. The protocol was the same as the previous. **(D)** Mice were weighed every three days until they started mating. There was no difference in their weight besides the expected weight gain as they grew up. We considered the final day (graph at the right) as the last day before mating. The arrow marks the 14th day. Data represented as mean ± S.E.M. (**A and B**, n = 6 0.1 group/ 7 0.15 group/ 7 0.2 group/ 5 Ctrl group. **C and D**, n = 24 0.2 group/ 10 Ctrl group).

3.4.2 Low dose PNE did not induce hyperlocomotion but had small effects on anxiety-like behavior and short-term memory

The main feature of the PNE model is the hyperlocomotion (Paz et al., 2007; Zhu et al., 2012). Other authors reported a significant increase in the distance traveled and speed in male mice from dams treated with 0.1 mg/mL nicotine (Zhu et al., 2012). The model has been tested for anxiety-like behavior and memory, showing mixed results (Paz et al., 2007; Polli et al., 2020). To investigate these behaviors, we used OFT, EPM and ORT, in three different ages to also see if the changes were long-lasting (**Figure 3A**).

We did not observe increase in their distance traveled (6w: MedD = -0.7400, U = 126, p = 0.7098; 9w: MD = -2.562, 95% CI = -6.833 to 1.710, $t_{(34)}$ = 1.219, p = 0.2313; 12w: MD = -1.805, 95% CI = -7.501 to 3.892, $t_{(34)}$ = 0.6438, p = 0.5240, Student's t test or Mann-Whitney test. Interaction effect: $F_{(2, 68)}$ = 0.2181, p = 0.8046; Age effect: $F_{(1.882, 63.98)}$ = 23.22, p < 0.0001; Treatment effect: $F_{(1, 34)}$ = 0.7697, p = 0.3865; Animal effect: $F_{(34, 68)}$ = 3.711, p < 0.0001, two-way repeated-measures ANOVA; **Figure 3B**), mean speed (6w: MedD = -0.002000, U = 125, p = 0.6781; 9w: MD = 0.004127, 95% CI = -0.002975 to 0.01123, $t_{(34)}$ = 1.181, p = 0.2458; 12w: MD = -0.002825, 95% CI = -0.01229 to 0.006643, $t_{(34)}$ = 0.6064, p = 0.5482, Student's t test or Mann-Whitney test. Interaction effect: $F_{(2, 68)}$ = 0.1918, p = 0.8259; Age effect: $F_{(1.879, 63.90)}$ = 23.28, p < 0.0001; Treatment effect: $F_{(1, 34)}$ = 0.7217, p = 0.4015; Animal effect: $F_{(34, 68)}$ = 3.729, p < 0.0001, two-way repeated-measures ANOVA; **Figure 3C**) or max speed (6w: MD = -0.01595, 95% CI = -0.05270 to 0.02081, $t_{(34)}$ = 0.8819, p = 0.3840; 9w: MD = -0.008156, 95% CI = -0.04322 to 0.02691, $t_{(34)}$ = 0.4727, p =

0.6394; 12w: MD = 0.001927, 95% CI = -0.04756 to 0.05142, $t_{(34)}$ = 0.07914, p = 0.9374, Student's t test. Interaction effect: $F_{(2, 68)}$ = 0.2712, p = 0.7633; Age effect: $F_{(1.858, 63.17)}$ = 2.084, p = 0.1362; Treatment effect: $F_{(1, 34)}$ = 0.2622, p = 0.6119; Animal effect: $F_{(34, 68)}$ = 2.111, p = 0.0045, two-way repeated-measures ANOVA; **Figure 3D**) at any age tested.

We also did not observe anxiety-like behavior in either open field (OFT) and elevated plus maze (EPM) tests, in any age tested. For the OFT, anxiety-like behavior can be measured as less time spent in the center of the apparatus (Center time: 6w: MD = 21.06, 95% CI = 4.541 to 37.59, $t_{(28)}$ = 2.611, p = 0.0143; 9w: MD = -2.757, 95% CI = -17.57 to 12.05, $t_{(28)}$ = 0.3814, p = 0.7058; 12w: MD = -7.479, 95% CI = -18.17 to 3.207, $t_{(28)}$ = 1.434, p = 0.1627, Student's t test. Interaction effect: $F_{(2, 68)}$ = 2.398, p = 0.0986; Age effect: $F_{(2, 68)}$ = 9.595, p = 0.0002; Treatment effect: $F_{(1, 34)}$ = 1.558, p = 0.2204; Animal effect: $F_{(34, 68)}$ = 0.8491, p = 0.6948, two-way repeated-measures ANOVA; **Figure 3F**. Periphery time: 6w : MD = -20.48, 95% CI = -36.87 to -4.084, $t_{(28)}$ = 2.559, p = 0.0162; 9w: MedD = -3.500, U = 93, p = 0.6335; 12w: MD = 7.410, 95% CI = -3.315 to 18.13, $t_{(28)}$ = 1.415, p = 0.1680, Student's t test or Mann-Whitney test. Interaction effect: $F_{(2, 68)}$ = 2.265, p = 0.1116; Age effect: $F_{(1.388, 47.18)}$ = 10.60, p = 0.0007; Treatment effect: $F_{(1, 34)}$ = 1.399, p = 0.2452; Animal effect: $F_{(34, 68)}$ = 0.8881, p = 0.6411, two-way repeated-measures ANOVA; **Figure 3E**). We did however observe a decrease in both groups in all parameters analysed, except max speed, as the animals aged. The time spent in the periphery increased, as it is inversely proportional to the time spent in the center.

For the EPM, there was no difference in the distance traveled (6w: MD = -1.337, 95% CI = -5.896 to 3.222, $t_{(29)}$ = 0.5998, p = 0.5533; 9w: MD = -2.554, 95% CI = -6.299 to 1.191, $t_{(34)}$ = 1.386, p = 0.1748; 12w: MD = -0.8692, 95% CI = -5.576 to 3.837, $t_{(34)}$ = 0.3753, p = 0.7098, Student's t test. Interaction effect: $F_{(2, 58)}$ = 0.8545, p = 0.4308; Age effect: $F_{(1.635, 47.40)}$ = 0.07053, p = 0.8993; Treatment effect: $F_{(1, 29)}$ = 0.9595, p = 0.3354; Animal effect: $F_{(29, 58)}$ = 3.234, p < 0.0001, two-way repeated-measures ANOVA; **Figure 3G**) or in the mean speed (6w: MD = -0.004440, 95% CI = -0.01967 to 0.01079, $t_{(29)}$ = 0.5961, p = 0.5558; 9w: MD = -0.008462, 95% CI = -0.02092 to 0.003991, $t_{(34)}$ = 1.381, p = 0.1763; 12w: MD = -0.002971, 95% CI = -0.01865 to 0.01271, $t_{(34)}$ = 0.3850, p = 0.7026, Student's t test. Interaction effect: $F_{(2, 58)}$ = 0.8161, p = 0.4472; Age effect: $F_{(1.637, 47.49)}$ = 0.06606, p = 0.9047; Treatment effect: $F_{(1, 29)}$ = 0.9624, p = 0.3347; Animal effect: $F_{(29, 58)}$ = 3.203, p < 0.0001, two-way

repeated-measures ANOVA; **Figure 3H**). In this test, anxiety-like behavior can be measured analysing the time spent in the open and closed arms of the apparatus. Six weeks old treated mice spent less time in the closed arms than controls, showing less anxiety-like behavior (MD = 12.64, 95% CI = -11.19 to 36.48, $t_{(29)}$ = 1.085, p = 0.2869, Student's t test; **Figure 3I**).

The same results were not observed with 9 and 12 weeks old (9w: MD = -15.83, 95% CI = -40.04 to 8.388, $t_{(34)}$ = 1.328, p = 0.1930; 12w: MD = -13.91, 95% CI = -32.07 to 4.256, $t_{(34)}$ = 1.556, p = 0.1290, Student's t test; **Figure 3I**). There was no differences in the time spent in the closed arms, at any of the ages tested (6w: MD = -23.58, 95% CI = -46.25 to -0.9116, $t_{(29)}$ = 2.127, p = 0.0420; 9w: MedD = 32.00, U = 85, p = 0.0738; 12w: MD = 20.54, 95% CI = -4.340 to 45.42, $t_{(34)}$ = 1.678, p = 0.1026, Student's t test or Mann-Whitney test; **Figure 3I**). We also observed in the EPM the same effect seen in the OFT, as the animals aged they explored less, spending more time in the periphery than in the center (Center time: Interaction effect: $F_{(2, 58)}$ = 4.484, p = 0.0155; Age effect: $F_{(1.871, 54.26)}$ = 40.71, p < 0.0001; Treatment effect: $F_{(1, 29)}$ = 0.9375, p = 0.3409; Animal effect: $F_{(29, 58)}$ = 3.229, p < 0.0001, two-way repeated-measures ANOVA; Periphery time: Interaction effect: $F_{(2, 58)}$ = 8.289, p = 0.0007; Age effect: $F_{(1.809, 52.46)}$ = 42.10, p < 0.0001; Treatment effect: $F_{(1, 29)}$ = 1.104, p = 0.3021; Animal effect: $F_{(29, 58)}$ = 3.385, p < 0.0001, two-way repeated-measures ANOVA; **Figure 3I**).

As for the object recognition test (ORT), both groups showed no preference for the identical objects (Ctrl B vs. A, MD = -0.1364, 95% CI = -5.531 to 5.258, $t_{(10)}$ = 0.05633, p = 0.9562; 0.1 B vs. A, MD = 1.548, 95% CI = -1.078 to 4.174, $t_{(22)}$ = 1.222, p = 0.2345, Student's t test. Interaction effect: $F_{(1, 34)}$ = 0.4609, p = 0.5018; Objects effect: $F_{(1, 34)}$ = 0.3288, p = 0.5701; Treatment effect: $F_{(1, 34)}$ = 0.2908, p = 0.5932; Animal effect: $F_{(34, 34)}$ = 3.238, p = 0.0005, two-way repeated-measures ANOVA; **Figure 3J**). After 2h, treated mice failed at recognizing the familiar object and explored it more than the novel one (Novel vs. Old, Median Difference (MedD) = 2.500, W = 139.0, p = 0.0465, Wilcoxon test; **Figure 3K**). However, control mice also did not discriminate between objects after 2h, and explored both equally (Novel vs. Old , MD = -3.467, 95% CI = -7.517 to 0.5835, $t_{(8)}$ = 1.974, p = 0.0838, Student's t test. Interaction effect: $F_{(1, 34)}$ = 0.2202, p = 0.6419; Novelty effect: $F_{(1, 34)}$ = 1.916, p = 0.1753; Treatment effect: $F_{(1, 34)}$ = 0.08740, p = 0.7693; Animal effect: $F_{(34, 34)}$ = 0.7812, p = 0.7623, two-way repeated-measures ANOVA; **Figure 3K**). The

discrimination index (DI) was statisticly the same between the groups, with both failing the task (Training 0.1 vs. Ctrl, MD = -0.02113, 95% CI = -0.1108 to 0.06851, $t_{(34)}$ = 0.4789, p = 0.6350; After 2h 0.1 vs. Ctrl, MedD = -0.1182, U = 94, p = 0.1856, Student's t test or Mann-Whitney test. Interaction effect: $F_{(1, 34)}$ = 0.3017, p = 0.5864; Trial Stage effect: $F_{(1, 34)}$ = 0.2522, p = 0.6188; Treatment effect: $F_{(1, 34)}$ = 1.830, p = 0.1850; Animal effect: $F_{(34, 34)}$ = 0.5418, p = 0.9608, two-way repeated-measures ANOVA; **Figure 3L**).

3.4.3 Lower and higher PNE doses, but not the intermediate, induced hyperlocomotion

After analyzing the previous results, we hypothesized that this dose might not be ideal, as we could not observe the expected results. But it could also be because the conditions were not ideal. So we decided to test both hypotheses by repeating the dose and also using two higher doses (0.1, 0.15 and 0.2 mg/mL) and improving the conditions during testing, especially regarding the circadian cycle. We kept experimental mice in an inverted light/dark cycle and did all the following tests during their active phase. We repeated the previous behavioral tests with the improvements (OFT, EPM and ORT) and added the Y-maze (YM), the Sociability Test (ST) and the Social Novelty Test (SNT) (**Figure 4A**).

A major improvement was the use of a longer OFT protocol, as their hyperlocomotion is spontaneous and might not be detected with the previous 10-minutes protocol (Zhu et al., 2012). Now, with a 5-hours long OFT during their active phase, the 0.1 mg/mL group showed longer distance traveled and higher mean speed, but only until the first 2 hours (**Table 1.1** and **Table 2.1**; Distance traveled: Ctrl vs. 0.1, MD = -93.41, 95% CI = -213.3 to 26.43, $t_{(15)}$ = 1.661, p = 0.1174, Student's t test. Mean Speed: Ctrl vs. 0.1, MD = -0.005167, 95% CI = -0.01197 to 0.001641, $t_{(15)}$ = 1.618, p = 0.1265, Student's t test; **Figure 4B** and **4C** respectively, graphs at the right). The higher dose-group, 0.2 mg/mL, also showed the same results up until 2 hours (**Table 1.3** and **Table 2.3**). Still, the difference maintained significant even considering the total time, while the previous group failed to do the same (Distance traveled: Ctrl vs. 0.2, MD = -142.0, 95% CI = -271.2 to -12.88, $t_{(14)}$ = 2.359, p = 0.0334, Student's t test. Mean Speed: Ctrl vs. 0.2, MD = -0.007750, 95%

CI = -0.01485 to -0.0006510, $t_{(14)}$ = 2.341, p = 0.0345, Student's t test; **Figure 4B** and **4C** respectively, graphs at the right).

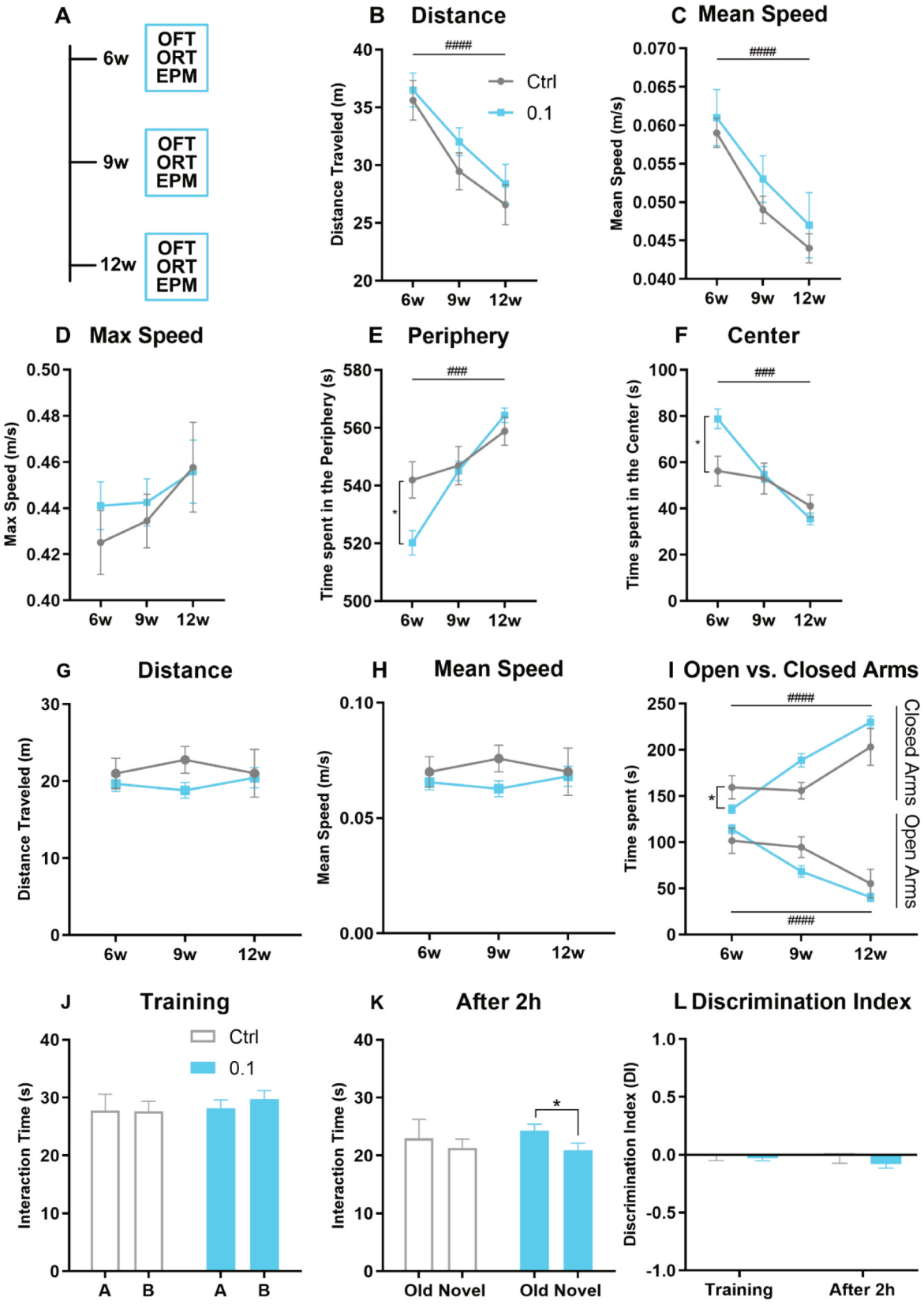

Figure 3. Low dose PNE did not induce hyperlocomotion but had small effects on anxiety-like behavior and short-term memory. **(A)** Experimental design showing behavioral assessments performed on 0.1 mg/mL group. **(B, C, D, E and F)** Evaluation of motor and anxiety-like parameters on the open field test (OFT): distance traveled **(B)**, mean speed **(C)**, max speed **(D)**, time spent in the periphery zone **(E)** and in the center zone **(F)**. **(G, H and I)** Evaluation of motor and anxiety-like parameters on the elevated plus maze (EPM): distance traveled **(G)**, mean speed **(H)** and time spent in the open vs. closed arms **(I)**. **(J, K and L)** Evaluation of short-term memory on the object recognition test (ORT): time interacting with the objects during training **(J)**, time interacting with the objects during the test **(K)**, discrimination index of both groups in the training and test **(L)**. Data represented as mean ± S.E.M. (OFT and ORT: n = 25 0.1 group/ 11 ctrl group; EPM: n = 25 0.1 group/ 6 ctrl group). *P < 0.05 (0.1 vs. Ctrl). #### P < 0.0001, ### P < 0.001 (two-way repeated-measures ANOVA).

