Sex-specific effects of psychoactive drugs on memory performance in an animal model of Attention-Deficit Hyperactivity Disorder

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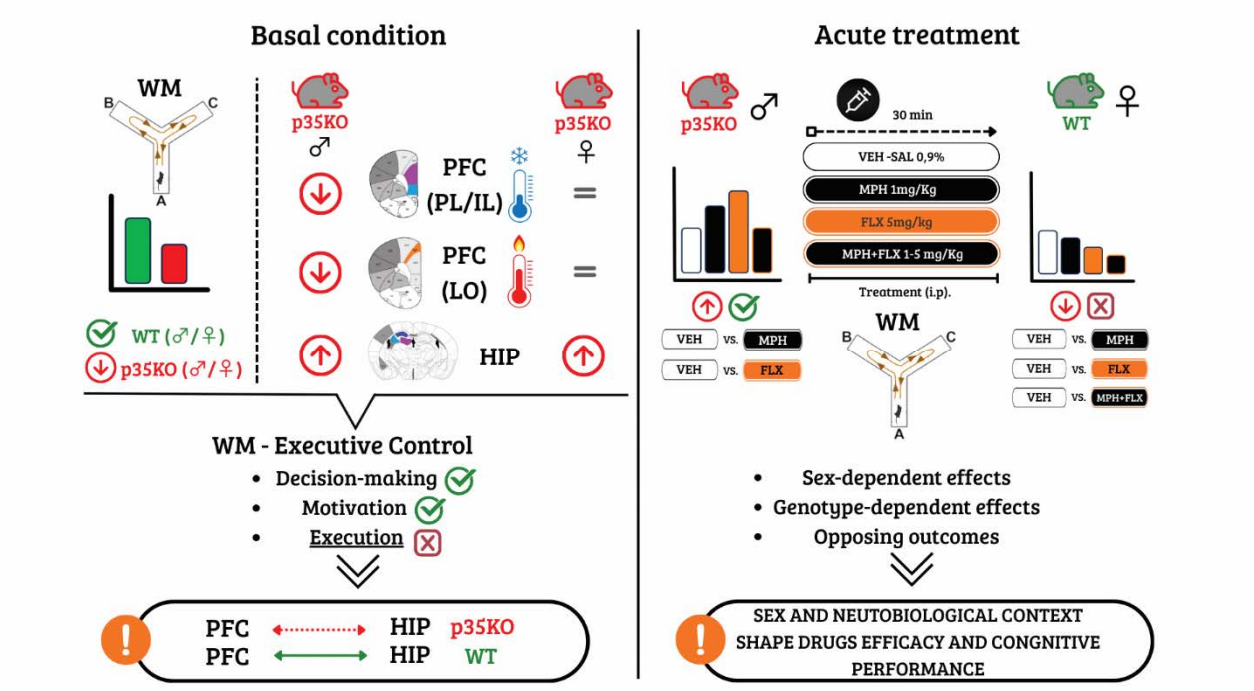
Sex-specific psychotropic effects on memory in an ADHD model

ABSTRACT

Attention-Deficit/Hyperactivity Disorder (ADHD) is a prevalent neurodevelopmental condition characterized by persistent deficits in working memory (WM) and executive control. Dysregulation of the Cyclin-dependent kinase 5 (Cdk5)/p35 signaling pathway has been implicated in ADHD pathophysiology due to its impact on neuronal connectivity and dopamine regulation. Using p35 knockout (p35KO) mice—a validated model exhibiting ADHD-like phenotypes—we investigated sex-specific WM performance, neuronal activation patterns, and responses to acute treatment with methylphenidate (MPH) or fluoxetine (FLX), administered alone or in combination. Under basal conditions, p35KO mice of both sexes exhibited significant WM impairment in the Y-maze test compared with wild type (WT) counterparts, whereas recognition memory remained intact. Analysis of neuronal activation (c-Fos-IR) 90 minutes after testing revealed region- and sex-dependent alterations. p35KO males showed significantly reduced c-Fos-IR expression in prefrontal cortical regions, while both sexes exhibited increased c-Fos-IR in hippocampal regions. Acute MPH or FLX treatment significantly improved WM in p35KO males, but this benefit was lost with the combined treatment (MPH+FLX). In contrast, p35KO females showed no WM improvement with any treatment. Notably, WT females exhibited a pronounced decline in WM after exposure to MPH, FLX, or their combination, indicating sex-specific pharmacological sensitivity in healthy animals. These findings highlight the critical role of Cdk5/p35 signaling in maintaining the functional balance between prefrontal and hippocampal networks. Our results demonstrate that pharmacological responses in this ADHD model are highly sensitive to both sex and neurobiological background, emphasizing the importance of considering sex as a biological variable in preclinical and translational ADHD research.



INTRODUCTION

Attention-Deficit/Hyperactivity Disorder (ADHD) is one of the most prevalent neurodevelopmental conditions in childhood and adolescence, with symptoms often persisting into adulthood. It is characterized by a persistent pattern of inattention, hyperactivity, and impulsivity, which interfere with academic performance, social functioning, and emotional regulation [1].

A deficit in working memory (WM) is one of the most consistently reported cognitive impairments in ADHD affecting the temporary storage and manipulation of information required for reasoning, planning, and goal-directed behavior [2]. WM involves the dynamic interaction of multiple brain regions, particularly the prefrontal cortex (PFC), which orchestrates attentional control and executive functions, and the hippocampus, which contributes to the encoding and retrieval of spatial and contextual information. Dysregulation within this fronto-hippocampal network has been associated with WM deficits observed in ADHD and related disorders [3].

The pharmacological treatment of ADHD typically involves the use of psychostimulants, such as methylphenidate (MPH), which enhance dopaminergic and noradrenergic signaling by blocking the reuptake of neurotransmitters. While MPH has proven efficacy in reducing core symptoms of ADHD, its effects are not uniform across individuals, with biological sex emerging as a relevant factor. In cases where ADHD presents with comorbid mood disorders, the selective serotonin reuptake inhibitor fluoxetine (FLX) is sometimes used as an adjunct treatment. However, the cognitive effects of FLX [4], alone or in combination with MPH, remain poorly characterized, particularly in developing individuals or animal models representing ADHD-like phenotypes.

A key molecular player in neurodevelopment and cognitive processing is Cyclin-dependent kinase 5 (Cdk5). Unlike other Cdks, Cdk5 requires association with a non-cyclin activator, p35 or p39, to become functionally active [5–8]. Alterations in the Cdk5/p35 signaling have been implicated in a variety of neurological and psychiatric conditions [9–14], including ADHD [15–17], due to its impact on dopamine signaling and neuronal connectivity. Mutant mouse lacking p35 (p35 knockout mice, p35KO) represents a valuable model for studying the neurobiological basis of ADHD-like symptoms [18,19]. Results from our laboratory have shown that these mice exhibit alterations in dopaminergic function, hyperactivity and the decrease of locomotor activity following MPH or amphetamine treatment—features that align with diagnostic criteria for ADHD [16,17]. However, the pharmacological improvement of symptoms, as well as the influence of psychoactive drug type and biological sex, are still poorly understood, despite their potential relevance to treatment efficacy. Given the interplay between neurotransmitter systems, molecular signaling pathways, and cognitive functions, it is imperative to explore how pharmacological agents like MPH, FLX, and their combination influence WM, particularly in the context of ADHD. To address this, we employed the p35KO mice—a validated model of ADHD-like phenotypes

characterized by Cdk5/p35 dysregulation—to examine the memory performance and neuronal activation patterns under basal conditions, as well as the effects of acute drugs administration. Furthermore, given the growing evidence of sex-specific treatment responses, we examined these effects in both male and female subjects to better understand the biological basis of individual variability that may contribute to differential therapeutic outcomes in ADHD.

MATERIAL AND METHODS

Murine model of ADHD

Male and female wild type (WT) and p35 (p35 KO) mutant mice were generated by breeding heterozygous mutants (kind gifts of Dr. L.H. Tsai) [20] maintained a C57BL/6J background via brother-sister mating in the vivarium of the INIMEC-CONICET-UNC (Cordoba, Argentina). Animals were weaned at 21 postnatal and housed up to six per cage under a 12-h light/12-h dark cycle and a constant temperature (22° C) with free access to food and water. All the experiments were performed during the light cycle, between 10:00 AM to 3:00 PM in a separated behavioral room. During the test, the room was quiet and slightly lit and mice were allowed to acclimatize for 30 min before the onset of each experiment. All animal procedures and care were approved by the National Department of Animal Care and Health (SENASA – ARGENTINA) and complied with the National Institute of Health guidelines for the Care and Use of Laboratory Animals. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (CICUAL) at INIMEC-CONICET-UNC. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Experiment 1: Working memory, exploratory behavior, and neuronal activation (Y-maze test)

WT and p35KO mice (both males and females) underwent daily handling at postnatal day (PND) 22 to habituate the animals to human contact and minimize stress during subsequent behavioral assessments. On PND 23 mice were evaluated in the Y-maze, a well-established paradigm used to assess spontaneous alternation as a measure of spatial WM and total arm entries as an index of exploratory behavior. Each animal was placed in the maze for 8 minutes, and spontaneous alternation behavior was recorded as an index of WM performance. 90 minutes after completing the behavioral test, mice were anesthetized and transcardially perfused with fixative solutions for brain collection (Fig. 1a.). Brains were coronally sectioned and processed for c-Fos-IR immunohistochemistry to quantify region-specific neuronal activation induced by Y-maze exploration.

Experiment 2: Recognition memory assessment (Novel Object Recognition Test)

Another group of mice, including WT and p35KO animals of both sexes, received daily handling starting at PND 22 and were subsequently (PND 23) subjected to the Novel Object Recognition (NOR) paradigm, which evaluates recognition memory based on the innate preference of rodents for novelty. On PND 23 (day 2), animals were habituated to the testing arena. On PND 24 (day 3), the task consisted of a 10-minute familiarization trial with two identical objects (A–A), followed by a 1-hour inter-trial interval. Mice were then reintroduced to the arena for a 10-minute test phase in which one familiar object was replaced by a novel object (A–B) (Fig.1b). The time spent exploring the novel versus the familiar object was recorded as a measure of recognition memory and preference for novelty.

Experiment 3: Psychoactive drugs treatment

Drug treatments were randomly assigned and consisted of a single intraperitoneal (i.p.) injection administered to mice of both sexes (male and female) and genotypes (p35KO and WT) 30 minutes

prior to the Y-maze test (Fig. 1c.). Animals received either methylphenidate (MPH; 1 mg/kg), fluoxetine (FLX; 5 mg/kg), a combination of MPH + FLX (1-5 mg/kg), or Vehicle (VEH; 0.9% saline solution). The MPH used was Ritalin® (Novartis), and FLX was Foxetin® (Gador). The selected doses were based on previous studies. Regarding MPH, this dose increased locomotor activity in WT mice while producing a marked decrease in locomotion in p35KO mice relative to VEH-treated controls [16]. The FLX dose has been shown to alter serotonergic tone and modulate cognitive and emotional behaviors without inducing anxiogenic or sedative effects in rodents [21–23]. All procedures involving substance administration were approved by resolution 021-2017B of the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) at the Instituto de Investigación Médica Mercedes y Martín Ferreyra.

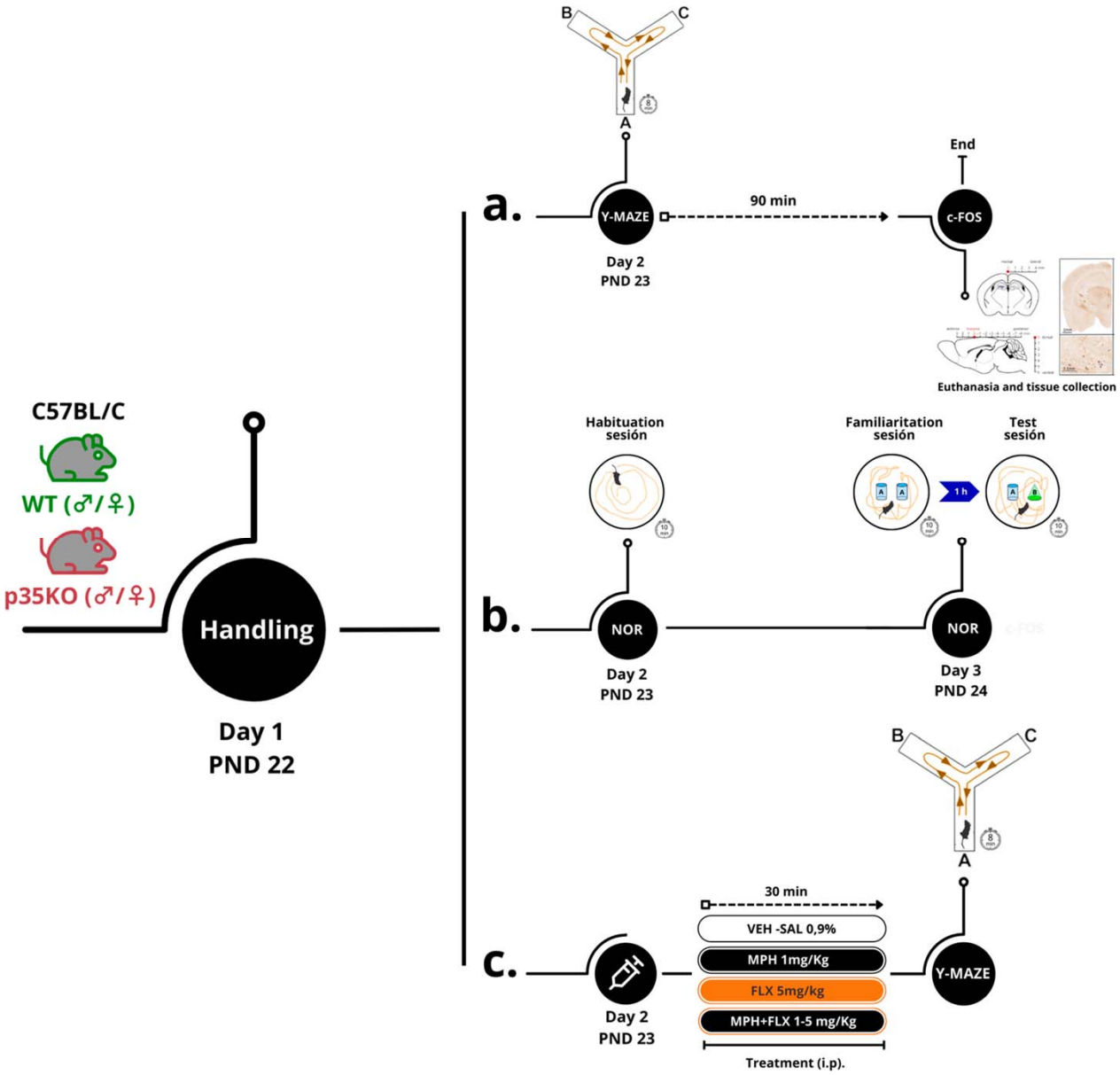


Figure 1. Experimental timelines. (**a**) ***Y-maze task and brain processing for c-Fos-IR immunohistochemistry.*** WT and p35KO mice (males and females) were handled daily starting at PND 22. At PND 23, animals were tested in the Y-maze for 8 min. 90 min after behavioral testing, mice were anesthetized and transcardially perfused for brain collection and preparation of coronal slices for c-Fos-IR immunostaining **(Experiment 1). (b)** ***Novel Object Recognition (NOR) task.*** WT and p35KO mice (males and females) were handled daily starting at PND 22. On day 2 (PND 23), mice were habituated to the testing arena. On day 3 (PND24), they underwent the NOR task: a 10-min familiarization phase with two identical objects (A–A), a 1-h inter-phase interval, and a 10-min test trial (A–B). Exploration time of the novel versus the familiar object was quantified as an index of recognition memory **(Experiment 2).** (**c**) ***Y-maze task with psychoactive drug administration***. WT and p35KO mice (males and females) were handled daily starting at PND 22.

At PND 23, animals received psychoactive drug treatments **(Experiment 3).** 30 min after drug administration, mice were tested in the Y-maze for 8 min.