The intermediate dose-group, 0.15 mg/mL PNE, did not show an increase in both parameters, either considering the total time (Distance traveled: Ctrl vs. 0.15, MD = -73.89, 95% CI = -162.9 to 15.15, $t_{(17)}$ = 1.751, p = 0.0980, Student's t test. Mean Speed: Ctrl vs. 0.15, MD = -0.004045, 95% CI = -0.009093 to 0.001002, $t_{(17)}$ = 1.691, p = 0.1091, Student's t test; **Figure 4B** and **4C** respectively, graphs at the right) or only until 2 hours (**Table 1.2** and **Table 2.2**). All groups decreased the activity across time (Distance traveled: Interaction effect: $F_{(27, 288)}$ = 1.839, p = 0.0082; Time effect: $F_{(2.212, 70.77)}$ = 130.4, p < 0.0001; Treatment effect: $F_{(3, 32)}$ = 2.189, p = 0.1085; Animal effect: $F_{(32, 288)}$ = 9.518, p < 0.0001, two-way repeated-measures ANOVA. Mean Speed: Interaction effect: $F_{(27, 288)}$ = 1.842, p = 0.0080; Time effect: $F_{(2.227, 71.28)}$ = 129.4, p < 0.0001; Treatment effect: $F_{(3, 32)}$ = 2.175, p = 0.1102; Animal effect: $F_{(32, 288)}$ = 9.501, p < 0.0001, two-way repeated-measures ANOVA; **Figure 4B** and **4C** respectively).

There was no difference in the max speed between groups, only the general decrease in the activity across time (Interaction effect: $F_{(27, 288)}$ = 1.920, p = 0.0049; Time effect: $F_{(4.230, 135.4)}$ = 7.547, p < 0.0001; Treatment effect: $F_{(3, 32)}$ = 0.03979, p = 0.9892; Animal effect: $F_{(32, 288)}$ = 3.234, p < 0.0001, two-way repeated-measures ANOVA; **Figure 4D**. Total accumulated time: Ctrl vs. 0.1, MD = -0.01097, 95% CI = -0.3045 to 0.2825, $t_{(15)}$ = 0.07968, p = 0.9375; Ctrl vs. 0.15, MD = 0.2224, 95% CI = 0.05356 to 0.3911, $t_{(16)}$ = 2.793, p = 0.0130; Ctrl vs. 0.2, MD = 0.2381, 95% CI = 0.03625 to 0.4400, $t_{(13)}$ = 2.548, p = 0.0243, Student's t test; **Figure 4D**, graph at the right. Analysis across time; **Table 3.1-3.3**). There were no differences in the time spent in the periphery (Ctrl vs. 0.1, MD = -369.1, 95% CI = -1683 to 945.1, $t_{(15)}$ = 0.5986, p = 0.5583; Ctrl vs. 0.15, MD = 526.1, 95% CI = -1310 to 2362, $t_{(17)}$ = 0.6046, p = 0.5534; Ctrl vs. 0.2, MD = 1459, 95% CI = -287.9 to 3206, $t_{(14)}$ = 1.791, p =

0.0949, Student's *t* test. Treatment effect: $F_{(3,\ 32)}$ = 1.844, *p* = 0.1591, ordinary one-way ANOVA; **Figure 4E**) or in the center of the apparatus (Ctrl vs. 0.1, MD = 370.1, 95% CI = -943.0 to 1683, $t_{(15)}$ = 0.6007, *p* = 0.5570; Ctrl vs. 0.15, MD = -524.9, 95% CI = -2361 to 1311, $t_{(17)}$ = 0.6031, *p* = 0.5544; Ctrl vs. 0.2, MD = -1459, 95% CI = -3206 to 287.2, $t_{(14)}$ = 1.792, *p* = 0.0948, Student's *t* test. Treatment effect: $F_{(3,\ 32)}$ = 1.846, *p* = 0.1588, ordinary one-way ANOVA; **Figure 4E**), contradicting the previous findings, in which 0.1 mg/mL group showed increased time spent in the center (**Figure 3F**).

Even though it is not the main purpose of the EPM, 0.2 mg/mL PNE mice showed an increased distance traveled (MD = 3.677, 95% CI = 0.01180 to 7.343, $t_{(15)}$ = 2.138, *p* = 0.0494, Student's *t* test; **Figure 4F**) and mean speed (MD = 0.01232, 95% CI = 3.256e-005 to 0.02461, $t_{(15)}$ = 2.137, *p* = 0.0495, Student's *t* test; **Figure 4G**). Both other groups failed to show the same results (Distance: 0.1 vs. Ctrl, MD = 1.660, 95% CI = -1.466 to 4.786, $t_{(16)}$ = 0.2769, *p* = 0.2769; 0.15 vs. Ctrl, MD = 1.718, 95% CI = -2.041 to 5.477, $t_{(19)}$ = 0.9565, *p* = 0.3508, Student's *t* test. Treatment effect: $F_{(3,\ 34)}$ = 1.563, *p* = 0.2162, ordinary one-way ANOVA; **Figure 4F**. Mean Speed: 0.1 vs. Ctrl, MD = 0.005556, 95% CI = -0.004911 to 0.01602, $t_{(16)}$ = 1.125, *p* = 0.2771; 0.15 vs. Ctrl, MD = 0.005611, 95% CI = -0.007029 to 0.01825, $t_{(19)}$ = 0.3645, *p* = 0.3645, Student's *t* test. Treatment effect: $F_{(3,\ 34)}$ = 1.558, *p* = 0.2174, ordinary one-way ANOVA; **Figure 4G**).

No group showed anxiety-like behavior in this test (Open Arms: 0.1 vs. Ctrl, MD = -2.562, 95% CI = -11.65 to 26.32, $t_{(16)}$ = 0.8188, *p* = 0.4249; 0.15 vs. Ctrl, MD = 3.097, 95% CI = -15,44 to 21,63, $t_{(19)}$ = 0.3497, *p* = 0.7304; 0.2 vs. Ctrl, MD = -11.92, 95% CI = -35.50 to 11.66, $t_{(15)}$ = 1.078, *p* = 0.2983, Student's *t* test. Treatment effect: $F_{(3,\ 34)}$ = 1.626, *p* = 0.2015, ordinary one-way ANOVA. Closed Arms: 0.1 vs. Ctrl, MD = -2.078, 95% CI = -22.00 to 17.85, $t_{(16)}$ = 0.2211, *p* = 0.8278; 0.15 vs. Ctrl, MD = 2.739, 95% CI = -13.35 to 18.83, $t_{(19)}$ = 0.3563, *p* = 0.7255; 0.2 vs. Ctrl, MD = -14.71, 95% CI = -39.14 to 9.710, $t_{(15)}$ = 1.284, *p* = 0.2186, Student's *t* test. Treatment effect: $F_{(3,\ 34)}$ = 1.078, *p* = 0.3715, ordinary one-way ANOVA; **Figure 4H**). In the previous test, the 0.1 mg/mL group spent significantly less time in the closed arms than controls (**Figure 3I)**, but the group did not replicate the results. There were no differences between the treated groups (Tukey's multiple comparisons tests, data not shown).

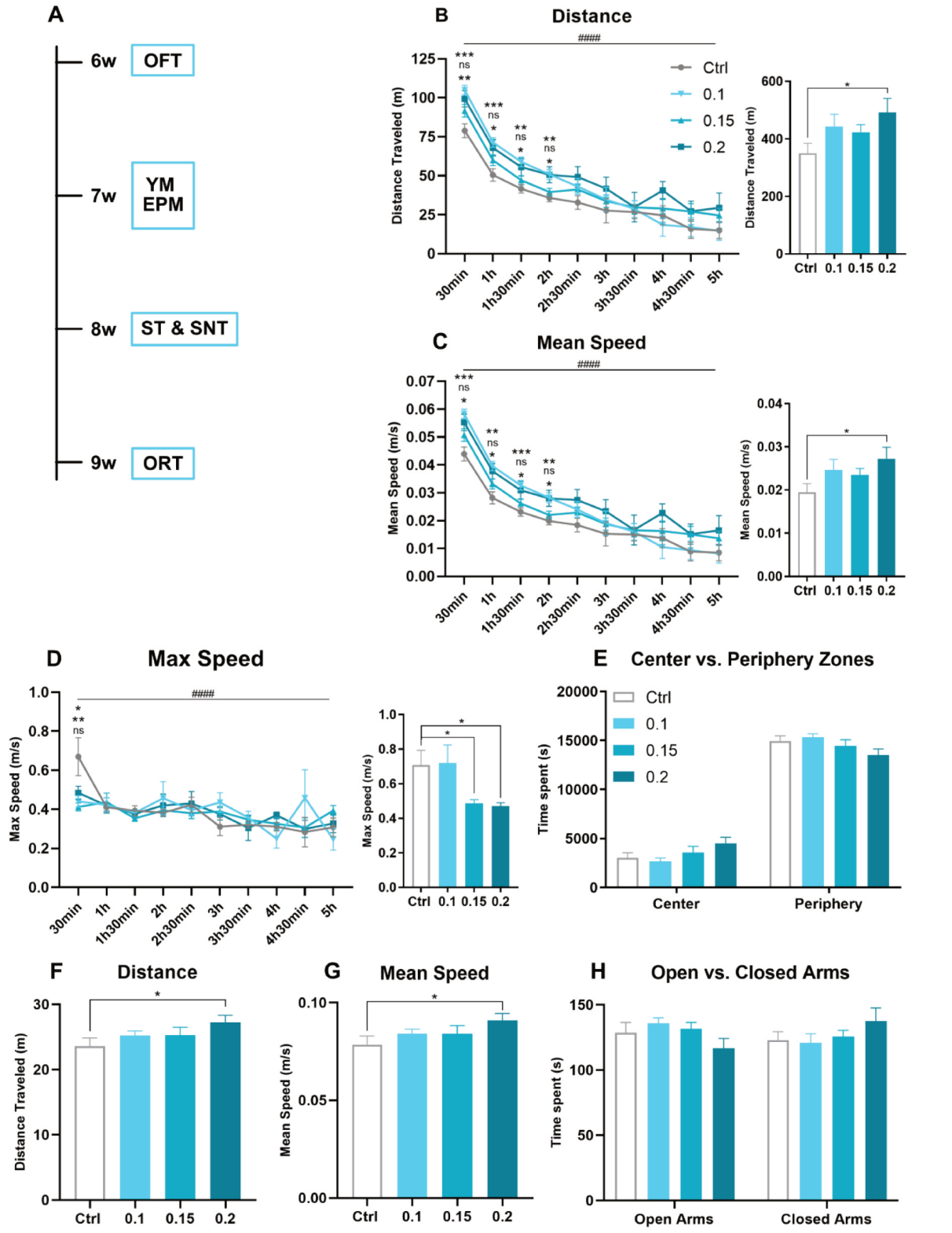

Figure 4. Lower and higher PNE doses, but not the intermediate, induced hyperlocomotion. **(A)** Experimental design showing behavioral assessments performed. **(B, C, D and E)** Evaluation of motor and anxiety-like parameters on the open field test (OFT): distance traveled **(B)**, mean speed **(C)**, max speed **(D)**, time spent in the center vs. periphery zones. P value representatives (asterisks or ns) are, from top to bottom: Ctrl vs. 0.1, Ctrl vs. 0.15 and Ctrl vs. 0.2. **(F, G and H)** Evaluation of motor and anxiety-like parameters on the elevated plus maze (EPM): distance traveled **(F)**, mean speed **(G)** and time spent in the open vs. closed arms **(H)**. Data represented

as mean ± S.E.M. (OFT: n = 9 0.1 group/ 11 0.15 group/ 8 0.2 group/ 8 Ctrl group; EPM: n = 9 0.1 group/ 12 0.15 group/ 8 0.2 group/ 9 Ctrl group). ***P < 0.001, **P < 0.01, *P < 0.05 (single variable comparison). #### P < 0.0001 (two-way repeated-measures ANOVA).

3.4.4 PNE treatment increased general activity but had a small effect on long-term memory

In the previous ORT, only short-term memory was tested. This time, we tested their long-term memory after 24h. Again, all groups showed no preference for the identical objects (Ctrl A vs. B, MD = -7.325, 95% CI = -23.59 to 8.938, $t_{(7)}$ = 1.065, p = 0.3222; 0.1 A vs. B, MD = -5.978, 95% CI = -17.21 to 5.250, $t_{(8)}$ = 1.228, p = 0.2544; 0.15 A vs. B, MD = 3.955, 95% CI = -3.967 to 11.88, $t_{(10)}$ = 1.112, p = 0.2920; 0.2 A vs. B, MedD = -0.3000, W = -8.000, p = 0.6406, Student's t test or Wilcoxon test; **Figure 5A**). We observed that all treated groups interacted less with the objects than the controls (Interaction time with object A, KW = 8.658, p = 0.0342; Interaction time with object B, KW = 6.201, p = 0.1022, Kruskal-Wallis test; **Figure 5A**), specially the 0.2 mg/mL group interaction with the object A (Interaction effect: $F_{(3, 32)}$ = 1.305, p = 0.2899; Objects effect: $F_{(1, 32)}$ = 1.620, p = 0.2122; Treatment effect: $F_{(3, 32)}$ = 3.809, p = 0.0193; Animal effect: $F_{(32, 32)}$ = 3.884, p < 0.0001, two-way repeated-measures ANOVA; Ctrl A vs. 0.2 A, MrD = 8.597, p = 0.0342, Dunn's Multiple comparisons test; **Figure 5A**).

After 24 hours, the only group to succeed in the task was 0.1 mg/mL (Old vs. Novel, MD = 24.73, 95% CI = 5.493 to 43.97, $t_{(8)}$ = 2.964, p = 0.0180, Student's t test. Interaction effect: $F_{(3, 32)}$ = 1.169, p = 0.3368; Objects effect: $F_{(1, 32)}$ = 12.64, p = 0.0012; Treatment effect: $F_{(3, 32)}$ = 1.371, p = 0.2692; Animal effect: $F_{(32, 32)}$ = 0.8883, p = 0.6302, two-way repeated-measures ANOVA; **Figure 5B**). Even though it was not statistically significant, we can see a success trend with the control group (Ctrl Old vs. Novel, MD = 19.71, 95% CI = -6.978 to 46.40, $t_{(7)}$ = 1.746, p = 0.1242, Student's t test; **Figure 5B**), and a smaller one with the 0.15 mg/mL group (Old vs. Novel, MedD = 15.70, W = 44.00, p = 0.0537, Wilcoxon test; **Figure 5B**). As for the 0.2 mg/mL group, mice spent the same time exploring both objects, failing the task (Old vs. Novel, MD = 3.088, 95% CI = -11.96 to 18.14, $t_{(7)}$ = 0.4851, p = 0.6424, Student's t; **Figure 5B**).