Behavioral tests

Y-maze test (Y-MAZE)

The Y-maze apparatus was constructed of white-painted plywood, with each arm measuring 50 cm in length, 12 cm in height and 4 cm in width, positioned at equal angles (120º). Each mouse was placed at the end of one arm, facing the center of the maze, and allowed to explore freely for an 8-min session. The sequence of arm entries was recorded manually. Spontaneous alternation behavior was defined as consecutive entries into all three arms in overlapping triplet sets. The percent of spontaneous alternation was calculated as the ratio of actual to possible alternations, where possible alternations equal the total number of arm entries minus two, multiplied by 100, as described previously [21,24]. The maze was cleaned with 70% ethanol between trials to eliminate olfactory cues.

Novel object recognition task (NOR)

The NOR test was performed to evaluate short-term recognition memory based on spontaneous exploratory behavior. The task consisted of three phases: habituation (10 min free exploration of an empty arena), familiarization/acquisition (10 min exploration of two identical objects), and test/retention (1 h later, one familiar object replaced with a novel one). The objects differed in shape but were otherwise identical in size, material, color, and odor, and were not climbable. Trials were conducted in a circular arena (40 cm diameter under dim light conditions (~40 lx). Exploration was defined as the time the animal's nose was within ≤3 cm of an object, accompanied by active vibrissae movement. A recognition index was calculated as the ratio of time spent exploring the

novel object to the total exploration time of both objects. The arena and objects were cleaned with 70% ethanol between trials to eliminate olfactory cues.

c-Fos-IR immunohistochemistry and quantification

Neuronal activation was assessed through c-Fos-IR immunoreactivity following established procedures [25]. WT and p35KO mice were anesthetized with 30% chloral hydrate and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were post-fixed, cryoprotected, and cut into 40-µm coronal sections. Sections were incubated with a rabbit anti–c-Fos antibody (Ab-5, Oncogene Science) and processed using a biotinylated secondary antibody, avidin–biotin complex, and DAB for visualization. Brain section images were acquired using a PhenoImager™ Fusion microscope (Akoya Biosciences) and visualized with Phenochart software (v1.2.0), which incorporates multirange spectral detection and multispectral imaging (MSI) technology. Quantification was performed using a custom ImageJ macro that automated image preprocessing, ROI alignment, and particle detection. c-Fos-IR nuclei were quantified across four predefined anteroposterior levels relative to Bregma, each associated with a specific set of cortical or hippocampal regions. The macro generated standardized outputs for nuclei count, nuclear area, and ROI size. Full details of anatomical ROI definitions, preprocessing steps, image-processing parameters, and the complete macro code are provided in the Supplementary Material and Methods.

Statistical analysis

Statistical analyses were performed using Statistica Stat-Soft Inc. version 8.0 Tulsa, OK, USA. Assumptions of homoscedasticity and normality were tested using Levene and Shapiro-Wilks tests, respectively. Data were analyzed using one-, two-, or three-way ANOVAs as appropriate, followed by Tukey's post hoc tests for multiple comparisons. The factors included in the analyses were

genotype (WT vs. p35KO), sex (male vs. female), and treatment (saline, MPH, FLX, MPH+FLX). Statistical significance was set at $p < 0.05$. Data are presented as the mean ± standard error of the mean (SEM).

RESULTS

Impairment of Working Memory in Mice Lacking Cdk5-activator p35

Given the relevance of WM deficits in ADHD, we first investigated this cognitive domain in WT and p35KO mice under basal conditions. To this end, we analyzed possible sex- and genotype-dependent differences in cognitive performance using the spontaneous Y-maze test. Healthy animals (WT) showed sex-dependent differences in the Y-maze, with WT females exhibiting a higher percent of alternation compared to males. In addition, genotype-related differences were observed in both sexes. Male and female p35KO mice exhibited impairment of WM, as evidenced by a significant reduction in the percent of alternation compared to their WT counterparts (Fig. 2a). Additionally, p35KO mice of both sexes showed a significant increase in the total number of arm entries (Fig. 2b), denoting an enhanced exploratory motivation in this animal model of ADHD.

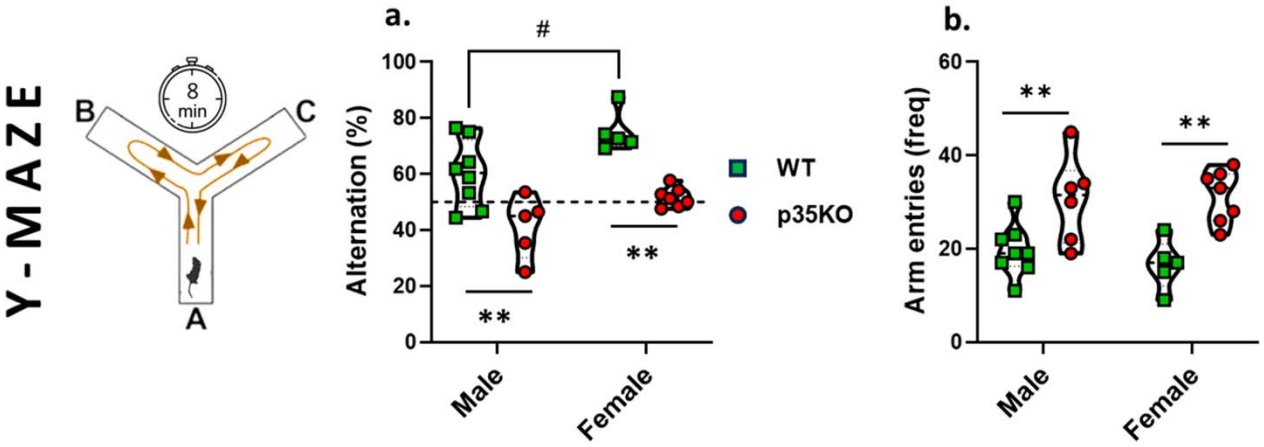


Figure 2. Cognitive and exploratory performance of WT and p35KO mice of both sexes in the Y-maze task, evaluating working memory through alternation rate and exploratory motivation through arm entry frequency.

Alternation percentage (a): Both male and female p35KO mice displayed decreased alternation performance compared with their WT controls, indicating impaired working memory (n = 9–10, **p<0.01). **Arm entry frequency (b)** Both male and female p35KO mice exhibited an increased number of arm entries relative to WT mice, suggesting enhanced exploratory motivation (n = 9–10, **p<0.01). Data are expressed as mean ± SEM. Statistical analyses were performed using ANOVA followed by Tukey's post hoc test.

Sex-Dependent Differences in Exploratory Behavior but Preserved Recognition Memory in p35 KO and WT Mice

To assess hippocampal-sensitive tasks in p35 KO and WT mice, we employed the NOR memory and exploration test, which leverages rodents' innate preference for novelty. This task is widely used to evaluate responses to discrete novel stimuli and the ability to distinguish familiar from unfamiliar objects and is considered to involve hippocampal processing [26,27]. Exploration behavior during the training and test sessions was analyzed across sex and genotype. A significant main effect of sex was observed on total exploration time ($F(1, 63) = 15.62$, ***$p = 0.0002$), with female mice—regardless of genotype—spending less time exploring the objects compared to males (Fig 3a). In the test phase, all groups exhibited a preference for the novel object over the familiar one. Analysis of the preference index showed no significant genotype effect; however, a significant effect of sex was again observed on total exploration time during the test session ($F(1, 62) = 25.71$, ***$p < 0.0001$), as shown in Fig. 3b, c and d. Taken together, these findings suggest that while exploratory behavior is modulated by sex, recognition memory—as assessed by NOR—is not significantly altered by the absence of p35.

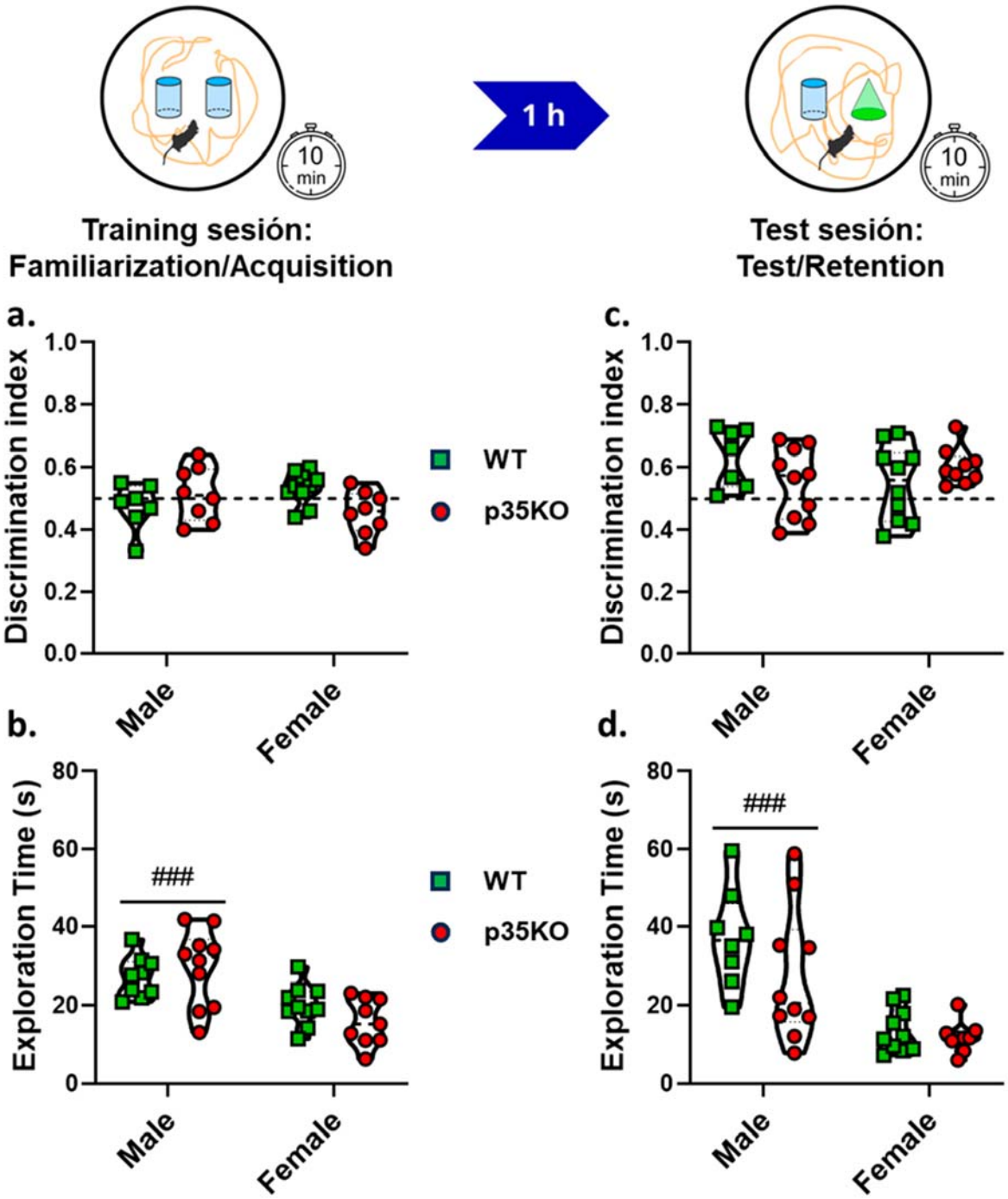


Figure 3: Behavioral performance of WT and p35KO mice of both sexes in the Novel Object Recognition (NOR) task, assessing discrimination and exploratory behavior.

Discrimination index for the novel object during the training (a) and test (c) sessions across experimental groups: No significant effects of genotype or sex were observed.

Exploration time (in seconds) during the training (b) and test (d) sessions across experimental groups. A significant main effect of sex was found both in the training phase ($F(1,63) = 15.62$; ###$p = 0.0002$) and in

the test phase ($F(1,62) = 25.71$; ###$p = 0.0001$). Data are expressed as mean ± SEM for WT males, WT females, p35KO males, and p35KO females. Statistical analyses were performed using ANOVA followed by Tukey's post hoc test.

Hippocampal and cortical neuronal activation in WT and p35KO female and male mice

Neuronal activation was evaluated through the determination of c-Fos-IR transcription factor expression in different regions of prefrontal cortex and hippocampus, 90 min after the Y-maze test (Fig. 4 and S1, S2). The quantification of the number of c-Fos-IR positive cells was performed in CA1, CA3 and dentate gyrus of hippocampus (Fig 4 a, b, c and S1) and prelimbic (PL), infralimbic (IF), Lateral Orbitofrontal cortex (LO), Cingulate (Cg1) and Ventro orbital (VLO) of prefrontal cortex (Fig 4 d, e, f, g, h and S2).

In WT animals, c-Fos-IR expression was significantly higher in males than in females across all prefrontal cortex regions analyzed (Fig.4 d, e, f, g, h and S2) whereas no sex-related differences were detected in the hippocampal regions, including CA1, CA3, and dentate gyrus (Fig. 4 a, b, c and S1).

Comparison of c-Fos-IR expression between WT and p35KO mice revealed a significant reduction in the PL, IF, and LO regions of the prefrontal cortex in p35KO male mice. In contrast, no significant differences were observed between WT and p35KO females across any of the cortical regions analyzed (Fig. 4 d, e, f, g and h). Conversely, analysis of c-Fos-IR expression in the hippocampus revealed that both male and female p35KO mice exhibited significantly higher immunostaining in the CA1 and CA3 regions compared with WT counterparts, indicating enhanced neuronal activity in these areas of the dorsal hippocampus. Moreover, a significant increase in c-Fos-IR expression was observed in dentate gyrus exclusively in female p35KO compared with WT female mice (Fig. 4 c).

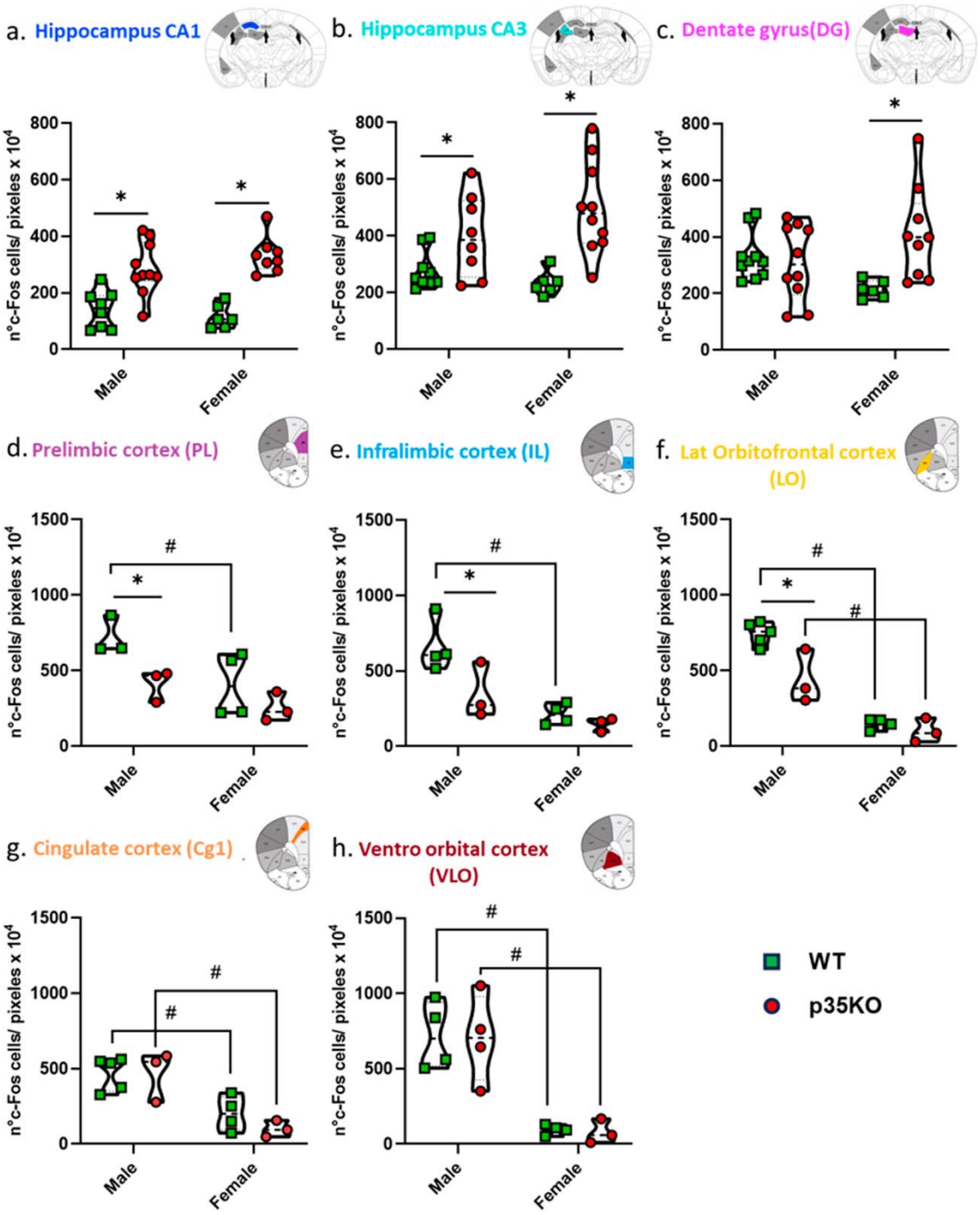


Figure 4. Neuronal activation in the hippocampus and prefrontal cortex of WT and p35KO mice (males and females) assessed by c-Fos-IR immunostaining.