There was no difference in the interaction time between the objects (Interaction time with Old object, *KW* = 0.8658, *p* = 0.8337; Interaction time with Novel object, $F_{(3, 32)}$ = 1.926, *p* = 0.1452, ordinary one-way ANOVA or Kruskal-Wallis test, data not shown). The same trend can be seen with the DI (Training: 0.1 vs. Ctrl, MD = 0.004410, 95% CI = -0.1283 to 0.1372, $t_{(15)}$ = 0.07081, *p* = 0.9445; 0.15 vs. Ctrl, MD = -0.07770, 95% CI = -0.1815 to 0.02612, $t_{(17)}$ = 1.579, *p* = 0.1328; 0.2 vs. Ctrl, MD = -0.01703, 95% CI = -0.1304 to 0.09632, $t_{(14)}$ = 0.3222, *p* = 0.7520, Student's *t* test. After 24h: 0.1 vs. Ctrl, MD = 0.7003, 95% CI = -0.1769 to 0.2567, $t_{(15)}$ = 0.3924, *p* = 0.7003; 0.15 vs. Ctrl, MD = -0.01837, 95% CI = -0.2190 to 0.1823, $t_{(17)}$ = 0.1931, *p* = 0.8492; 0.2 vs. Ctrl, MD = -0.09490, 95% CI = -0.3238 to 0.1340, $t_{(14)}$ = 0.8893, *p* = 0.3888, Student's *t* test. Interaction effect: $F_{(3, 32)}$ = 0.8038, *p* = 0.5010; Trial Stage effect: $F_{(1, 32)}$ = 7.620, *p* = 0.0095; Treatment effect: $F_{(3, 32)}$ = 0.8962, *p* = 0.4538; Animal effect: $F_{(32, 32)}$ = 1.436, *p* = 0.1556, two-way repeated-measures ANOVA; **Figure 5C**). There were no differences between the treated groups (Sidak's multiple comparisons tests, data not shown).

Secondly, we tested the effect of PNE on the working memory using the YM. We observed that both 0.15 and 0.2 mg/mL groups showed increased distance traveled (0.15 vs. Ctrl, MD = 4.881, 95% CI = 2.167 to 7.595, $t_{(22)}$ = 3.730, *p* = 0.0012; 0.2 vs. Ctrl, MD = 6.254, 95% CI = 3.254 to 9.006, $t_{(21)}$ = 4.432, *p* = 0.0002, Student's *t* test. Treatment effect: $F_{(3, 40)}$ = 9.577, *p* < 0.0001, ordinary one-way ANOVA; **Figure 5D**), mean speed (0.15 vs. Ctrl, MD = 0.01617, 95% CI = 0.007071 to 0.02526, $t_{(22)}$ = 3.686, *p* = 0.0013; 0.2 vs. Ctrl, MD = 0.009581, 95% CI = 0.009581 to 0.03017, $t_{(18)}$ = 4.056, *p* = 0.0007, Student's *t* test. Treatment effect: $F_{(3, 37)}$ = 8.736, *p* = 0.0002, ordinary one-way ANOVA; **Figure 5E**) and number of entries in the arms of the apparatus (0.15 vs. Ctrl, MD = 8.667, 95% CI = 2.897 to 14.44, $t_{(22)}$ = 3.115, *p* = 0.0050; 0.2 vs. Ctrl, MD = 12.33, 95% CI = 6,019 to 18,65, $t_{(18)}$ = 4.104, *p* = 0.0007, Student's *t* test. Treatment effect: $F_{(3, 37)}$ = 6.489, *p* = 0.0012, ordinary one-way ANOVA; **Figure 5F**), showing an increase in the general activity.

The 0.1 mg/mL did not differ from controls in any of the parameters mentioned (Distance: 0.1 vs. Ctrl, MD = 1.808, 95% CI = -1.331 to 4.948, $t_{(19)}$ = 1.206, *p* = 0.2428; Mean Speed: 0.1 vs. Ctrl, MD = 0.005917, 95% CI = -0.004675 to 0.01651, $t_{(19)}$ = 1.169, *p* = 0.2568; Total Number of Entries: 0.1 vs. Ctrl, MD = 3.583, 95% CI = -3.550 to 10.72, $t_{(19)}$ = 1.051, *p* = 0.3062, Student's *t* test; **Figure 5D-F**, respectively).

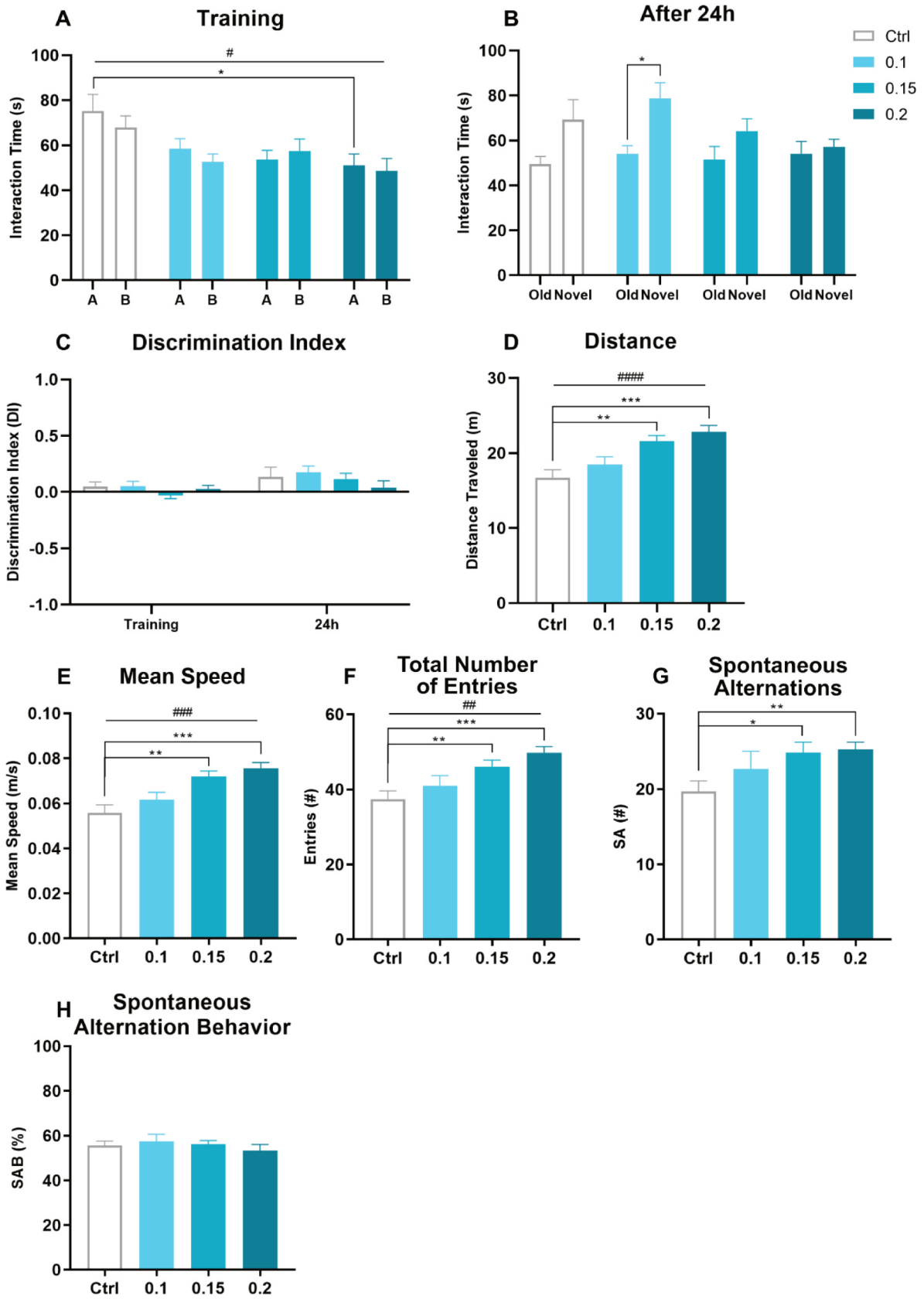

Figure 5. PNE treatment increased general activity, but had a small effect on long-term memory. **(A, B and C)** Evaluation of short-term memory on the object recognition test (ORT): time interacting with the objects during training **(A)**, time interacting with the objects during the test **(B)**, discrimination index of both groups in the training and test **(C)**. **(D, E, F, G and H)** Evaluation of motor and working memory parameters on the y-maze (YM):

distance traveled **(D)**, mean speed **(E)**, total number of entries in the arms **(F)**, number of spontaneous alternations **(G)** and percentage of spontaneous alternation behavior **(H)**. Data represented as mean ± S.E.M. (ORT: n = 9 0.1 group/ 11 0.15 group/ 8 0.2 group/ 8 Ctrl group; YM: n = 9 0.1 group/ 12 0.15 group/ 11 0.2 group/ 12 Ctrl group). ***P < 0.001, **P < 0.01, *P < 0.05 (single variable comparison). #### P < 0.0001, ### P < 0.001, ## P < 0.01, #P < 0.05 (two-way repeated-measures ANOVA).

Both 0.15 and 0.2 mg/mL groups also showed a higher number of spontaneous alternations (0.15 vs. Ctrl, MD = 5.167, 95% CI = 1.121 to 9.212, $t_{(22)}$ = 2.649, p = 0.0147; 0.2 vs. Ctrl, MD = 5.583, 95% CI = 1.616 to 9.551, $t_{(18)}$ = 2.956, p = 0.0084, Student's t test. Treatment effect: $F_{(3,\ 37)}$ = 2.803, p = 0.0532, ordinary one-way ANOVA; **Figure 5G**), but since their number of entries was also increased, the percentage of spontaneous alternations behavior (%SAB) did not differ from controls (0.15 vs. Ctrl, MD = -0.5808, 95% CI = -5.925 to 4.764, $t_{(22)}$ = 0.2254, p = 0.8238; 0.2 vs. Ctrl, MD = 2.226, 95% CI = -4.715 to 9.166, $t_{(18)}$ = 0.6738, p = 0.5090, Student's t test. Treatment effect: $F_{(3,\ 37)}$ = 0.4717, p = 0.7038, ordinary one-way ANOVA; **Figure 5H**). Again, the 0.1 mg/mL did not differ from controls (Number of Spontaneous Alternations: 0.1 vs. Ctrl, MD = 3.000, 95% CI = -2.393 to 8.393, $t_{(19)}$ = 1.164, p = 0.2587; %SAB: 0.1 vs. Ctrl, MD = -1.932, 95% CI = -9.368 to 5.504, $t_{(19)}$ = 0.5438, p = 0.5929, Student's t test; **Figure 5G** and **5H**, respectively). There were no differences between the treated groups (Tukey's multiple comparisons tests, data not shown).

3.4.5 Only 0.2 mg/mL group had impaired sociability but not social novelty preference

We also tested the effect of PNE on mice sociability and social novelty preference with the three-chamber sociability and social novelty test (3-CSSNT). The test consists of two parts. The first part evaluates the mice's sociability, as they are placed in the apparatus with an unknown non-aggressive mouse (Stranger 1; S1) on one side and an empty cage (E) on the other side. The second part evaluates the mice's social novelty preference, as they are placed in the apparatus with the same mouse from before (S1) on one side and an unknown non-aggressive mouse (Stranger 2; S2) on the other side (**Figure 6A**).

Generally, typical mice spend more time interacting with S1 than exploring the E, as seen with controls (S1 vs. E, MD = 56.76, 95% CI = 18.07 to 95.44, $t_{(8)}$ = 3.383,

p = 0.0096, Student's t test; **Figure 6B**). The 0.15 mg/mL group also preferred to interact with the S1 (S1 vs. E, MD = 34.60, 95% CI = 4.574 to 64.63, $t_{(7)}$ = 2.725, p = 0.0296, Student's t test; **Figure 6B**). The 0.1 mg/mL group did not have statistically significant preference for the S1, but they still spent more time interacting with them (S1 vs. E, MedD = 44.35, W = 18.0, p = 0.2500, Wilcoxon test; **Figure 6B**).

However, the 0.2 mg/mL group explored both cages equally (S1 vs. E, MD = -1.400, 95% CI = -38.59 to 35.79, $t_{(7)}$ = 0.08901, p = 0.9316, Student's t test. Interaction effect: $F_{(3,\ 29)}$ = 1.924, p = 0.1478; Sociability effect: $F_{(1,\ 29)}$ = 11.62, p = 0.0019; Treatment effect: $F_{(3,\ 29)}$ = 0.8379, p = 0.4841; Animal effect: $F_{(29,\ 29)}$ = 0.2033, p > 0.9999, two-way repeated-measures ANOVA; **Figure 6B**). This group also spent significantly less time exploring the S1 chamber than controls (S1 Ctrl vs. 0.2, Predicted (LS) MD = 36.57, 95% CI = 0.1953 to 72.95, $t_{(58)}$ = 2.738, p = 0.0481, Sidak's Multiple comparisons test; **Figure 6B**). All groups entered more in the empty chamber, with only the 0.15 mg/mL group being statistically significant (Ctrl S1 vs. E, MedD = -5.000, W = -27.00, p = 0.1211; 0.1 S1 vs. E, MD = -2.250, 95% CI = -10.80 to 6.296, $t_{(7)}$ = 0.6225, p = 0.5533; 0.15 S1 vs. E, MD = -7.375, 95% CI = -14.05 to -0.7027, $t_{(7)}$ = 2.614, p = 0.0347; 0.2 S1 vs. E, MD = -8.375, 95% CI = -17.18 to 0.4266, $t_{(7)}$ = 2.250, p = 0.0592, Student's t test or Wilcoxon's test. Interaction effect: $F_{(3,\ 29)}$ = 0.6340, p = 0.5991; Sociability effect: $F_{(1,\ 29)}$ = 12.47, p = 0.0014; Treatment effect: $F_{(3,\ 29)}$ = 0.8271, p = 0.4898; Animal effect: $F_{(29,\ 29)}$ = 0.6978, p = 0.8309, two-way repeated-measures ANOVA; **Figure 6C**).

As for social novelty preference, typical mice spend more time interacting with S2 than with the familiar S1, as seen with controls (S2 vs. S1, MD = 107.7, 95% CI = 60.93 to 154.5, $t_{(8)}$ = 5.310, p = 0.0007, Student's t test; **Figure 6D**). All groups demonstrated preference for the S2, but as the nicotine dose was higher, less significant was the difference (0.1 S2 vs. S1, MD = 104.8, 95% CI = 65.89 to 143.6, $t_{(7)}$ = 6.374, p = 0.0004; 0.15 S2 vs. S1, MD = 49.41, 95% CI = 18.67 to 80.16, $t_{(7)}$ = 3.800, p = 0.0067; 0.2 S2 vs. S1, MD = 49.56, 95% CI = 8.422 to 90.70, $t_{(7)}$ = 2.849, p = 0.0247, Student's t test. Interaction effect: $F_{(3,\ 29)}$ = 3.621, p = 0.0247; Social Novelty effect: $F_{(1,\ 29)}$ = 81.30, p < 0.0001; Treatment effect: $F_{(3,\ 29)}$ = 0.8565, p = 0.4747; Animal effect: $F_{(29,\ 29)}$ = 0.6634, p = 0.8626, two-way repeated-measures ANOVA; **Figure 6D**).

The 0.15 mg/mL group also interacted more with the S1 than controls (S1 0.15 vs. Ctrl, MD = 27.44, 95% CI = 9.337 to 45.54, $t_{(15)}$ = 3.231, p = 0.0056, Student's t

test; **Figure 6D**). All groups entered more in the S2 chamber than in the S1, but were only statistically significant for control and 0.1 mg/mL groups (Ctrl S2 vs. S1, MD = 8.000, 95% CI = 2.887 to 13.11, $t_{(8)}$ = 3.608, p = 0.0069; 0.1 S2 vs. S1, MD = 10.25, 95% CI = 3.778 to 16.72, $t_{(7)}$ = 3.745, p = 0.0072; 0.15 S2 vs. S1, MD = 4.250, 95% CI = -1.538 to 10.04, $t_{(7)}$ = 1.736, p = 0.1261; 0.2 S2 vs. S1, MD = 6.625, 95% CI = -0.4398 to 13.69, $t_{(7)}$ = 2.217, p = 0.0621, Student's t test. Interaction effect: $F_{(3, 29)}$ = 0.9138, p = 0.4464; Social Novelty effect: $F_{(1, 29)}$ = 31.45, p < 0.0001; Treatment effect: $F_{(3, 29)}$ = 1.186, p = 0.3321; Animal effect: $F_{(29, 29)}$ = 0.7720, p = 0.7548, two-way repeated-measures ANOVA; **Figure 6E**).