Differences in neuronal activity were observed across hippocampal and prefrontal cortex subregions between WT and p35KO mice of both sexes. **(a) CA1 region**; *p<0.01 indicates a significant genotype effect (WT males vs. p35KO males; WT females vs. p35KO females). **(b) CA3 region**; *p<0.01 indicate significant genotype effects (WT males vs. p35KO males; WT females vs. p35KO females). **(c) Dentate**

gyrus (DG); *p<0.01 indicates a significant genotype effect (WT females vs. p35KO females). **(d) Prelimbic cortex** (PL); #p<0.05 indicates a significant sex effect (WT males vs. WT females), *p<0.05 indicates a significant genotype effect (WT males vs. p35KO males). **(e) Infralimbic cortex (IL)**; ##p<0.01, sex effect (WT males vs. WT females), *p<0.05, genotype effect (WT males vs. p35KO males). **(f) Lateral orbitofrontal cortex (LO)**; ##p<0.01, sex effect (WT males vs. WT females); #p<0.05, sex effect (p35KO males vs. p35KO females); *p<0.05, genotype effect (WT males vs. p35KO males). **(g) Cingulate cortex (Cg1)**; ##p<0.01, sex effect (WT males vs. WT females); #p<0.01, sex effect (p35KO males vs. p35KO females). **(h) Ventrolateral orbitofrontal cortex (VLO)**; ##p<0.01, sex effect (WT males vs. WT females); #p<0.05, sex effect (p35KO males vs. p35KO females). Data are expressed as mean ± SEM. Statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc test.

The treatment with psychoactive drugs improves working memory in p35KO male mice

Working memory was assessed in the Y-maze test 30 minutes after treatment with psychoactive drugs administered either alone (MPH or FLX) or in combination (MPH+FLX). Male p35KO mice treated with MPH or FLX showed a significant improvement in WM, as evidenced by a higher number of alternations compared with vehicle-treated p35KO. In contrast, the combined treatment (MPH+FLX) did not enhance performance in p35KO males, whose alternation scores remained comparable to vehicle-treated p35KO mice and to WT animals receiving the drug combination (Fig. 5 a).

Psychoactive drugs treatment impaired working memory in WT female mice

In female mice, p35KO animals showed no significant differences in WM performance between vehicle- and drug-treated groups (Fig. 5 c). Surprisingly, WT females (healthy) exhibited a significant decline in WM after treatment with MPH, FLX, or their combination (MPH+FLX) compared with vehicle-treated WT controls (Fig. 5 c).

Additionally, the increased motivation to explore the arms in p35 KO animals of both sexes remained unaffected by any drug treatments (Fig. 5 b and d).

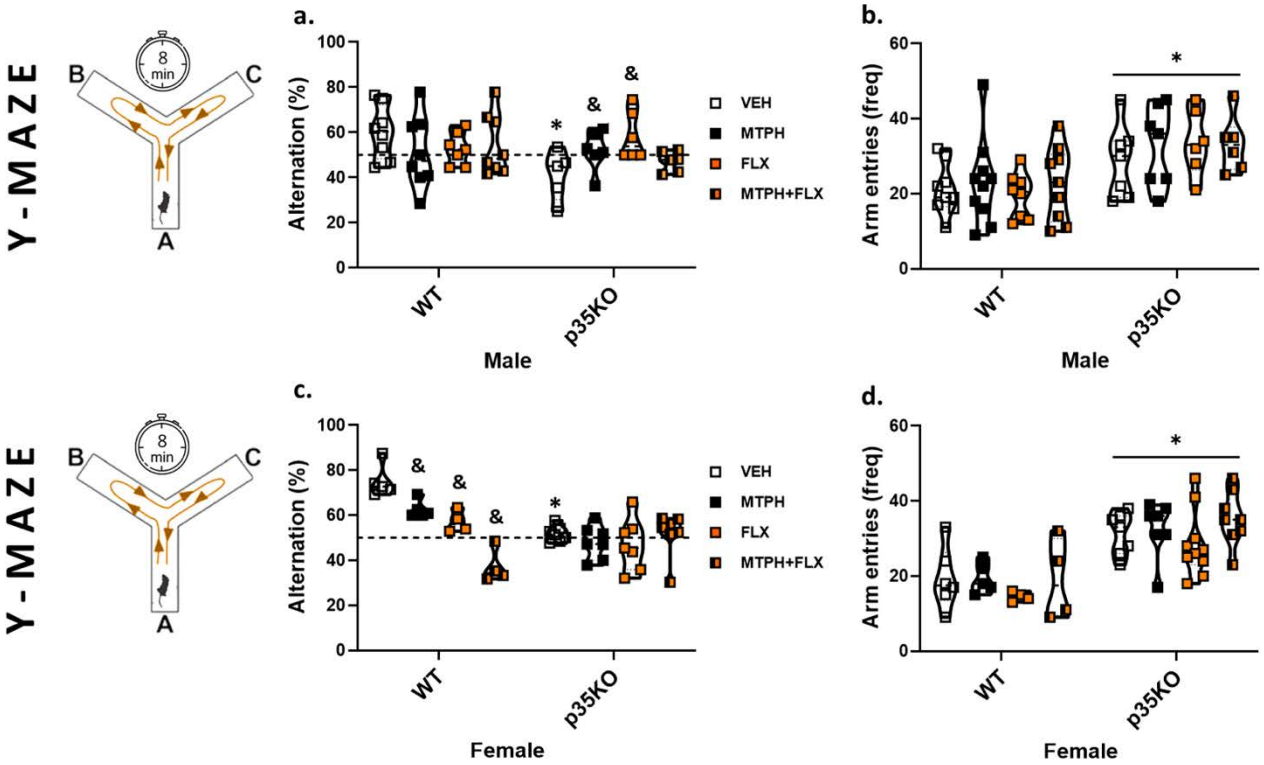


Figure 5. Effects of psychotropic drug treatments on working memory performance in the Y-maze. Males: (a) Alternation percentage; (b) Arm entry frequency. p35KO mice showed reduced alternation, indicating working memory impairment. **Genotype effect: *p<0.05 (p35KO-VEH males WT-VEH males).* WT male mice showed no significant differences in alternation percentage across treatments. In contrast, p35KO male mice exhibited improved working memory following MPH or FLX treatment alone, but not after the combined administration (MPH+FLX). *&Treatment effect: &p<0.05 (p35KO-VEH males vs. p35KO-MPH males or p35KO-FLX males).* p35KO males showed increased arm entries under all treatments, indicating increased motivation to explore the arms (**p < 0.05).*

Females: (c) Alternation percentage; (d) Arm entry frequency. p35KO mice showed reduced alternation, indicating working memory impairment. **Genotype effect: *p<0.05 (p35KO-VEH females WT-VEH females).* WT female mice exhibited impaired working memory when treated with MPH, FLX, or the combination (MPH+FLX), whereas p35KO female mice showed no improvement under any treatment condition. *&Treatment effect: &p<0.05 (WT-VEH female vs. WT-MPH, WT-FLX, WT-MPH+FLX females).* p35KO females showed increased arm entries under all treatments, consistent with the increased motivation to explore the arms (**p < 0.05).*

Data are expressed as mean ± SEM. Statistical comparisons were performed using three-way ANOVA followed by Tukey's post hoc test.