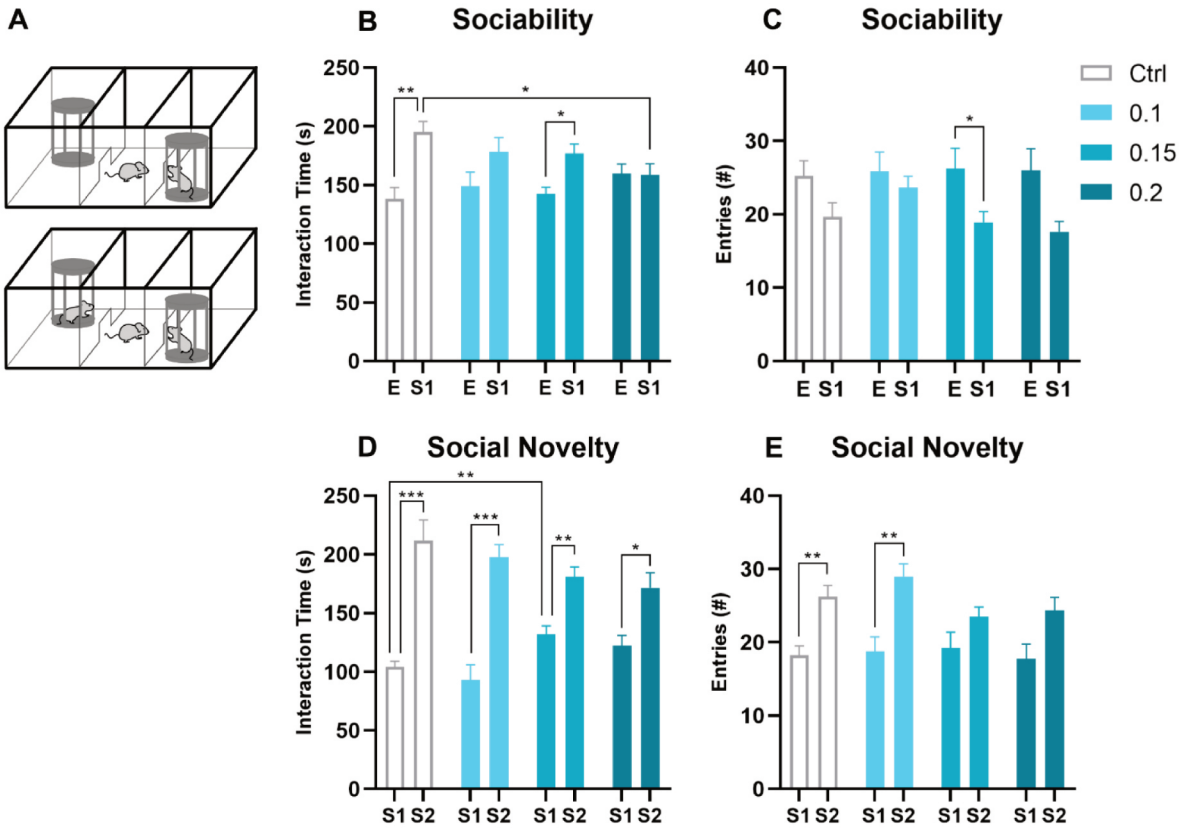

Figure 6. Only 0.2 mg/mL group had impaired sociability, but not social novelty preference. **(A)** Illustration representing the three-chamber sociability and social novelty test (3-CSSNT). **(B and C)** Evaluation of mice's sociability considering the time interacting with the S1 vs. E **(B)** and the number of entries made in the closer zone to the S1 and E cages **(C)**. **(D and E)** Evaluation of mice's sociability considering the time interacting with the S2 vs. S1 **(D)** and the number of entries made in the closer zone to the S2 and S1 cages **(E)**. Data represented as mean ± S.E.M. (n = 8 0.1 group/ 8 0.15 group/ 8 0.2 group/ 9 Ctrl group). ***P < 0.001, **P < 0.01, *P < 0.05 (single variable comparison).

3.4.6 The 0.2 mg/ml group reliably presents hyperlocomotion at a young age

Considering the previous results, we decided to do the last two rounds of behavioral testing with only the higher dose, the 0.2 mg/mL. The previous PNE protocol brought satisfying results, so we repeated it. With this reproduction, we divided all pups (treated and controls) into two groups (**Figure 7A**). With the first one, we repeated the OFT, as it is the confirmatory test of the hyperlocomotion, and proposed a last assessment of memory and learning in this model, the Barnes maze (**Figure 7A**). With the second one, we also performed the OFT and tested impulsivity, anxiety and depressive-like behaviors (**Figure 7A**). After analyzing the previous OFT results, we noticed that it was not necessary to test for 5 hours, as they only showed differences until the first 2h. We also assessed the hyperlocomotion with mice at a younger age, 6-7 weeks old, and at an older age, with 8-9 weeks old (**Figure 7A**), but as the median of the ages was 6 and 9 weeks respectively, we address the groups in this shorter manner (6w and 9w, respectively).

We observed that the treated group with 6 weeks old presented an increase in the distance traveled (Interaction effect: $F_{(4, 164)} = 3.231$, $p = 0.0139$; Age effect: $F_{(1.133, 46.47)} = 689.6$, $p < 0.0001$; Treatment effect: $F_{(1, 41)} = 4.206$, $p = 0.0467$; Animals effect: $F_{(41, 164)} = 10.40$, $p < 0.0001$, two-way repeated-measures ANOVA; **Figure 7B**) and mean speed (Interaction effect: F(4, 164) = 0.4021, p = 0.8069; Age effect: F(1.500, 61.48) = 431.2, p < 0.0001; Treatment effect: F(1, 41) = 4.026, p = 0.0514; Animals effect: F(41, 164) = 26.82, p < 0.0001, two-way repeated-measures ANOVA; **Figure 7E**) when compared to their controls. The difference is especially from 30 minutes to 1 hour and 30 minutes in both distance traveled and mean speed (**Supplementary Table 4** and **Supplementary Table 5**, respectively).

However, with mice at 9 weeks old, there were no differences in the distance traveled, except at 1 hour and 30 minutes (Interaction effect: $F_{(4, 164)} = 9.055$, $p < 0.0001$; Age effect: $F_{(1.676, 68.72)} = 726.8$, $p < 0.0001$; Treatment effect: $F_{(1, 41)} = 2.567$, $p = 0.1168$; Animals effect: $F_{(41, 164)} = 10.74$, $p < 0.0001$, two-way repeated-measures ANOVA. 1h30 min: Ctrl vs. 0.2, MD = -36.46, 95% CI = -62.72 to -10.20, $t_{(33.18)} = 3.782$, p = 0.0031, Sidak's Multiple comparisons test; **Figure 7C** and **Supplementary Table 4**). There were also no differences in the mean speed at 9 weeks old

(Interaction effect: $F_{(4,\ 164)}$ = 0.5285, p = 0.7149; Age effect: $F_{(1.564,\ 64.13)}$ = 312.3, p < 0.0001; Treatment effect: $F_{(1,\ 41)}$ = 0.4215, p = 0.5198; Animals effect: $F_{(41,\ 164)}$ = 29.21, p < 0.0001, two-way repeated-measures ANOVA; **Figure 7F** and **Supplementary Table 5**).

Both groups significantly reduced the distance traveled and mean speed when comparing the different ages (Distance: Ctrl 9w vs. 6w, MD = -31.71, 95% CI = -49.41 to -14.02, $t_{(20)}$ = 3.739, p = 0.0013; 0.2 9w vs. 6w, MedD = -49.83, W = -213.0, p = 0.0002, Student's t test or Wilcoxon's test. Interaction effect: $F_{(1,\ 41)}$ = 1.873, p = 0.1786; Age effect: $F_{(1,\ 41)}$ = 33.04, p < 0.0001; Treatment effect: $F_{(1,\ 41)}$ = 2.608, p = 0.1140; Animals effect: $F_{(41,\ 41)}$ = 3.052, p = 0.0003, two-way repeated-measures ANOVA; **Figure 7D**. Mean Speed: Ctrl 9w vs. 6w, MD = -0.004524, 95% CI = -0.006982 to -0.002066, $t_{(20)}$ = 3.839, p = 0.0010; 0.2 9w vs. 6w, MedD = -0.007500, W = -213.0, p = 0.0002, Student's t test or Wilcoxon's test. Interaction effect: $F_{(1,\ 41)}$ = 1.722, p = 0.1967; Age effect: $F_{(1,\ 41)}$ = 34.31, p < 0.0001; Treatment effect: $F_{(1,\ 41)}$ = 2.407, p = 0.1285; Animals effect: $F_{(41,\ 41)}$ = 3.141, p = 0.0002, two-way repeated-measures ANOVA; **Figure 7G**).

There were no differences in the max speed (6w: Interaction effect: $F_{(4,\ 160)}$ = 0.4467, p = 0.7747; Age effect: $F_{(2.054,\ 82.17)}$ = 27.43, p < 0.0001; Treatment effect: $F_{(1,\ 40)}$ = 2.166, p = 0.1489; Animals effect: $F_{(40,\ 160)}$ = 39.46, p < 0.0001; 9w: $F_{(4,\ 160)}$ = 1.344, p = 0.2561; Age effect: $F_{(2.127,\ 85.07)}$ = 32.93, p < 0.0001; Treatment effect: $F_{(1,\ 40)}$ = 0.002303, p = 0.9620; Animals effect: $F_{(40,\ 160)}$ = 16.02, p < 0.0001, two-way repeated-measures ANOVA; **Figure 7H-I** and **Supplementary Table 6**), periphery time (6w: Interaction effect: $F_{(4,\ 164)}$ = 1.246, p = 0.2935; Age effect: $F_{(1.323,\ 54.26)}$ = 3717, p < 0.0001; Treatment effect: $F_{(1,\ 41)}$ = 1.213, p = 0.2771; Animals effect: $F_{(41,\ 164)}$ = 5.671, p < 0.0001; 9w: $F_{(4,\ 164)}$ = 2.010, p = 0.0954; Age effect: $F_{(1.304,\ 53.46)}$ = 3743, p < 0.0001; Treatment effect: $F_{(1,\ 41)}$ = 1.948, p = 0.1703; Animals effect: $F_{(41,\ 164)}$ = 4.477, p < 0.0001, two-way repeated-measures ANOVA; **Figure 7K-L** and **Supplementary Table 7**) or center time (6w: Interaction effect: $F_{(4,\ 164)}$ = 1.428, p = 0.2270; Age effect: $F_{(1.173,\ 48.08)}$ = 235.9, p < 0.0001; Treatment effect: $F_{(1,\ 41)}$ = 1.199, p = 0.2799; Animals effect: $F_{(41,\ 164)}$ = 6.185, p < 0.0001; 9w: $F_{(4,\ 164)}$ = 2.236, p = 0.0674; Age effect: $F_{(1.167,\ 47.84)}$ = 179.8, p < 0.0001; Treatment effect: $F_{(1,\ 41)}$ = 1.892, p = 0.1765; Animals effect: $F_{(41,\ 164)}$ = 4.804, p < 0.0001, two-way repeated-measures ANOVA; **Figure 7K-L** and **Supplementary Table 8**), in any of the ages tested.

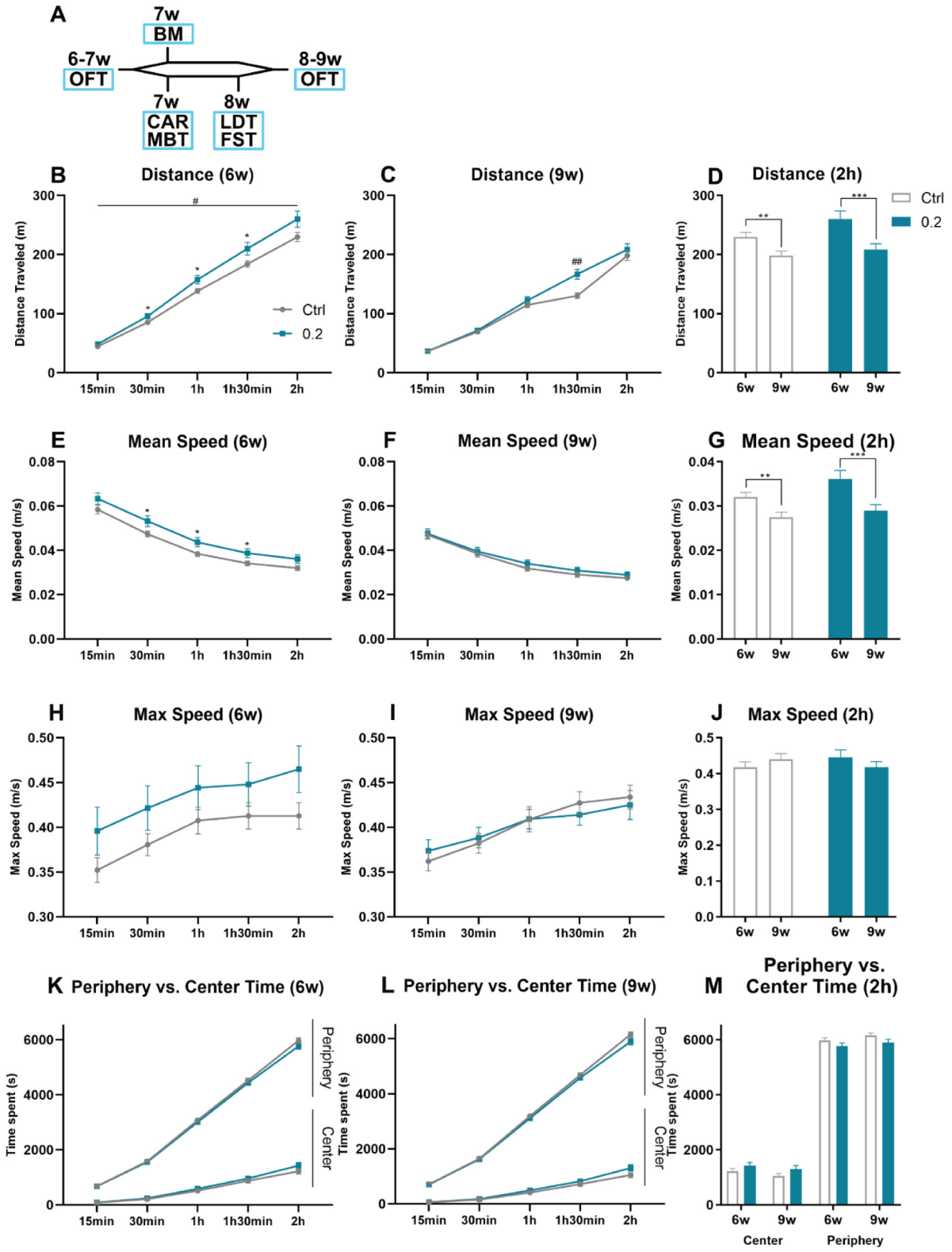

Figure 7. The 0.2 mg/ml group reliably presents hyperlocomotion at a young age shown in OFT. **(A)** Experimental design showing behavioral assessments performed. In the same breeding, we divided all pups (treated and controls) into two groups: the first one performed OFT, BM and repeated the OFT at an older age; the second one performed OFT, CAR, MBT, LDT, FST and repeated the OFT at an older age. **(B, C and D)** Evaluation of the distance traveled at 6 weeks old **(B)**, 9 weeks old **(C)**, 6 weeks vs. 9 weeks old (total time) **(D)**. **(E, F and G)** Evaluation of the mean speed at 6 weeks old **(E)**, 9 weeks old **(F)**, 6 weeks vs. 9 weeks old (total time) **(G)**. **(H, I**

and J) Evaluation of the max speed at 6 weeks old **(H)**, 9 weeks old **(I)**, 6 weeks vs. 9 weeks old (total time) **(J)**. **(K, L and M)** Evaluation of the time spent in the periphery vs. center zones at 6 weeks old **(K)**, 9 weeks old **(L)**, 6 weeks vs. 9 weeks old (total time) **(M)**. Data represented as mean ± S.E.M. (n = 22 0.2 group/ 21 Ctrl group). ***P < 0.001, **P < 0.01, *P < 0.05 (single variable comparison). ## P < 0.01, #P < 0.05 (two-way repeated-measures ANOVA and Sidak's Multiple comparisons test).