DISCUSION

Attention-Deficit/Hyperactivity Disorder (ADHD) is characterized by a persistent pattern of inattention, hyperactivity, and impulsivity that interferes with academic performance, social functioning, and emotional regulation. These behavioral symptoms arise from complex neurobiological mechanisms underlying the cognitive deficits associated with the disorder. Among the molecular pathways implicated, Cdk5/p35 signaling plays a pivotal role in prefrontal cortical function and WM. Here, we show that disruption of this pathway in p35KO mice, which have reduced Cdk5 activity, leads to altered neuronal activation and sex-specific response patterns to psychoactive drugs, supporting the relevance of this model for studying the cognitive dimension of ADHD.

ADHD is also strongly influenced by sex, with males diagnosed more frequently than females, showing male-to-female ratios of approximately 3:1 to 5:1 [28]. This imbalance partly reflects historical biases in diagnostic criteria and clinical assessment, as females often present with subtler or atypical symptoms that are easily overlooked [29,30]. Despite increasing awareness, women remain less likely to receive pharmacological treatment unless symptoms are severe. Considering these disparities, incorporating sex as a biological variable has become essential for understanding the neurobiological and pharmacological heterogeneity of ADHD. In this context, our study provides experimental evidence addressing sex-specific response patterns in cognitive performance, neuronal activation, and drug response using the p35KO mouse model for studying ADHD.

Given the clinical evidence that cognitive symptoms in ADHD vary between sexes, we first examined WM performance in male and female p35KO mice under basal conditions to determine whether the absence of p35 differentially affects this prefrontal-dependent function. p35KO mice showed significant impairment in WM, evidenced by a reduction in spontaneous alternations in the

Y-maze test compared to WT counterparts, consistent with the executive dysfunction frequently reported in ADHD patients. These deficits were present in both sexes, indicating that the loss of p35—and consequent reduction in Cdk5 activity—impairs prefrontal-dependent cognitive processing regardless of sex. In contrast, recognition memory, as assessed by the NOR test, remained intact in all groups. Additionally, p35KO mice of both sexes exhibited increased exploratory motivation, a behavioral feature that may reflect enhanced novelty seeking associated with ADHD-like traits. Interestingly, females spent less time exploring novel objects than males, regardless of genotype, underscoring the importance of incorporating sex as a biological variable in ADHD research.

To further explore the neural mechanisms underlying the observed behavioral alterations, we analyzed patterns of neuronal activation in prefrontal cortical and hippocampal regions by assessing c-Fos-IR expression. Given the central role of these structures in WM and exploratory behavior, this approach allowed us to determine whether the absence of p35 is associated with region- and sex-specific changes in brain activation that could explain the cognitive and motivational differences observed in p35KO mice.

The c-Fos-IR analysis revealed that p35 deletion induces a robust impairment in WM, a “cold” executive function typically associated with the medial prefrontal cortex [31–33]. In parallel, p35KO animals exhibited altered cortical layering, consistent with a delay in prefrontal neurodevelopment similar to that described in ADHD patients, suggesting an early impact on the organization of cortical circuits required for cognitive control. Integrated into the conceptual framework of cold and hot executive functions proposed by Salehinejad et al. (2021) [33], our data provide a more precise delineation of how different prefrontal subregions contribute to this phenotype.

In male p35KO mice, we observed marked post-task hypoactivation in the prelimbic and infralimbic cortices, regions fundamental for maintaining and manipulating online information. We also detected reduced activation in the orbitofrontal cortex, which is involved in value integration, motivational evaluation, and other hot executive functions. This pattern aligns with the model of Friedman & Robbins (2022) in which these areas constitute essential nodes for controlled cognition, and their dysfunction compromises the capacity to select and execute goal-directed behaviors [34].

In contrast to the prefrontal alterations, we found that the anterior cingulate cortex (ACC)—considered a functional bridge between cold and hot functions and responsible for conflict monitoring, error evaluation, and signaling the need for control—showed similar activation levels in p35KO and WT mice, in both males and females. This preserved activation suggests that monitoring and detection mechanisms for cognitive demands remain intact. Thus, the deficit may not lie in conflict evaluation or motivational signaling, but rather in the prefrontal cortex's ability to implement appropriate behavioral adjustments.

Male p35KO mice also exhibited hippocampal hyperactivation (CA1, CA3, and dentate gyrus), concurrent with prefrontal hypoactivation. This dissociation between a hyperresponsive hippocampus and a less-engaged prefrontal cortex supports the hypothesis of a functional disruption in PFC-hippocampal integration: the animals may retrieve relevant spatial or contextual information yet fail to translate it into efficient decision-making due to impaired executive control. While the ACC likely continues to detect the need for adjustments, the medial PFC—the structure responsible for implementing them—fails to sustain the appropriate strategy.

Females did not exhibit the same pattern of prefrontal hypoactivation, possibly due to compensatory mechanisms or distinct baseline activation thresholds in WT controls. This

interpretation is consistent with previous evidence of sex dimorphism in the functional organization of prefrontal networks. Large-scale neuroimaging studies have demonstrated that intrinsic brain networks exhibit consistent sexual dimorphism, particularly within task-positive control networks that underlie cognitive control and attentional processes [35]. At a more local level, females show enhanced glutamatergic transmission in the mPFC—reflected in higher amplitude and frequency of spontaneous excitatory postsynaptic currents and greater inward rectification—which suggests a stronger excitatory drive that may buffer against reductions in prefrontal activation [36]. Additionally, sex differences in intracortical network activity have been observed ex vivo from prepubertal stages through aging, indicating stable and developmentally conserved divergence in prefrontal network dynamics [37].

In summary, p35 deletion selectively affects prefrontal circuits involved in cold and partially hot executive functions, preserves cingulate monitoring, and generates a desynchronization with hippocampal activity. This integrative profile—prefrontal hypoactivation, preserved ACC activation, and hippocampal hyperactivation—provides a robust explanatory framework for the WM deficits observed and positions the p35KO model as a valuable tool for investigating the neurobiological mechanisms of neurodevelopmental disorders such as ADHD. In this context, WM relies on coordinated activity across these regions [31,38,39], and thus Cdk5/p35 deficiency could lead to dysregulated neuronal signaling within these networks, contributing to the cognitive impairments associated with ADHD.

The pharmacological results highlight genotype-dependent effects with distinct within-sex response patterns to psychoactive drugs in the p35KO model of ADHD. Improvements in WM were restricted to male p35KO mice treated with either MPH or FLX but not when both drugs were

combined, while females showed no comparable benefit, suggesting sex-specific expression of genotype-related effects.

This improvement parallels the "paradoxical response" to psychostimulants previously described in male mice of this model, where drugs such as MPH or amphetamine reduce hyperactivity and normalize dopaminergic signaling, in contrast to their activating effects in WT animals [16]. Previous evidence indicates that Cdk5/p35 dysfunction alters dopamine homeostasis by increasing striatal dopamine content while reducing dopamine turnover and surface DAT expression [16,17]. Thus, the beneficial effects of MPH on WM likely reflect a restoration of dopaminergic tone within fronto-striatal circuits [40], a mechanism that may also underlie the partial efficacy of FLX through serotonergic modulation of prefrontal and hippocampal pathways.

The absence of improvement with the combined MPH+FLX treatment is particularly noteworthy. Several studies have shown that co-administration of SSRIs with psychostimulants can potentiate gene regulation in striatal regions, increase stereotyped behaviors, and facilitate cocaine self-administration [41–43]. Such molecular and behavioral potentiation may counteract the therapeutic benefits of each drug when given alone, possibly through excessive activation of overlapping monoaminergic pathways (dopaminergic and serotonergic) or by promoting maladaptive plasticity within reward-related networks. In this context, the lack of cognitive improvement observed in male p35KO mice after combined treatment may reflect a detrimental over-activation of dopaminergic–serotonergic interactions, consistent with these preclinical findings.

Together, these findings emphasize that sex and neurobiological variability critically shape responses to psychoactive drugs, and caution against assuming that drug combinations effective in one experimental or clinical context will yield similar benefits across sexes or within the heterogeneous ADHD population.

An important finding emerged from the evaluation of WT females, which showed a pronounced decline in WM after treatment with MPH, FLX, or their combination. In contrast, WT males did not exhibit significant changes in WM, revealing a sex-specific pharmacological sensitivity in healthy animals. Previous reports have emphasized that the effects of MPH differ markedly between control and ADHD models, which is crucial for distinguishing its therapeutic versus misuse/abuse consequences. For instance, Coelho-Santos et al. (2018) demonstrated that MPH misuse negatively affects rat brain function and behavior [44]. Furthermore, these authors reported that healthy animals exposed to high doses of MPH, support a non-linear, inverted-U relationship between catecholaminergic activity and cognitive performance [45]. In such cases, supra-optimal doses elevate dopamine and norepinephrine levels beyond the range necessary for optimal prefrontal functioning, leading to cognitive disruption. The acute dosing used here may have exceeded the optimal threshold in WT females, resulting in impaired WM performance. Moreover, sex differences in monoaminergic regulation and receptor expression could further modulate drug sensitivity, as previously shown in both rodents and humans [46,47].

Finally, the finding that none of the drug treatments altered the increased exploratory motivation of p35KO mice indicates that the pharmacological effects observed were specific to cognitive domains, without affecting general motivational components.

Overall, our results highlight the pivotal role of Cdk5/p35 signaling in maintaining the functional balance between prefrontal and hippocampal networks that support WM and executive control. The loss of p35, resulting in reduced Cdk5 activity, disrupts this coordination and leads to dysregulated dopaminergic and serotonergic signaling, contributing to the cognitive deficits characteristic of ADHD. The psychoactive drug responses are highly influenced by both sex and genotype, emphasizing that identical pharmacological manipulations can yield opposite effects

depending on the underlying neurobiological state. These findings provide experimental evidence for the necessity of incorporating sex as a biological variable in preclinical and clinical ADHD research, as well as for reconsidering combination therapies that simultaneously target multiple monoaminergic systems.

Understanding how Cdk5/p35 signaling interfaces with neurotransmitter regulation and sex-dependent mechanisms may offer new perspectives for the development of personalized, mechanism-based interventions aimed at improving cognitive outcomes in ADHD.

DATA AVAILABILITY

Datasets generated during the current study are available from the corresponding author [gpaglini@immf.uncor.edu] upon reasonable request.

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AUTHOR CONTRIBUTIONS

MGP and SL conceived and designed the study. FD and OMB performed the behavioral and pharmacological experiments, conducted c-Fos immunohistochemistry, and conducted data curation and statistical analyses. DYS and MES conducted the NOR experiments. GB quantified c-Fos immunohistochemistry images and FAH analyzed behavioral videos. Supervision and project administration were done by MGP and funding's acquisition by MGP. JV, FD and MGP interpreted the data and drafted the manuscript. All authors critically revised the manuscript, approved the final version, and agree to be accountable for all aspects of the work.

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COMPETING INTERESTS

The authors declare no competing interests.

Sex-specific effects of psychoactive drugs on memory performance in an animal model of Attention-Deficit Hyperactivity Disorder

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RUNNING TITLE:

Sex-specific psychotropic effects on memory in an ADHD model

Supplementary Material and Methods

c-Fos-IR immunohistochemistry

Neuronal activation was assessed through c-Fos-IR immunohistochemistry, following previously described procedures [25]. Briefly, WT and p35KO mice were anesthetized by i.p. injection with an overdose of 30% chloral hydrate (0.1 mL/100 g) and transcardially perfused with isotonic saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 (Riedel-de Haën, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany). The brains were then removed, fixed overnight in the fixative solution, and stored at 4°C in 30% sucrose until processing and coronal sections of 40 um were cut using a freezing microtome (Reicher-Jung Hn40, Leica Microsystems, Wetzlar, Germany). The Fos antibody used in this study was raised in rabbits against a synthetic 14-amino acid sequence corresponding to residues 4–17 of human Fos (Ab-5, batch no. 60950101; Oncogene Science, Manhasset, NY). Following primary antibody incubation, sections were washed three times in PBS and placed in 1:200 goat anti-mouse IgG biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) in 1% NHS PBS for 2 h. The sections were then washed 3 times in PBS and incubated in avidin-biotin complex (Vectastain, ABC Kit, Vector Laboratories) for 2 h. After washing 3 times in PBS, sections were immersed in 0.05% 3,3´-diaminobenzidine tetrahydrochloride and 0.005% H2O2 to visualize the reaction product. The reaction was stopped with PBS, and sections were mounted on subbed slides, dried on a slide warmer, dehydrated and coverslipped with DPX (Fluka, Biochemika, Sigma Aldrich, Chemie GmbH, Steinheim, Germany).

Anatomical Context and Region Selection

Coronal sections were analyzed at four distinct anteroposterior (AP) levels relative to Bregma, each corresponding to a predefined analysis category within the macro (Table 1). The "Analyze Image" function automatically determined the number of ROIs for quantification based on the user's initial input specifying the anatomical level of the section.

Table 1. Anatomical regions and corresponding ROI count defined by the macro based on the selected anteroposterior level.

AP Level (Bregma)	Macro Label (Internal Name)	N_{ROIs}	Target Regions Analyzed
+2.22 mm	Cortex	5	Cingulate Cortex (Cg), Prelimbic Cortex (PrL), Infralimbic Cortex (IL), Lateral Orbital Cortex (LO), Ventral Orbital Cortex (VO)
-1.58 mm	Hippocampus	13	CA1, CA3, Dentate Gyrus (DG),

Image Preprocessing and Quantification

Images were acquired using an Akoya Bioscences Phenoimager Fusion equipped with a UPlanXApo 10×/0.40 objective (OFN 26.5). Selected brain regions were preprocessed using the *image preprocess* function, which automated the generation of a binary mask of c-Fos–immunoreactive (c-Fos-IR) nuclei while incorporating user-assisted quality control procedures. To prepare the images, the original files were duplicated and subjected to a Bandpass Filter (large = 40, small = 3) followed by Unsharp Masking (radius = 3, mask = 0.70) to enhance nuclear signal intensity and suppress low-frequency background variation. A semi-automated thresholding step was then applied using the Intermodes algorithm,

allowing manual adjustment of the threshold prior to conversion of the image into a binary mask.

The resulting mask was refined through standard morphological image analysis to accurately delineate individual nuclei. Morphological opening was first performed to separate aggregated particles, followed by size filtering (20–500 pixels), Watershed segmentation, and hole filling to complete nuclear contours. A final particle-filtering step retained only objects within a defined size range (30–500 pixels) and shape range (circularity = 0.80–1.00). Manual quality control (QC) was conducted in two phases: during the nuclei-inclusion phase, users could manually add missed particles using a 15-pixel white pencil, whereas during the artifact exclusion phase, incorrect or spurious objects could be removed using a 15-pixel black pencil.

Region-specific quantification was subsequently performed using the *analyze image* function. The corresponding region of interest (ROI) file was first loaded, allowing manual adjustment of the polygonal ROI boundaries to ensure precise anatomical alignment. Thereafter, both the occupied area and the number of c-Fos-IR nuclei were automatically measured for each ROI. Finally, the procedure generated two CSV output files—one containing the nuclei count and size data, and another containing the total ROI areas—along with a ZIP archive containing the processed image and the final set of ROIs used for quantification.

The ImageJ macro code implementing the *image preprocess* and *analyze image* functions is available under request.