When comparing the different ages within the groups, we also did not find differences in the max speed (Ctrl 9w vs. 6w, MD = 0.2711, 95% CI = -0.01647 to 0.05521, $t_{(20)}$ = 1.135, p = 0.2711; 0.2 9w vs. 6w, MedD = -0.01800, W = -55.0, p = 0.3509, Student's t test or Wilcoxon's test. Interaction effect: $F_{(1,\ 38)}$ = 4.002, p = 0.0526; Age effect: $F_{(1,\ 38)}$ = 0.03892, p = 8447; Treatment effect: $F_{(1,\ 38)}$ = 0.01455, p = 0.9046; Animals effect: $F_{(38,\ 38)}$ = 2.675, p = 0.0016, two-way repeated-measures ANOVA; **Figure 7J**), periphery time (Ctrl 9w vs. 6w, MedD = 123.7, W = 107.0, p = 0.0646; 0.2 9w vs. 6w, MD = 128.0, 95% CI = -56.80 to 312.8, $t_{(21)}$ = 1.441, p = 0.1645, Student's t test or Wilcoxon's test. Interaction effect: $F_{(1,\ 41)}$ = 0.1112, p = 0.7405; Age effect: $F_{(1,\ 41)}$ = 3.944, p = 0.0537; Treatment effect: $F_{(1,\ 41)}$ = 2.913, p = 0.0954; Animals effect: $F_{(41,\ 41)}$ = 3.102, p = 0.0002, two-way repeated-measures ANOVA; **Figure 7M**) or center time (Ctrl 9w vs. 6w, MedD = -118.4, W = -105.0, p = 0.0701; 0.2 9w vs. 6w, MD = -124.5, 95% CI = -309.2 to 60.19, $t_{(21)}$ = 1.402, p = 0.1756, Student's t test or Wilcoxon's test. Interaction effect: $F_{(1,\ 41)}$ = 0.1070, p = 0.7453; Age effect: $F_{(1,\ 41)}$ = 3.736, p = 0602; Treatment effect: $F_{(1,\ 41)}$ = 2.931, p = 0.0945; Animals effect: $F_{(41,\ 41)}$ = 3.094, p = 0.0002, two-way repeated-measures ANOVA; **Figure 7M**).

3.4.7 PNE had no effects on visuospatial learning and memory in Barnes maze

We did observe a small effect in the short-term memory (**Figure 3K**) and long-term memory (**Figure 5B**) with the ORT, but it is still unclear if they have a deficit, since they could identify the S1 mouse as familiar with the SN test (**Figure 6D**). Thus we opted for a more complex memory test, the Barnes maze (BM). It is a behavioral test developed to evaluate visuospatial learning, strategies and memory learning (Barnes, 1979; Gawel et al., 2019). The test's main goal is for the mice to learn how to escape the apparatus and is divided into three stages: habituation, training and test (see METHODS for more details). We did not observe any

differences in the habituation stage, in either distance traveled (MD = -0.5096, 95% CI = -1.909 to 0.8901, $t_{(18)}$ = 0.7649, p = 0.4542, Student's t test; **Figure 8A**), mean speed (MD = -0.002500, 95% CI = -0.01327 to 0.008270, $t_{(18)}$ = 0.4877, p = 0.6317, Student's t test; **Figure 8B**) or total errors made (MD = -2.000, 95% CI = -10.91 to 6.914, $t_{(18)}$ = 0.4714, p = 0.6430, Student's t test; **Figure 8C**).

As for the training stage, we found differences in the test duration and in the distance traveled within the groups, as it decreased as the days of training progressed in both groups (Test Duration: D1: MedD = -0.3670, U = 46, p = 0.7959; D2: MD = 2.440, 95% CI = -5.992 to 10.87, $t_{(18)}$ = 0.6080, p = 0.5508; D3: MedD = -3.500, U = 46.50, p = 0.8097, Student's t test or Mann-Whitney's test. Interaction effect: $F_{(2, 36)}$ = 0.09180, p = 0.9125; Day of training effect: $F_{(1.180, 21.24)}$ = 35.75, p < 0.0001; Treatment effect: $F_{(1, 18)}$ = 0.4105, p = 0.5298; Animal effect: $F_{(18, 36)}$ = 1.307, p = 0.2407, two-way repeated-measures ANOVA; **Figure 8D** and **Supplementary Table 9**. Distance: D1: MD = 0.1019, 95% CI = -1.883 to 2.087, $t_{(18)}$ = 0.1078, p = 0.9153; D2: MD = 0.2800, 95% CI = -0.3030 to 0.8630, $t_{(18)}$ = 1.009, p = 0.3263; D3: MD = 0.8000, 95% CI = -0.4229 to 0.5829, $t_{(18)}$ = 0.3342, p = 0.7421, Student's t test. Interaction effect: $F_{(2, 36)}$ = 0.03385, p = 0.9667; Day of training effect: $F_{(1.192, 21.45)}$ = 17.07, p = 0.0003; Treatment effect: $F_{(1, 18)}$ = 0.2248, p = 0.6411; Animal effect: $F_{(18, 36)}$ = 0.8901, p = 0.5929, two-way repeated-measures ANOVA; **Figure 8E** and **Supplementary Table 10**).

There were no differences in the mean speed, either between or within the groups, however we can notice an increasing trend, as they got faster at finding the escape (Mean Speed: D1: MD = -0.004100, 95% CI = -0.01535 to 0.007151, $t_{(18)}$ = 0.7656, p = 0.4538; D2: MD = 0.002100, 95% CI = -0.01345 to 0.01765, $t_{(18)}$ = 0.2837, p = 0.7799; D3: MedD = -0.001500, U = 43, p = 0.6174, Student's t test or Mann-Whitney's test. Interaction effect: $F_{(2, 36)}$ = 0.3109, p = 0.7348; Day of training effect: $F_{(1.968, 35.42)}$ = 7.907, p = 0.0015; Treatment effect: $F_{(1, 18)}$ = 0.005747, p = 0.9404; Animal effect: $F_{(18, 36)}$ = 2.429, p = 0.0115, two-way repeated-measures ANOVA; **Figure 8F**).

As for error measures, there was no difference in the number of primary errors (D1: MedD = -0.9995, U = 41.50, p = 0.5402; D2: MD = 2.500, 95% CI = -1.825 to 6.825, $t_{(18)}$ = 1.214, p = 0.2403; D3: MedD = -2.000, U = 39, p = 0.4244, Student's t test or Mann-Whitney's test. Interaction effect: $F_{(2, 36)}$ = 0.4164, p = 0.6626; Day of training effect: $F_{(1.712, 30.82)}$ = 2.809, p = 0.0830; Treatment effect: $F_{(1, 18)}$ = 0.3372, p =

0.5686; Animal effect: $F_{(18,\ 36)}$ = 1.061, p = 0.4251, two-way repeated-measures ANOVA; **Figure 8G**), either between or within the groups, however there was difference in the total number of errors within the groups, as they learned the task and made less mistakes (D1: MD = 0.7667, 95% CI = -9.141 to 10.67, $t_{(18)}$ = 0.1626, p = 0.8727; D2: MD = 0.7003, 95% CI = -3.516 to 4.917, $t_{(18)}$ = 0.3489, p = 0.7312; D3: MD = 1.000, 95% CI = -3.213 to 5.213, $t_{(18)}$ = 0.4987, p = 0.6240, Student's t test. Interaction effect: $F_{(2,\ 36)}$ = 0.002479, p = 0.9975; Day of training effect: $F_{(1.247,\ 25.69)}$ = 14.49, p = 0.0002; Treatment effect: $F_{(1,\ 18)}$ = 0.1969, p = 0.6625; Animal effect: $F_{(18,\ 36)}$ = 1.031, p = 0.4525, two-way repeated-measures ANOVA; **Figure 8H** and **Supplementary Table 11**).

There were also no differences between treated and control groups in the primary or total latency, only differences within the groups, as they learned the task (Primary Latency: D1: MedD = -1.834, U = 47, p = 0.8534; D2: MD = 3.190, 95% CI = -3.382 to 9.762, $t_{(18)}$ = 1.020, p = 0.3213; D3: MedD = -4.333, U = 34, p = 0.2388, Student's t test or Mann-Whitney's test. Interaction effect: $F_{(2,\ 36)}$ = 0.8181, p = 0.4493; Day of training effect: $F_{(1.354,\ 24.38)}$ = 12.18, p = 0.0008; Treatment effect: $F_{(1,\ 18)}$ = 1.292, p = 0.2706; Animal effect: $F_{(18,\ 36)}$ = 1.255, p = 0.2731, two-way repeated-measures ANOVA; **Figure 8I** and **Supplementary Table 12**. Total Latency: D1: MedD = -0.8335, U = 46, p = 0.7801; D2: MD = 2.567, 95% CI = -5.875 to 11.01, $t_{(18)}$ = 0.6388, p = 0.5310; D3: MedD = -3.500, U = 47, p = 0.8387, Student's t test or Mann-Whitney's test. Interaction effect: $F_{(2,\ 36)}$ = 0.08366, p = 0.9199; Day of training effect: $F_{(1.185,\ 21.32)}$ = 35.66, p < 0.0001; Treatment effect: $F_{(1,\ 18)}$ = 0.4140, p = 0.5280; Animal effect: $F_{(18,\ 36)}$ = 1.302, p = 0.2440, two-way repeated-measures ANOVA; **Figure 9A** and **Supplementary Table 13**).

We also analysed the strategy they used to learn the task, and again there was no difference between the groups (Random: MD = 1.000, 95% CI = -1.060 to 3.060, $t_{(16)}$ = 1.029, p = 0.3188; Serial: MD = -0.4444, 95% CI = -2.122 to 1.234, $t_{(16)}$ = 0.5615, p = 0.5822; Direct: MD = -0.5556, 95% CI =-1.856 to 0.7453, $t_{(16)}$ = 0.9054, p = 0.3787, Student's t test. Interaction effect: $F_{(2,\ 32)}$ = 0.7734, p = 0.4699; Treatment effect: $F_{(1,\ 16)}$ = 0.000, p > 0.9999; Strategy effect: $F_{(2,\ 21.45)}$ = 9.014, p = 0.0008; Animal effect: $F_{(16,\ 32)}$ = 0.000, p > 0.9999, two-way repeated-measures ANOVA; **Figure 9B**), only the controls had a significant higher use of the serial strategy over the random (Ctrl Random vs. Serial, MD = -3.556, 95% CI = -5.980 to -1.131, $q_{(32)}$ = 5.096, p = 0.0029, Tukey's Multiple comparisons test; **Figure 9B**).

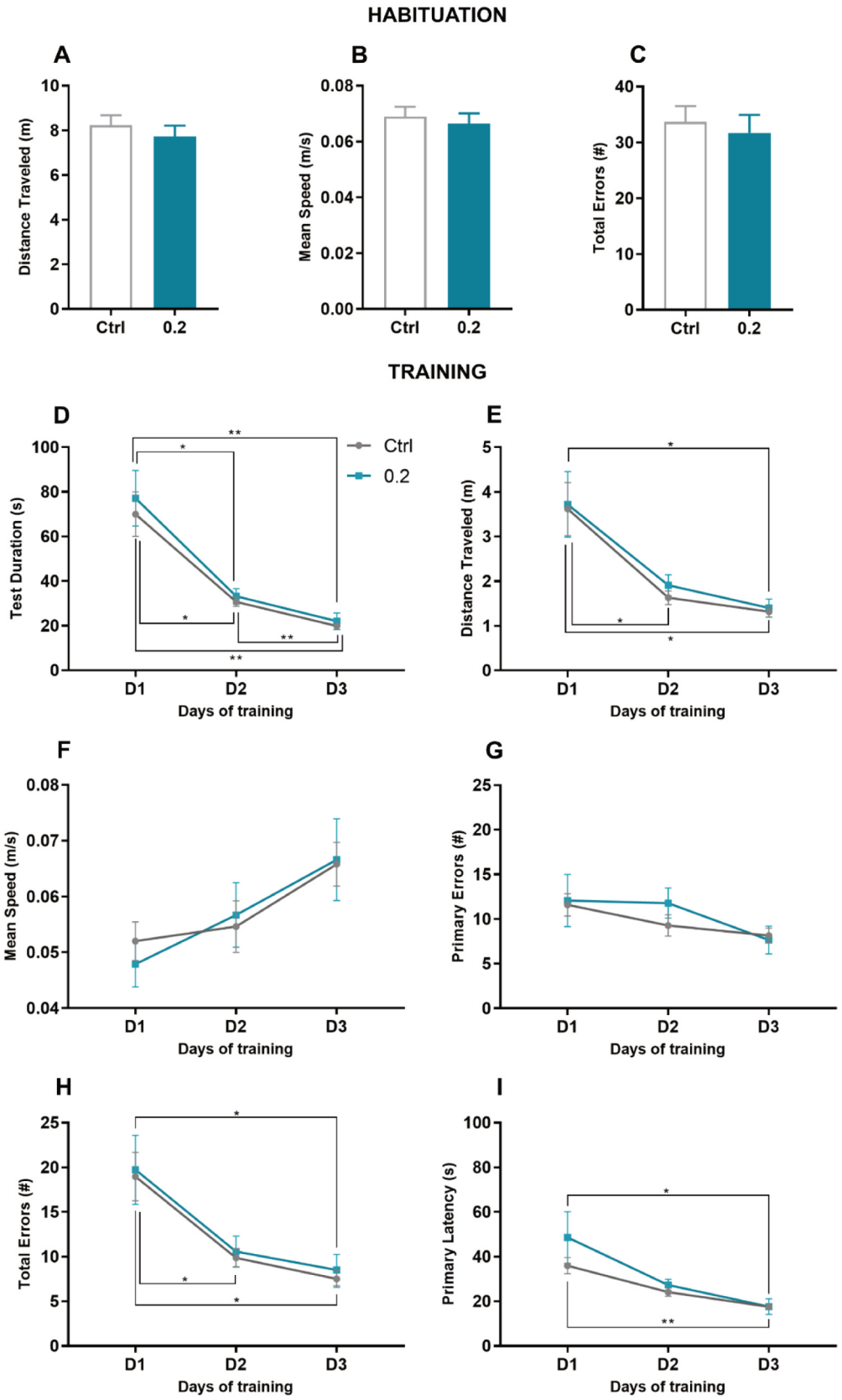

Figure 8. PNE had no effects on learning and memory in Barnes maze (part one). **(A, B and C)** Habituation stage: Evaluation of the distance traveled **(A)**, mean speed **(B)**, total errors **(C)**. **(E, F, G, H and I)** Training stage: Evaluation of the test duration **(D)**, distance traveled **(E)**, mean speed **(F)**, primary errors **(G)**, total errors **(H)**,

primary latency **(I)**. Data represented as mean ± S.E.M. (n = 10 0.2 group/ 10 Ctrl group). **P < 0.01, *P < 0.05 (single variable comparison).

During the test stage, the escape chamber is replaced with a fake escape hole, and we evaluate mainly the number of errors made and latency to find where the correct hole was. There were no differences in the primary errors (MedD = 0.5000, U = 44.50, p = 0.6973, Mann-Whitney's test; **Figure 9C**), the primary latency (MD = 1.890, 95% CI = -7.429 to 11.21, $t_{(18)}$ = 0.4261, p = 0.6751, Student's t test; **Figure 9D**) and in the strategy used (Treatment effect: $F_{(1, 2)}$ = 0.000, p > 0.9999; Strategy effect: $F_{(2, 2)}$ = 24.33, p = 0.0395, two-way ANOVA; **Figure 9E**). Analysing the use of a strategy (serial or direct) versus no strategy (random) used during the whole BM test, there was only difference within the groups in the test stage (Ctrl: Serial + Direct vs. Random, MD = 7.000, 95% CI = 5.450 to 8.550, $t_{(18)}$ = 9.487, p < 0.0001; **Figure 9F**. 0.2: Serial + Direct vs. Random, MD = 5.000, 95% CI = 2.819 to 7.181, $t_{(18)}$ = 4.816, p = 0.0001, Student's t test; **Figure 9G**).

3.4.8 The 0.2 mg/ml group presents increased impulsivity, with no signs of anxiety-like or depressive-like behaviors

Impulsivity is a characteristic feature of ADHD, and for a better understanding of the disorder using animal models, it is preferable that the model also show it alongside the other features. To evaluate impulsivity-like behavior in the PNE model, we also used the CAR test. Treated mice showed an increased number of falls (MD = 2.989, 95% CI = 0.1114 to 5.866, $t_{(17)}$ = 2.191, p = 0.0426, Student's t test; **Figure 10A**) and a shorter latency to first fall (MedD = -158.0, U = 5, p = 0.0008, Mann Whitney test; **Figure 10B**). We also tested how they would behave in the marble-burying test (MBT). It is usually used in studies involving obsessive-compulsive disorder and anxiety. However, treated mice did not show a difference in the number of buried marbles compared to controls (MD = 0.2889, 95% CI = -1.696 to 2.274, $t_{(17)}$ = 0.3070, p = 0.7625, Student's t test; **Figure 10C**).

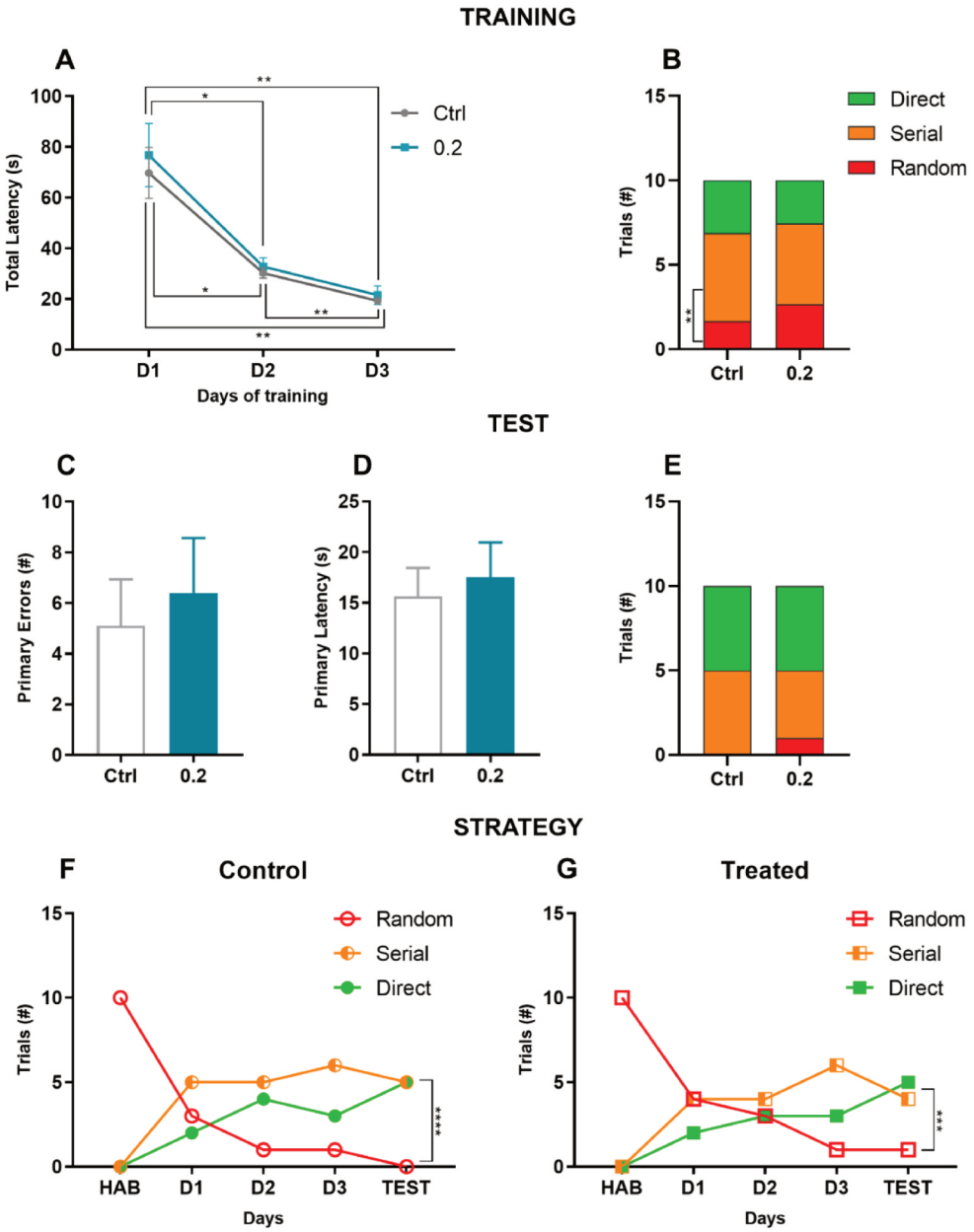

Figure 9. PNE had no effects on learning and memory in Barnes maze (part two). **(A and B)** Training stage: Evaluation of the total latency **(A)** and total strategy **(B)**. **(C, D and E)** Test stage: Evaluation of the primary errors **(C)**, primary latency **(D)** and strategy **(E)**. **(F and G)** Strategy analysis: Evaluation of the strategy used by controls **(F)** and treated **(G)**. Data represented as mean ± S.E.M. (n = 10 0.2 group/ 10 Ctrl group). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05 (single variable comparison).

We tested anxiety-like behaviors previously and the 0.2 mg/mL group showed small and not statistically significant signs of it. As a last test, we opted for an extreme anxiety inducing test, the light/dark box. There were no differences in the latency to first entry in the light zone (MD = 9.303, 95% CI = -8.265 to 26.87, $t_{(17)}$ = 1.117, p = 0.2794, Student's t test; **Figure 10D**), in the total number of entries in the light zone (MD = 0.1333, 95% CI = -2.283 to 2.549, $t_{(17)}$ = 0.1164, p = 0.9087,

Student's *t* test; **Figure 10E**) or in the time spent in the light zone vs. dark zone (Time in the LZ: MD = -5.398, 95% CI = -29.77 to 18.98, $t_{(17)}$ = 0.4672, p = 0.6463; Time in the DZ: MD = 3.462, 95% CI = -18.38 to 25.30, $t_{(17)}$ = 0.3345, p = 0.7421, Student's *t* test; Light Zone vs. Dark Zone: Interaction effect: $F_{(1, 17)}$ = 0.1653, p = 0.6894; Zone effect: $F_{(1, 17)}$ = 233.0, p < 0.0001; Treatment effect: $F_{(1, 17)}$ = 0.6004, p = 0.4491; Animals effect: $F_{(17, 17)}$ = 0.01314, p > 0.9999, two-way repeated-measures ANOVA; **Figure 10F**).

There were also no differences in the latency to first risk assessment (MD = 2.226, 95% CI = -8.993 to 13.44, $t_{(17)}$ = 0.4185, p = 0.6808, Student's *t* test; **Figure 10G**) or in the total number of risk assessments (MD = 1.411, 95% CI = -2.254 to 5.076, $t_{(17)}$ = 0.8123, p = 0.4278, Student's *t* test; **Figure 10H**). Other authors have reported that some PNE models may show depressive-like behaviors (Paz et al., 2007; Zhang M. et al., 2018). We tested using the forced swim test.

Treated mice did not differ from controls in time swimming (MD = 16.48, 95% CI = -11.72 to 44.69, $t_{(17)}$ = 1.233, p = 0.2344, Student's *t* test; **Figure 10I**), time immobile (MD = -16.48, 95% CI = -44.69 to 11.72, $t_{(17)}$ = 1.233, p = 0.2344, Student's *t* test; **Figure 10I**) and longest swimming episode (MD = 7.803, 95% CI = -3.519 to 19.13, $t_{(17)}$ = 1.454, p = 0.1641, Student's *t* test; **Figure 10J**). Both groups spent more time swimming (Swimming vs. Immobile: Interaction effect: $F_{(1, 17)}$ = 1.520, p = 0.2344; Activity effect: $F_{(1, 17)}$ = 24.08, p = 0.0001; Treatment effect: $F_{(1, 17)}$ = 2.381, p = 0.1412; Animals effect: $F_{(17, 17)}$ = 1.186e-031, p > 0.9999, two-way repeated-measures ANOVA, data not shown; **Figure 10I**).

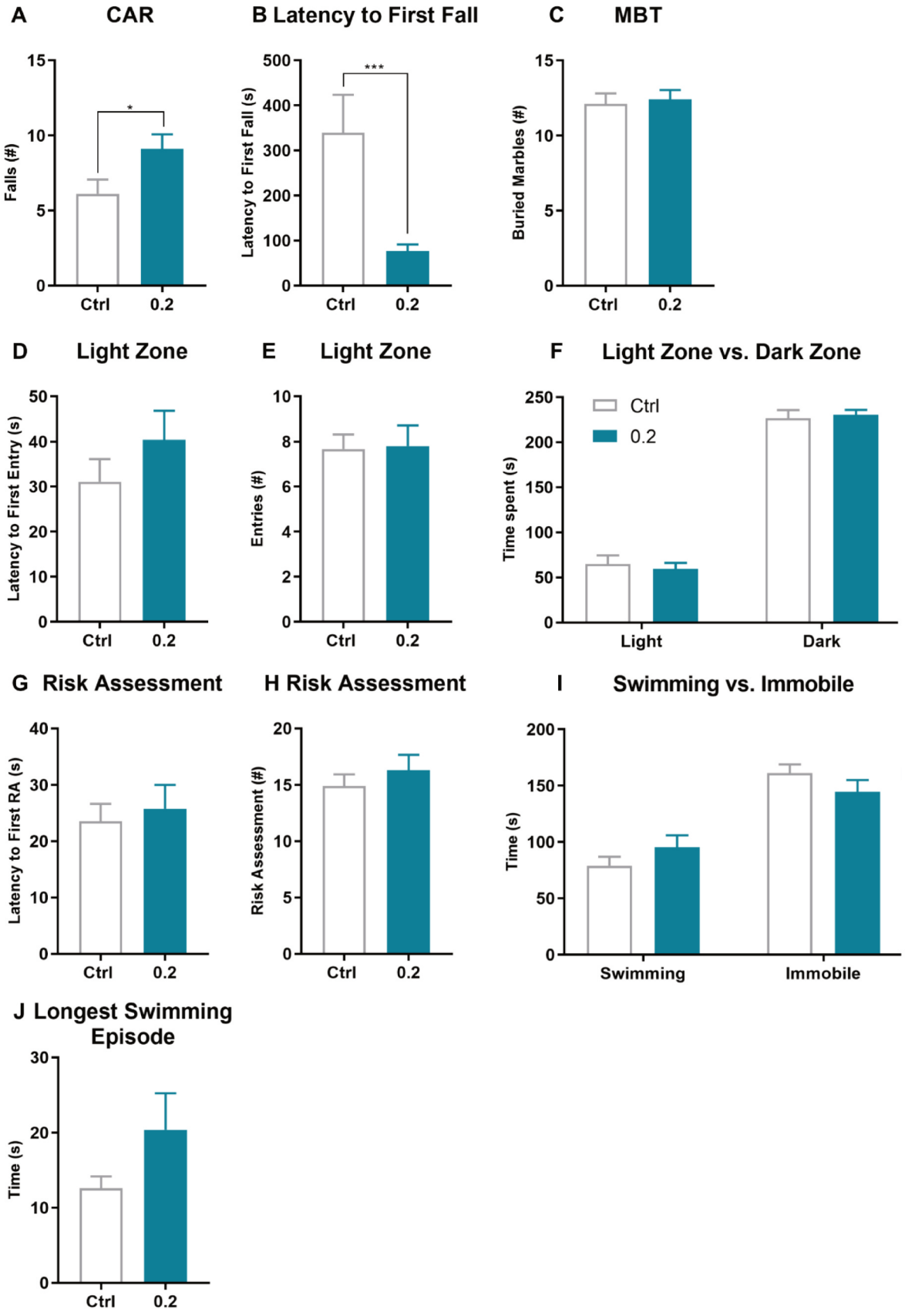

Figure 10. The 0.2 mg/ml group presents increased impulsivity, with no signs of anxiety-like or depressive-like behaviors. **(A and B)** CAR: Evaluation of the number of falls **(A)** and latency to first fall **(B)**. MBT: Evaluation of the number of buried marbles **(C)**. **(D, E, F, G and H)** LDT: Evaluation of the latency to the first entry in the light

zone **(D)**, number of entries in the light zone **(E)**, time spent in the light zone vs. dark zone **(F)**, latency to first risk assessment **(G)** and number of risk assessments **(H)**. **(I and J)** FST: Evaluation of the time spent swimming vs. immobile **(I)** and longest swimming episode **(J)**. Data represented as mean ± S.E.M. (n = 10 0.2 group/ 9 Ctrl group). ***P < 0.001, *P < 0.05 (single variable comparison).

3.5 DISCUSSION

3.5.1 The PNE model

The most commonly used PNE protocol, by oral exposure, includes offering an aqueous solution containing nicotine to female mice and saccharin for a defined period before mating and during pregnancy (Paz et al., 2007; Zhu et al., 2012; Zhu et al., 2017; Zhang L. et al., 2018; Polli et al., 2020). We tested the protocol, introducing nicotine slowly and adding 2% of sugar, to avoid rejection of the nicotine solutions and dehydration (Paz et al., 2007). Our results showed no difference in the solution intake or weight between the females who received solutions with nicotine and sugar or sugar only (**Figure 1**). We did not test if there was a difference in introducing nicotine gradually or full dose from the start because our main goal was to reduce the risks, so further research is needed. However, adding 2% sugar was efficient to prevent a reduced fluid intake (Paz et al., 2007; Zhu et al., 2012; Alkam et al., 2013; Zhu et al., 2014; Balsevich et al., 2014).

The females in the first experiment were individually housed (**Figure 1**), but we decided to house the next females together (**Figure 2**), in 4-5 mice per cage, during treatment to reduce social separation-induced stress (Nih et al., 2011). The reproduction design we later used was also very common (two females for each male) and, as other studies suggest, we observed that it improved their motherly instincts, for example, with better-built nests and less stress during handling (Nih et al., 2011; Brennan, 2018; Braden et al., 2017). All sires in our experiment also drank the nicotine solutions as they were housed with the females for breeding. Some PNE protocols include treatment for the sires, or even just for the sires, and have shown similar results as the protocols with dam's treatments (McCarthy et al., 2018). We do not consider that our protocol had an effect coming from the males since their contact with nicotine was short before the copulation.

Regarding the removal of nicotine after birth, some authors did not remove the solutions until the pups were weaned, as to offer nicotine during their full development (Balsevich et al., 2014; Zhang L. et al., 2018; Buck et al., 2019). Other papers cross-fostered the pups to drug-naive nursing mothers, so the pups did not receive nicotine through the milk (Zhu et al., 2012; Zhu et al., 2014; Zhu et al., 2017). We choose to remove it gradually after birth as to not let the pups drink it through the milk for too long but also trying to avoid stressing the dams (Paz et al., 2007). The sudden removal of nicotine might stress the dams, lowering the chances of the pups surviving. Cross fostering could also be a source of stress to the pups, although one study did not find differences in body weight gain or locomotor activity between cross-fostered and non cross-fostered pups (Zhu et al., 2012). The natality of the 0.2 mg/mL group has evidence of being low (Zhu et al., 2012), however, several other studies used the same dose or even higher (0.3 mg/mL) and did not report small litters (Alkam et al., 2013; Balsevich et al., 2014; Buck et al., 2019; Polli et al., 2020), as we also did not observe in our study.

3.5.2 Spontaneous hyperactivity

ADHD is a developmental disorder characterized by hyperactivity, impulsivity and inattention, especially in children and adolescents (APA, 2013), so an ADHD mice model must exhibit these features. We observed that the lower and higher dose tested (0.1 and 0.2 mg/mL, respectively) showed increased distance traveled and mean speed during longer OFT trials (5 and 2 hours long), with no significant differences with the intermediate dose (0.15 mg/mL) (0.1 and 0.2 mg/mL: **Figure 4A-B**; 0.2 mg/mL: **Figure 7B and 7E**). The 0.2 group also presented an increase in the same parameters in tests that do not aim to evaluate locomotor features, such as EPM (**Figure 4F-G**) and YM (**Figure 5D-E**), which was impressive for such short tests. Although the 0.15 group showed increased activity in the YM (**Figure 5D-E**), it does not seem like a reliable model for hyperactivity. Several studies report hyperactivity in the nicotine exposure models, so such findings were expected (Paz et al., 2007; Zhu et al., 2012; McCarthy et al., 2018; Zhang M. et al., 2018; Buck et al., 2019; Polli et al., 2020). Other ADHD rodent models also present hyperactivity, such as neonatal rats damaged by 6-hydroxydopamine (Erinoff et al., 1979; Miller et al., 1981; Archer et al., 1988), spontaneously strained rats (SHR; Wultz et al., 1990;

Sagvolden et al., 1992), coloboma mutant mice (Hess et al., 1996) and dopamine transporter knockout mice (DAT-KO; Giros et al., 1996).

Another finding was the reduced general activity (OFT: **Figure 3B-C**, **Figure 7D and 7G**) and the reduced time exploring the center (OFT: **Figure 3E-F**; EPM, **Figure 3I**) in the tests repeated at older ages. This is most likely due to the loss of interest in exploring the apparatus/performing the task as the novelty impact has worn off. To verify if the effect is due to the model losing the phenotype, the experimental design should include new mice at an older age.

3.5.3 Memory deficits

In addition to common ADHD symptoms, patients exhibit impairment in several cognitive abilities, such as some executive functions, learning and short-term memory (Arnsten, 2009; Lubow et al., 2014; Fried et al., 2016). We observed a small effect in short-term memory (**Figure 4K**) and long-term memory with the ORT (**Figure 5B**). In the short-term, 0.1 group explored the familiar object more than the novel one, failing at recognizing the familiar object (**Figure 4K**). However, the controls explored both objects equally, also failing at recognizing the familiar object, which made us question the validity of this test. It is important to notice that, at this time point, the tests were still done during the inactive phase, in a well-lit room, and that could have interfered with the results. Another contribution to the doubt is that when we repeated the test with the 0.1 group, they successfully discriminated between the familiar and novel object after 24h of the first contact (**Figure 5B**).