Supplementary Figure S1

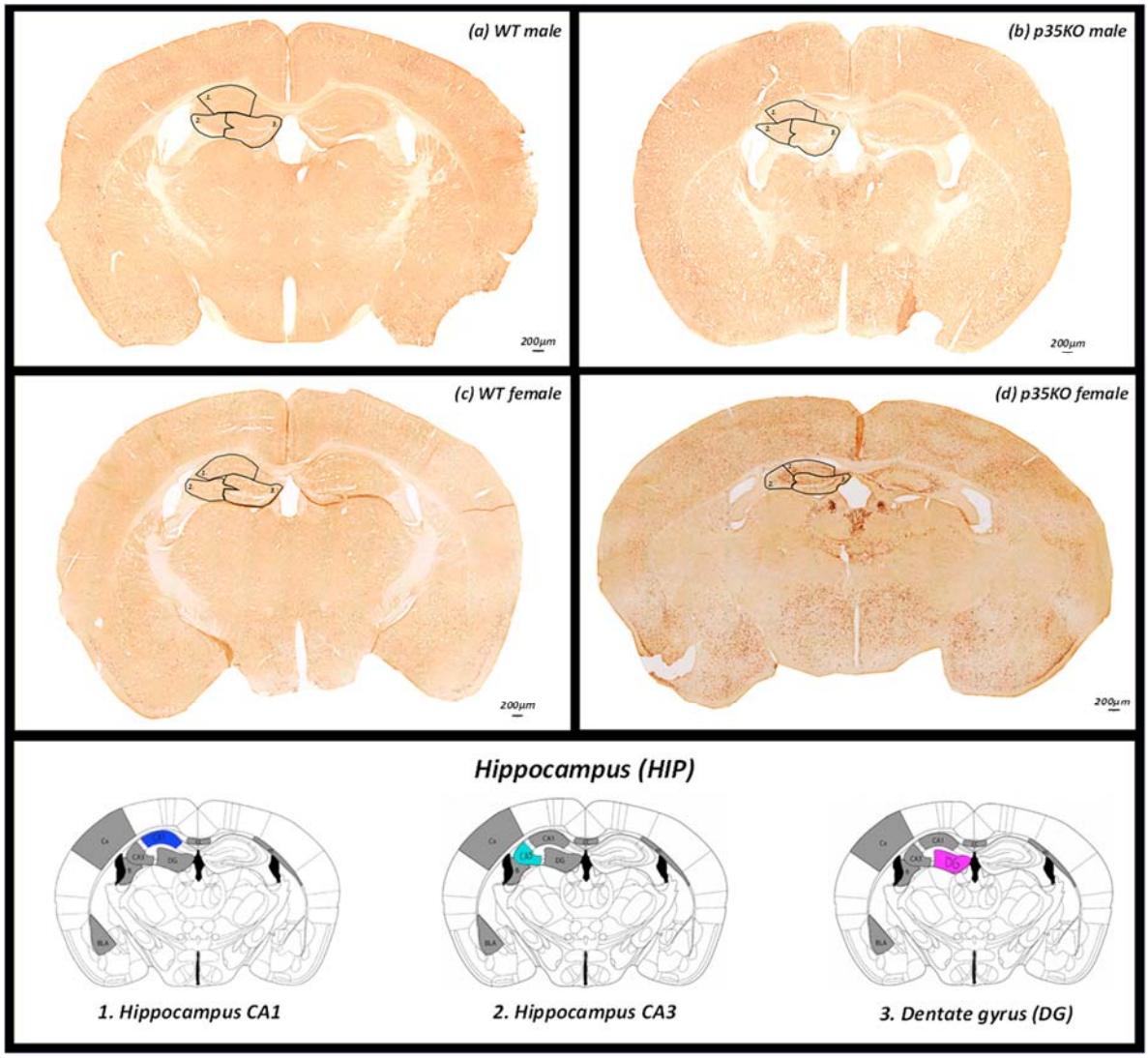

Figure S1. Representative c-Fos-IR immunostaining in the hippocampus of WT and p35KO mice (males and females).

Representative photomicrographs show neuronal activation patterns across hippocampal subregions (CA1, CA3, and dentate gyrus, DG) in WT and p35KO mice of both sexes. Panels display c-Fos–immunoreactive nuclei in: *(a) WT male, (b) p35KO male, (c) WT female, and (d) p35KO female.* These images provide a qualitative comparison of regional activation across genotypes and sexes. All photomicrographs were captured using identical acquisition parameters to ensure cross-sample comparability. Olympus UPlanXApo 10x/0,40∞/0,17/OFN 26,5. Scale bar: 200 µm.

Supplementary Figure S2

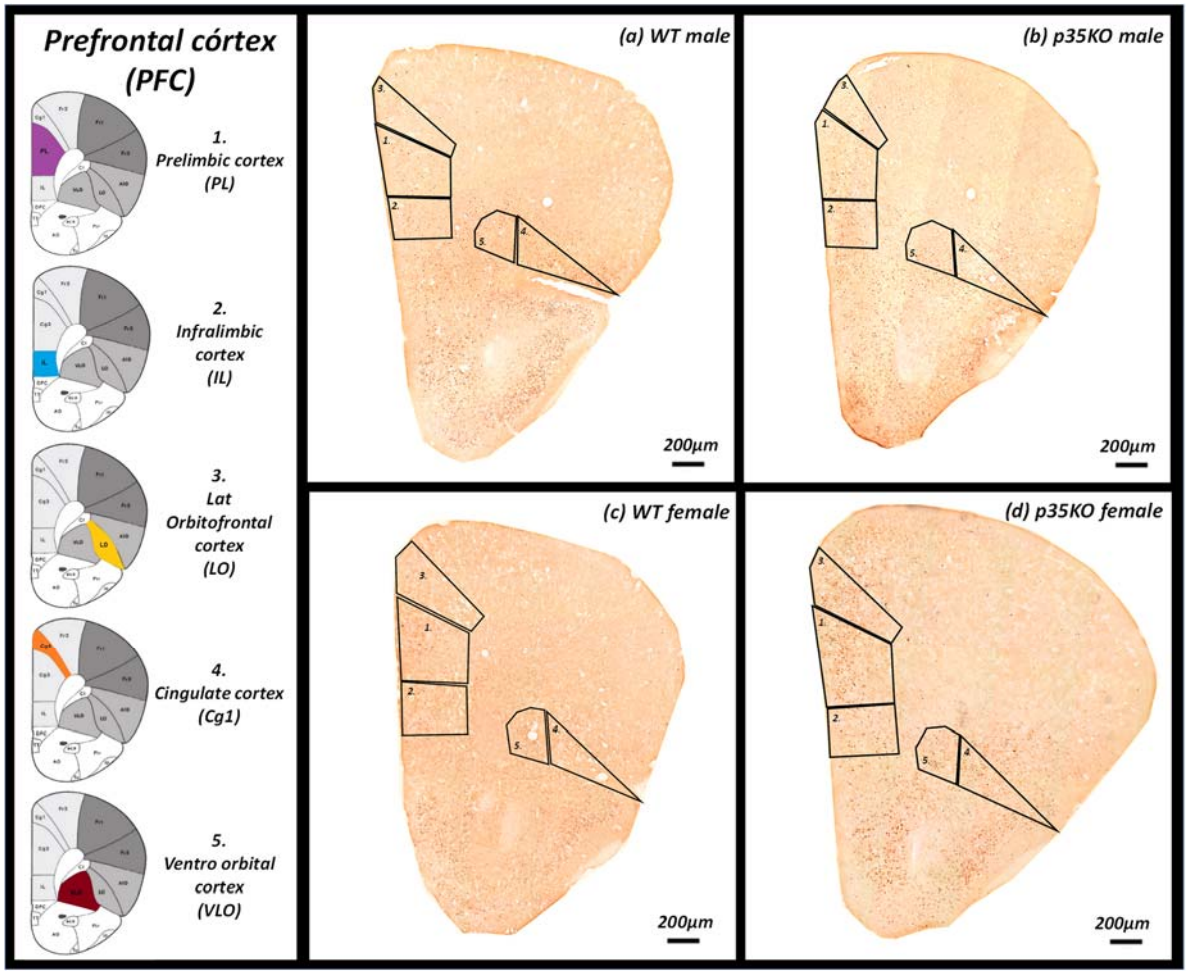

Figure S2. Representative c-Fos-IR immunostaining in the prefrontal cortex of WT and p35KO mice (males and females).

Representative photomicrographs show neuronal activation patterns across prefrontal cortex subregions, including the prelimbic (PL), infralimbic (IL), lateral orbitofrontal (LO), cingulate (Cg1), and ventrolateral orbitofrontal (VLO) cortices, in WT and p35KO mice of both sexes. Panels display c-Fos–immunoreactive nuclei in: *(a) WT male, (b) p35KO male, (c) WT female, and (d) p35KO female*. Images allow qualitative comparison of activation patterns across genotypes and sexes. All photomicrographs were acquired under identical microscope settings to ensure comparability. Olympus UPlanXApo 10x/0,40∞/0,17/OFN 26,5. Scale bar: 200 µm.