In the long-term ORT, as we said previously, the only group to succeed in the task was 0.1 mg/mL, showing no signs of long-term memory deficits. The control and 0.15 groups did not show a statistically significant difference in the interaction time between the two objects, but there was a visible success trend (**Figure 5B**). As for the 0.2 group, mice spent the same time exploring both objects, failing the task and showing signs of long-term memory deficits (**Figure 5B**). This time, the conditions were ideal, as the tests were performed during their active phase, in a dimly red light-lit room. To decide which result is valid for the 0.1 group, other tests should be done, in ideal conditions. We also observed that all treated groups interacted less with the objects during training than the controls, especially the 0.2 group (**Figure 5A**). This surprised us as we were expecting that PNE mice would be interested in

the novelty and more testing should be done to elucidate this. Object recognition-based tests have a wide range of protocols, so comparing studies can be dangerously misleading. Considering similar protocols, other studies found impairments in short-term memory in PNE mice but did not test long-term memory (Zhu et al., 2017; Zhang L. et al., 2018; McCarthy et al., 2018). Other ADHD rodent models also exhibit similar deficits (Li et al., 2010; Bouchatta et al., 2018).

The YM is a working memory assessment, also widely used with the nicotine exposure models. Besides the increase in the locomotor activity seen with the 0.15 and 0.2 groups discussed previously, we also found an increased number of entries in the arms and spontaneous alternations compared with controls (**Figure 5F-G**). These results corroborate with the increase in the activity while also demonstrating no signs of deficits in the working memory, as the percentage of spontaneous alternations behavior did not differ from controls (**Figure 5H**). All previous studies using maternal nicotine exposure models found reduced spontaneous alternations behavior, showing deficits in working memory (Alkam et al., 2013; Zhu et al., 2017; Zhang L. et al., 2018; Polli et al., 2020). McCarthy (2018) used parental prenatal nicotine exposure models and found no differences in the spontaneous alternations behavior in either males or females tested.

We also used the BM cognitive paradigm to evaluate mice's visuospatial learning and memory. There are a couple of protocols available, but the focus is on the learning process, with acquisition training trials, and in the memory test, with the use of the spatial location of the escape hole (O'Leary et al., 2011). Both groups successfully learned the task and used effective strategies, so there were no differences between the groups, only between the training days as they progressed (**Figure 8 and Figure 9**). We also found no differences in the probe trial (**Figure 9C-D**). We found no similar studies testing the model in the BM, only McCarthy (2018) with parental prenatal nicotine exposure. They also found no differences between the groups in the acquisition stage, however, the tested mice showed significant reversal learning deficits (McCarthy et al., 2018).

3.5.4 Sociability

ADHD patients also often face challenges such as social disabilities and emotional deficits (Barkley, 2002). We found that both 0.1 and 0.15 preferred to

spend time with stranger 1 (S1) than with the empty cage. However, the 0.2 group explored both cages equally and also spent significantly less time exploring the S1 cage than controls (**Figure 6B**). So far, the results suggest social anxiety. All groups entered more in the empty cage-chamber than in the S1 cage-chamber (**Figure 6C**), and that could be because they were searching for something as interesting as a mouse that was on the other side. As for social novelty, all groups showed a preference for stranger 2 (S2), but as the nicotine dose was higher, less significant was the difference (**Figure 6D**). Here the results suggest some level of social anxiety within the 0.2 group, as they interacted less with the S2, but they still showed more interest in the novelty than in the familiarity (S1). They also entered more in the S2 chamber than in the S1, as did all the other groups (**Figure 6E**).

There is not extensive research using three-chamber social tests or any other social tests with the PNE model. Alkam (2013) found no differences in sociability, but in social novelty, PNE mice showed a decreased preference for the S2 over S1. Zhang M. (2018) found sociability impairments with mice born from maternal only and maternal and paternal intraperitoneal PNE. McCarthy (2018) found impaired social interaction using paternal nicotine exposure. Tests in the tobacco smoke exposure model (Dai et al., 2017) and neonatal 6-OHDA lesion model (Bouchatta et al., 2018) also found similar results.

3.5.5 Anxiety and depression

The emotional deficits and poor emotion regulation presented by patients with ADHD, especially children and adolescents, can also lead to a higher diagnosis rate of anxiety and depression (Barkley, 2002). In the OFT, 0.1 group spent more time in the center at 6 weeks old than controls (**Figure 3F**), showing an anxiolytic-like effect. In the EPM, the same mice spent less time in the closed arms at 6 weeks old than controls (**Figure 3I**), showing less anxiety-like behavior. These tests were repeated but the 0.1 group did not replicate the results (OFT: **Figure 4E**; EPM: **Figure 4H**). We must recall that the first tests were performed during the inactive phase, in a well-lit room, that could have interfered with the results. The second EPM was still well illuminated because of the anxiogenic characteristics of the test, but the change in the active/inactive phase could still interfere.

The other treated groups also did not show signs of either anxiolytic-like or anxiogenic-like effects (OFT: **Figure 4E**; EPM: **Figure 4H**). Although we can visually see a trend with the 0.2 group, with a little more time spent in the center in the OFT (**Figure 4E**) and a little less time spent in the open arms in the EPM (**Figure 4H**) compared with their respective controls. Other authors have reported mixed results in similar PNE mice models using EPM or elevated zero maze (EZM), such as anxiogenic-like effect in both sexes (Alkam et al., 2013; Buck et al., 2019), an anxiogenic-like effect only in males (Balsevich et al., 2014; Polli et al., 2020) or no differences in both sexes (Zhang L. et al., 2018). Other ADHD models have also shown either an anxiety-like behavior (Neonatal 6-OHDA lesion model, Bouchatta et al., 2018) or no effect (Neonatal sevoflurane exposure, Xie et al., 2020).

We found no differences between the 0.2 group and controls in the MBT (**Figure 10C)**, LDT (**Figure 10D-H)** and FST (**Figure 10I-J)**, suggesting no signs of anxiety-like or depressive-like behavior. As these tests are not commonly used with this model, we found only a few reports. Two authors, using a similar PNE mice model, reported an increased number of buried marbles in both sexes (Polli et al., 2020) or only in males (Alkam et al., 2013). Alkam (2013) also found anxiogenic-like effects in both sexes with the LDT. In a cigarette smoke-exposed (CSE) mice model, the study found no differences in the MBT and an anxiolytic-like effect in the LDT (Amos-Kroohs et al., 2013). Zhang M. (2018) found that maternal intraperitoneal PNE can lead to depressive-like behaviors, tested with the FST and sucrose preference test (SPT).

3.5.6 Impulsivity

Impulsivity is a characteristic feature and criteria for the diagnosis of ADHD (APA, 2013). To evaluate impulsivity-like behavior in the PNE model, we used the CAR. Treated mice showed an increased number of falls (**Figure 10A**) and a shorter latency to first fall (**Figure 10B**) compared to controls. Zhu (2017) found, using a similar PNE mice model, impulsive-like behavior only in male mice. Zhang L. (2018) also used a similar PNE mice model but found no differences between treated and control mice, in a 60 minutes-long CAR protocol. Other ADHD mice models reported impulsivity using the cliff avoidance reaction (CAR) test (Yamashita et al., 2013; Xie et al., 2020; Bouchatta et al., 2020) and the 5-choice serial reaction time task test

(5-CSRTT; Bouchatta et al., 2018; Bouchatta et al., 2020). It is relevant to highlight that the hyperlocomotion/hyperactivity seen in the model could interfere with the CAR results, even if the act of falling off the edge itself shows a level of impulsivity and miscalculation of risks with no direct connection to hyperactivity. Contributing to this, a research group, using a similar PNE mice model, found hyperactivity in both sexes (Zhu et al., 2012; Zhu et al., 2014) but impulsivity only in males (Zhu et al., 2017).

CONCLUSION

Here we showed that the 0.1 and 0.15 mg/mL nicotine treatment did not provide a reliable ADHD model, as we had mixed or negative results. However, we found that the 0.2 mg/mL nicotine treatment provides a model with hyperlocomotion, social anxiety and impulsivity. We also found a small effect in the long-term memory and no signs of anxiety-like or depressive-like behaviors. Together, these findings are consistent with the symptoms and psychiatric disorders of ADHD, considering that only a subpopulation of patients has anxiety or depression. The development of the model (prenatal nicotine exposure) is also consistent with clinical studies correlating a higher risk of developing ADHD in children from mothers who smoked or used NRTs during pregnancy.

Therefore, this PNE model presents face, predictive and construct validity and can be a powerful tool to better understand the mechanisms underlying ADHD clinical manifestations. We suggest further behavioral investigations, especially involving impulsive behavior, as it is understudied in this model. Future studies with molecular, genetic and histologic investigations are also needed, as they are necessary to unravel the mechanisms underlying ADHD clinical manifestations.

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3.6 SUPPLEMENTARY MATERIAL

Supplementary Tables 1 - Effect of different doses in the prenatal nicotine exposure on the distance traveled, analysis across time

Ctrl vs. 0.1	Mean Difference	95% CI	$t_{(15)}$	Summary	*p* value
30min	**-26.08 ± 5.290**	**-37.35 to -14.80**	**4.930**	*******	**0.0002**
1h	**-20.97 ± 4.885**	**-31.39 to -10.56**	**4.294**	*******	**0.0006**
1h30min	**-17.24 ± 4.239**	**-26.28 to -8.206**	**4.067**	******	**0.0010**
2h	**-15.08 ± 4.230**	**-24.10 to -6.069**	**3.566**	******	**0.0028**
2h30min	-10.28 ± 6.773	-24.72 to 4.153	1.518	ns	0.1497
3h	-7.140 ± 11.07	-30.73 to 16.45	0.6452	ns	0.5285
3h30min	-1.959 ± 6.978	-16.83 to 12.91	0.2808	ns	0.7827
4h	6.159 ± 9.647	-14.40 to 26.72	0.6384	ns	0.5328
4h30min	-1.099 ± 8.601	-19.43 to 17.23	0.1278	ns	0.9000
5h	5.261 ± 6.585	-8.864 to 19.39	0.7988	ns	0.4377

Supplementary Table 1.1 - Effect of 0.1 mg/mL prenatal nicotine exposure on the distance traveled, analysis across time

Table showing the statistical results of Student's *t* test demonstrating the difference in the distance traveled at different time points between the 0.1 and Ctrl groups. Data correspondent to **Figure 4B**.

Ctrl vs. 0.15	Mean Difference	95% CI	$t_{(17)}$	Summary	*p* value
30min	-12.79 ± 6.072	-25.60 to 0.02063	2.106	ns	0.0503
1h	-9.463 ± 5.135	-20,30 to 1.372	1.843	ns	0.0829
1h30min	-5.436 ± 4.176	-14.25 to 3.375	1.302	ns	0.2104
2h	-3.723 ± 3.569	-11.25 to 3.806	1.043	ns	0.3114
2h30min	-8.332 ± 5.223	-19.35 to 2.688	1.595	ns	0.1291
3h	-6.099 ± 8.081	-23.15 to 10.95	0.7548	ns	0.4607
3h30min	-2.956 ± 6.237	-16.11 to 10.20	0.4739	ns	0.6416
4h	-4.392 ± 9.099	-23.59 to 14.80	0.4827	ns	0.6355
4h30min	-11.10 ± 7.893	-27.75 to 5.557	1.406	ns	0.1778
5h	-9.607 ± 6.996	-24.37 to 5.153	1.373	ns	0.1875

Supplementary Table 1.2 - Effect of 0.15 mg/mL prenatal nicotine exposure on the distance traveled, analysis across time

Table showing the statistical results of Student's *t* test demonstrating the difference in the distance traveled at different time points between the 0.15 and Ctrl groups. Data correspondent to **Figure 4B**.

Ctrl vs. 0.2	Mean Difference	95% CI	$t_{(14)}$	Summary	*p* value
30min	**-20,45 ± 6,821**	**-35,08 to -5,823**	**2.998**	**	**0.0096**
1h	**-17,42 ± 6,543**	**-31,46 to -3,390**	**2.663**	*	**0.0186**
1h30min	**-13,95 ± 6,346**	**-27,56 to -0,3356**	**2.198**	*	**0.0453**
2h	**-14,85 ± 5,704**	**-27,09 to -2,617**	**2.604**	*	**0.0208**
2h30min	-16,39 ± 7,972	-33,49 to 0,7034	2.057	ns	0.0589
3h	-14,08 ± 10,81	-37,27 to 9,106	1.303	ns	0.2137
3h30min	-3,086 ± 10,39	-25,38 to 19,21	0.2969	ns	0.7709
4h	-15,96 ± 8,395	-33,97 to 2,044	1.901	ns	0.0780
4h30min	-11,36 ± 8,841	-30,32 to 7,607	1.284	ns	0.2198
5h	-14,48 ± 10,82	-37,68 to 8,715	1.339	ns	0.2019

Supplementary Table 1.3 - Effect of 0.2 mg/mL prenatal nicotine exposure on the distance traveled, analysis across time

Table showing the statistical results of Student's *t* test demonstrating the difference in the distance traveled at different time points between the 0.2 and Ctrl groups. Data correspondent to **Figure 4B**.

Supplementary Tables 2 - Effect of different doses in the prenatal nicotine exposure on the mean speed, analysis across time

Ctrl vs. 0.1	Mean Difference	95% CI	$t_{(15)}$	Summary	*p* value
30min	**-0.01446 ± 0.002955**	**-0.02076 to -0.008159**	**4.892**	***	**0.0002**
1h	**-0.01025 ± 0.002538**	**-0.01569 to -0.004806**	**4.038**	**	**0.0012**
1h30min	**-0.009542 ± 0.002337**	**-0.01452 to -0.004561**	**4.083**	***	**0.0010**
2h	**-0.008347 ± 0.002335**	**-0.01332 to -0.003370**	**3.575**	**	**0.0028**
2h30min	-0.005625 ± 0.003730	-0.01358 to 0.002325	1.508	ns	0.1523
3h	-0.003861 ± 0.006119	-0.01690 to 0.009180	0.6310	ns	0.5375
3h30min	-0.001000 ± 0.003867	-0.009242 to 0.007242	0.2586	ns	0.7995
4h	0.003306 ± 0.005370	-0.008139 to 0.01475	0.6156	ns	0.5474
4h30min	-0.0004583 ± 0.004776	-0.01064 to 0.009721	0.0959	ns	0.9248
5h	0.003125 ± 0.003650	-0.004703 to 0.01095	0.8563*	ns	0.4063

*$t_{(14)}$

Supplementary Table 2.1 - Effect of 0.1 mg/mL prenatal nicotine exposure on the mean speed, analysis across time

Table showing the statistical results of Student's *t* test demonstrating the difference in the mean speed at different time points between the 0.1 and Ctrl groups. Data correspondent to **Figure 4C**.

Ctrl vs. 0.15	Mean Difference	95% CI	$t_{(17)}$	Summary	*p* value
30min	-0.006852 ± 0.003417	-0.01406 to 0.0003559	2.006	ns	0.0611
1h	-0.005057 ± 0.002816	-0.01100 to 0.0008851	1.796	ns	0.0904
1h30min	-0.003057 ± 0.002294	-0.007898 to 0.001784	1.332	ns	0.2004
2h	-0.002125 ± 0.001987	-0.006317 to 0.002067	1.069	ns	0.2998
2h30min	-0.004534 ± 0.002875	-0.01060 to 0.001532	1.577	ns	0.1332
3h	-0.003477 ± 0.004471	-0.01291 to 0.005955	0.7778	ns	0.4474
3h30min	-0.001545 ± 0.003438	-0.008799 to 0.005708	0.4495	ns	0.6587
4h	-0.002523 ± 0.005070	-0.01322 to 0.008173	0.4976	ns	0.6251
4h30min	-0.006216 ± 0.004382	-0.01546 to 0.003029	1.419	ns	0.1741
5h	-0.005136 ± 0.003872	-0.01331 to 0.003033	1.326	ns	0.2022

Supplementary Table 2.2 - Effect of 0.15 mg/mL prenatal nicotine exposure on the mean speed, analysis across time

Table showing the statistical results of Student's *t* test demonstrating the difference in the mean speed at different time points between the 0.15 and Ctrl groups. Data correspondent to **Figure 4C**.

Ctrl vs. 0.2	Mean Difference	95% CI	$t_{(14)}$	Summary	*p* value
30min	**-0.01138 ± 0.003888**	**-0.01971 to -0.003037**	**2.926**	*	**0.0111**
1h	**-0.009625 ± 0.003552**	**-0.01724 to -0.002008**	**2.710**	*	**0.0169**
1h30min	**-0.007750 ± 0.003535**	**-0.01533 to -0.0001684**	**2.192**	*	**0.0457**
2h	**-0.008125 ± 0.003193**	**-0.01497 to -0.001277**	**2.545**	*	**0.0234**
2h30min	-0.009000 ± 0.004476	-0.01860 to 0.0005993	2.011	ns	0.0640
3h	-0.008125 ± 0.005978	-0.02095 to 0.004697	1.359	ns	0.1956
3h30min	-0.001625 ± 0.005809	-0.01408 to 0.01083	0.2797	ns	0.7838
4h	-0,009000 ± 0,004673	-0.01902 to 0.001023	1.926	ns	0.0747
4h30min	-0,006250 ± 0,004935	-0.01683 to 0.004334	1.267	ns	0.2260
5h	-0,008000 ± 0,006030	-0.02093 to 0.004932	1.327	ns	0.2058

Supplementary Table 2.3 - Effect of 0.2 mg/mL prenatal nicotine exposure on the mean speed, analysis across time

Table showing the statistical results of Student's *t* test demonstrating the difference in the mean speed at different time points between the 0.2 and Ctrl groups. Data correspondent to **Figure 4C**.

Supplementary Tables 3 - Effect of different doses in the prenatal nicotine exposure on the max speed, analysis across time

Ctrl vs. 0.1	Mean Difference	95% CI	$t_{(15)}$	Summary	*p* value
30min	**0.2292 ± 0.09582**	**0.02499 to 0.4335**	**2.392**	*	**0.0303**
1h	-0.01153 ± 0.04246	-0.1020 to 0.07897	0.2715	ns	0.7897
1h30min	0.01017 ± 0.02923	-0.05213 to 0.07246	0.3479	ns	0.7328
2h	0.004875 ± 0.03945	-0.07973 to 0.08948	0.1236*	ns	0.9034
2h30min	0.02814 ± 0.04508	-0.06795 to 0.1242	0.6242	ns	0.5419
3h	-0.1235 ± 0.06847	-0.2694 to 0.02244	1.804	ns	0.0914
3h30min	-0.03710 ± 0.03813	-0.1184 to 0.04419	0.9728	ns	0.3461
4h	0.06172 ± 0.05452	-0.05448 to 0.1779	1.132	ns	0.2753
4h30min	-0.1755 ± 0.1690	-0.5357 to 0.1847	1.039	ns	0.3154
5h	0.06107 ± 0.07394	-0.09654 to 0.2187	0.8259	ns	0.4218

* $t_{(14)}$

Supplementary Table 3.1 - Effect of 0.1 mg/mL prenatal nicotine exposure on the max speed, analysis across time

Table showing the statistical results of Student's *t* test demonstrating the difference in the max speed at different time points between the 0.1 and Ctrl groups. Data correspondent to **Figure 4D**.

Ctrl vs. 0.15	Mean Difference	95% CI	$t_{(17)}$	Summary	*p* value
30min	**0.2578 ± 0.08518**	**0.07809 to 0.4375**	**3.027**	**	**0.0076**
1h	0.01745 ± 0.02439	-0.03426 to 0.06916	0.7154*	ns	0.4847
1h30min	0.03914 ± 0.02777	-0.01945 to 0.09772	1.409	ns	0.1767
2h	-0.01151 ± 0.02797	-0.07051 to 0.04749	0.4116	ns	0.6858
2h30min	0.04261 ± 0.04625	-0.05497 to 0.1402	0.9213	ns	0.3698
3h	-0.07686 ± 0.05013	-0.1826 to 0.02890	1.533	ns	0.1436
3h30min	-0.02597 ± 0.02350	-0.07554 to 0.02361	1.105	ns	0.2845
4h	-0.04380 ± 0.02498	-0.09676 to 0.009157	1.753*	ns	0.0987
4h30min	-0.02241 ± 0.08717	-0.2063 to 0.1615	0.2571	ns	0.8002
5h	-0.08628 ± 0.05023	-0.1923 to 0.01969	1.718	ns	0.1040

*$t_{(16)}$

Supplementary Table 3.2 - Effect of 0.15 mg/mL prenatal nicotine exposure on the max speed, analysis across time

Table showing the statistical results of Student's *t* test demonstrating the difference in the max speed at different time points between the 0.15 and Ctrl groups. Data correspondent to **Figure 4D**.

Ctrl vs. 0.2	Mean Difference	95% CI	$t_{(14)}$	Summary	*p* value
30min	0.1858 ± 0.1028	-0.03474 to 0.4062	1.807	ns	0.0923
1h	-0.008625 ± 0.04389	-0.1028 to 0.08552	0.1965	ns	0.8470
1h30min	0.01575 ± 0.03399	-0.05716 to 0.08866	0.4633	ns	0.6502
2h	-0.03750 ± 0.05995	-0.1661 to 0.09107	0.6256	ns	0.5417
2h30min	-0.006250 ± 0.07334	-0.1635 to 0.1510	0.0852	ns	0.9333
3h	-0.06513 ± 0.05595	-0.1851 to 0.05489	1.164	ns	0.2639
3h30min	0.01638 ± 0.06667	-0.1266 to 0.1594	0.2456	ns	0.8095
4h	-0.05788 ± 0.02736	-0.1166 to 0.0008161	2.115	ns	0.0528
4h30min	-0.01638 ± 0.08714	-0.2033 to 0.1705	0.1879	ns	0.8536
5h	-0.02000 ± 0.06724	-0.1642 to 0.1242	0.2975	ns	0.7705

Supplementary Table 3.3 - Effect of 0.2 mg/mL prenatal nicotine exposure on the max speed, analysis across time

Table showing the statistical results of Student's *t* test demonstrating the difference in the max speed at different time points between the 0.2 and Ctrl groups. Data correspondent to **Figure 4D**.

Age	0.2 vs. Ctrl	Mean or Median Difference	95% CI	$t_{(41)}$	Summary	*p* value
6w	15min	3.864 ± 3.791	-3.792 to 11.52	1.019	ns	0.3140
	30min	10.36 ± 5.101	0.05894 to 20.66	2.031	*	0.0488
	1h	19.15 ± 8.566	1.855 to 36.45	2.236	*	0.0308
	1h30min	23.93	-	-	*	0.0416
	2h	22.78	-	-	ns	0.0963
9w	15min	0.4766 ± 3.459	-6.508 to 7.461	0.1378	ns	0.8911
	30min	2.011 ± 4.370	-6.814 to 10.84	0.4603	ns	0.6477
	1h	7.465 ± 7.686	-8.056 to 22.99	0.9714	ns	0.3371
	1h30min	10.01 ± 10.62	-11.44 to 31.45	0.9425	ns	0.3515
	2h	10.52 ± 12.70	-15.12 to 36.16	0.8285	ns	0.4122

Supplementary Table 4 - Effect of 0.2 mg/mL prenatal nicotine exposure on the distance traveled, analysis across time

Table showing the statistical results of Student's *t* test (mean difference) or Mann-Whitney test (median difference) demonstrating the difference in the distance traveled at different time points between the 0.2 and Ctrl groups. Data correspondent to **Figure 7B and Figure 7C**.

Age	0.2 vs. Ctrl	Mean or Median Difference	95% CI	$t_{(41)}$	Summary	*p* value
6w	15min	0.007000	-	-	ns	0.0946
	30min	0.005846 ± 0.002830	0.0001320 to 0.01156	2.066	*	0.0452
	1h	0.005251 ± 0.002411	0.0003830 to 0.01012	2.178	*	0.0352
	1h30min	0.004000	-	-	*	0.0499
	2h	0.003000	-	-	ns	0.1155
9w	15min	-0.002500	-	-	ns	0.9376
	30min	0.0009740 ± 0.002428	-0.003929 to 0.005877	0.4012	ns	0.6904
	1h	0.002143 ± 0.002130	-0.002159 to 0.006444	1.006	ns	0.3203
	1h30min	0.001814 ± 0.001952	-0.002127 to 0.005755	0.9295	ns	0.3581
	2h	0.001431 ± 0.001776	-0.002157 to 0.005018	0.8054	ns	0.4252

Supplementary Table 5 - Effect of 0.2 mg/mL prenatal nicotine exposure on the mean speed, analysis across time

Table showing the statistical results of Student's *t* test (mean difference) or Mann-Whitney test (median difference) demonstrating the difference in the mean speed at different time points between the 0.2 and Ctrl groups. Data correspondent to **Figure 7E and Figure 7F**.

Age	0.2 vs. Ctrl	Mean Difference	95% CI	$t_{(40)}$	Summary	*p* value
6w	15min	0.008090 ± 0.02001	-0.03238 to 0.04856	0.4044*	ns	0.6882
	30min	0.01576 ± 0.02111	-0.02690 to 0.05842	0.7467	ns	0.4596
	1h	0.01024 ± 0.02388	-0.03803 to 0.05851	0.4287	ns	0.6704
	1h30min	0.009190 ± 0.02364	-0.03858 to 0.05696	0.3888	ns	0.6995
	2h	0.03510 ± 0.02555	-0.01657 to 0.08677	1.374*	ns	0.1772
9w	15min	0.003965 ± 0.01778	-0.03195 to 0.03988	0.2230**	ns	0.8247
	30min	-0.000450 ± 0.0168	-0.03441 to 0.03351	0.02678**	ns	0.9788
	1h	-0.00510 ± 0.01794	-0.04134 to 0.03113	0.2846**	ns	0.7774
	1h30min	-0.01321 ± 0.01710	-0.04778 to 0.02135	0.7726	ns	0.4443
	2h	-0.02050	-	-	ns	0.3326

*$t_{(39)}$ **$t_{(41)}$

Supplementary Table 6 - Effect of 0.2 mg/mL prenatal nicotine exposure on the max speed, analysis across time

Table showing the statistical results of Student's *t* test (mean difference) or Mann-Whitney test (median difference) demonstrating the difference in the max speed at different time points between the 0.2 and Ctrl groups. Data correspondent to **Figure 7H and Figure 7I**.

Age	0.2 vs. Ctrl	Mean Difference	95% CI	$t_{(41)}$	Summary	*p* value
6w	15min	-36.45	-	-	ns	0.9521
	30min	-31.05 ± 27.76	-87.11 to 25.02	1.118	ns	0.2700
	1h	-68.64 ± 72.80	-215.7 to 78.38	0.9429	ns	0.3512
	1h30min	-90.13 ± 116.0	-324.4 to 144.1	0.7770	ns	0.4416
	2h	-156.9	-	-	ns	0.1958
9w	15min	26.25	-	-	ns	0.5235
	30min	-10.40	-	-	ns	0.3168
	1h	-80.87 ± 63.07	-208.2 to 46.49	1.282	ns	0.2069
	1h30min	-119.8	-	-	ns	0.3922
	2h	-286.3	-	-	ns	0.1411

Supplementary Table 7 - Effect of 0.2 mg/mL prenatal nicotine exposure on the time spent in the periphery, analysis across time

Table showing the statistical results of Student's *t* test (mean difference) or Mann-Whitney test (median difference) demonstrating the difference in the time spent in the periphery zone at different time points between the 0.2 and Ctrl groups. Data correspondent to **Figure 7K and Figure 7L**.

Age	0.2 vs. Ctrl	Mean Difference	95% CI	$t_{(41)}$	Summary	*p* value
6w	15min	1.998 ± 12.04	-22.31 to 26.31	0.1660	ns	0.8690
	30min	32.10 ± 27.66	-23.76 to 87.97	1.160	ns	0.2526
	1h	69.67 ± 72.71	-77.18 to 216.5	0.9581	ns	0.3436
	1h30min	91.17 ± 115.9	-143.0 to 325.3	0.7864	ns	0.4362
	2h	156.4	-	-	ns	0.2043
9w	15min	9.700	-	-	ns	0.5882
	30min	11.00	-	-	ns	0.3168
	1h	80.94 ± 63.08	-46.44 to 208.3	1.283	ns	0.2066
	1h30min	119.5	-	-	ns	0.3922
	2h	286.3	-	-	ns	0.1411

Supplementary Table 8 - Effect of 0.2 mg/mL prenatal nicotine exposure on the time spent in the center, analysis across time

Table showing the statistical results of Student's *t* test (mean difference) or Mann-Whitney test (median difference) demonstrating the difference in the time spent in the center zone at different time points between the 0.2 and Ctrl groups. Data correspondent to **Figure 7K and Figure 7L**.

Group	Day of Training	Mean Difference	95% CI	$q_{(9)}$	Summary	*p* value
Ctrl	D1 vs. D2	39.25	9.639 to 68.87	5.234	*	0.0123
	D1 vs. D3	50.14	21.63 to 78.65	6.945	**	0.0022
	D2 vs. D3	10.89	3.558 to 18.22	5.864	**	0.0063
0.2	D1 vs. D2	43.99	11.19 to 76.78	5.296	*	0.0115
	D1 vs. D3	55.15	22.84 to 87.45	6.740	**	0.0026
	D2 vs. D3	11.16	-1.924 to 24.24	3.368	ns	0.0945

Supplementary Table 9 - Effect of 0.2 mg/mL prenatal nicotine exposure on the test duration, analysis across time

Table showing the statistical results of Tukey's Multiple comparisons test demonstrating the difference in the test duration at different time points (days of training) between the 0.2 and Ctrl groups. Data correspondent to **Figure 8D**.

Group	Day of Training	Mean Difference	95% CI	$q_{(9)}$	Summary	*p* value
Ctrl	D1 vs. D2	1.987	0.07484 to 3.900	4.103	*	0.0422
	D1 vs. D3	2.297	0.4717 to 4.123	4.696	*	0.0163
	D2 vs. D3	0.3100	-0.1468 to 0.7668	2.680	ns	0.1955
0.2	D1 vs. D2	1.809	-0.2805 to 3.899	3.419	ns	0.0894
	D1 vs. D3	2.319	0.2493 to 4.389	4.424	*	0.0296
	D2 vs. D3	0.5100	-0.3704 to 1.390	2.287	ns	0.2878

Supplementary Table 10 - Effect of 0.2 mg/mL prenatal nicotine exposure on the distance traveled, analysis across time

Table showing the statistical results of Tukey's Multiple comparisons test demonstrating the difference in the distance traveled at different time points (days of training) between the 0.2 and Ctrl groups. Data correspondent to **Figure 8E**.

Group	Day of Training	Mean Difference	95% CI	$q_{(9)}$	Summary	*p* value
Ctrl	D1 vs. D2	9.100	0.5827 to 17.62	4.219	*	0.0371
	D1 vs. D3	11.47	3.096 to 19.84	5.409	*	0.0102
	D2 vs. D3	2.367	-0.2379 to 4.971	3.588	ns	0.0743
0.2	D1 vs. D2	9.167	-3.085 to 21.42	2.954	ns	0.1471
	D1 vs. D3	11.23	0.5001 to 21.97	4.132	*	0.0408
	D2 vs. D3	2.067	-5.195 to 9.329	1.124	ns	0.7154

Supplementary Table 11 - Effect of 0.2 mg/mL prenatal nicotine exposure on the total number of errors, analysis across time

Table showing the statistical results of Tukey's Multiple comparisons test demonstrating the difference in the total number of errors at different time points (days of training) between the 0.2 and Ctrl groups. Data correspondent to **Figure 8H**.

Group	Day of Training	Mean Difference	95% CI	$q_{(9)}$	Summary	*p* value
Ctrl	D1 vs. D2	11.87	-1.492 to 25.23	3.508	ns	0.0812
	D1 vs. D3	18.44	8.018 to 28.87	6.985	**	0.0021
	D2 vs. D3	6.577	-0.7753 to 13.93	3.532	ns	0.0790
0.2	D1 vs. D2	21.31	-9.482 to 52.10	2.733	ns	0.1852
	D1 vs. D3	30.98	0.1685 to 61.78	3.970	*	0.0488
	D2 vs. D3	9.666	-4.435 to 23.77	2.707	ns	0.1902

Supplementary Table 12 - Effect of 0.2 mg/mL prenatal nicotine exposure on the primary latency, analysis across time

Table showing the statistical results of Tukey's Multiple comparisons test demonstrating the difference in the primary latency at different time points (days of training) between the 0.2 and Ctrl groups. Data correspondent to **Figure 8I**.

Group	Day of Training	Mean Difference	95% CI	$q_{(9)}$	Summary	*p* value
Ctrl	D1 vs. D2	39.50	9.846 to 69.15	5.260	*	0.0120
	D1 vs. D3	50.43	21.74 to 79.13	6.940	**	0.0022
	D2 vs. D3	10.93	3.532 to 18.33	5.833	**	0.0065
0.2	D1 vs. D2	44.00	11.09 to 76.91	5.278	*	0.0117
	D1 vs. D3	55.27	22.78 to 87.76	6.717	**	0.0027
	D2 vs. D3	11.27	-2.058 to 24.59	3.339	ns	0.0976

Supplementary Table 13 - Effect of 0.2 mg/mL prenatal nicotine exposure on the total latency, analysis across time

Table showing the statistical results of Tukey's Multiple comparisons test demonstrating the difference in the total latency at different time points (days of training) between the 0.2 and Ctrl groups. Data correspondent to **Figure 9A**.

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