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Addressing the maturation of higher-order cognitive functions relevant to psychiatric disorders in mice

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Chapter 1

1. General introduction

Psychiatric disorders are a large class of debilitating mental illnesses that affect everyday life of patients and people around them. In fact, they result in alteration of thinking, moods, behavior and increased risk of disability, pain, death, or loss of freedom [1]. Nevertheless, the exact mechanisms behind these diseases are still unknown. Over the last few years, researchers focused on the study of abnormalities in brain neurodevelopment [2] genetic mutations, impact of traumatic events [3], [4] and the interaction between these factors [5]–[7]. In particular, both genetic and environmental factors may influence brain developmental process throughout childhood, adolescence and adulthood. Previous studies investigated how genetic and environmental risk factors act during sensitive brain developmental periods whereby altering adult behavior and possibly causing vulnerability to neuropsychiatric disorders [8]. Different brain systems have been involved in the development of psychiatric disorders. However, for disorders such as attentional deficit hyperactivity disorder (ADHD), schizophrenia, and post-traumatic stress disorder (PTSD) there are consistent evidence of a major implication of the dopaminergic and endocannabinoid systems [9]–[14].

Dopamine (DA) plays an important role acting as a trophic factor, in the development of neuronal cyto-architecture and also modulating neurodevelopmental processes during the embryonic and postnatal period [8]. In particular, dopaminergic alterations within the prefrontal cortex (PFC) or *Striatum*, two brain area involved in cognition, learning and emotion, have been previously correlated to the etiology of neuropsychiatric disorders like schizophrenia, autism and ADHD [15]. On the other hand, several studies have related dysfunctions of endocannabinoid system to psychiatric disorders [16]. In fact, the relationship between cannabis consumption, especially during critical period of brain development, and schizophrenia onset has been demonstrated [17].

1.1 "Dopamine hypothesis" of schizophrenia

Schizophrenia is a debilitating psychiatric disorder that affect about 1% of world population [18]. The etiopathology of schizophrenia is still not clear, and both genetic and environmental factors are thought to be implicated. The most used drugs to treat schizophrenia are the antipsychotics [18] which act blocking dopamine 2 receptors (D2R) [9] and suppressing dopaminergic activity. These drugs ameliorate the positive symptoms (hallucinations, delusions and disorganized thinking) [1] related to an over activation of D2R, which results in increased subcortical dopamine release [19]. However, the core symptoms of schizophrenia include negative symptoms, as well as cognitive impairments [1]. In particular, anhedonia, social withdrawal and diminished emotional expression are negative symptoms [1] usually related to hypo-activation of dopamine 1 receptors (D1R) especially in the PFC [19]. Furthermore, cognitive impairment including working memory and attentional deficits are also correlated with imbalance of D1R and D2R in PFC [20], in particular with hypo-activation of dorsolateral PFC. In fact, DA plays a pivotal role in several functions including movement, memory and reward and in modulating higher-order cognitive functions like behavior and emotion in mammalian, regulating cognitive performance which a U-shaped relationship where too much or too little DA are related to worse performances [10]. Despite this, the newest hypothesis about schizophrenia etiology suggests a hyperactive DA transmission in mesolimbic areas and a hypoactive dopamine transmission in PFC, but a dopaminergic disruption may be also observed in brain areas related with emotional processing such as amygdala [21]. Indeed, DA is a neurotransmitter synthesized in dopaminergic neurons, whose cell bodies are located in ventral tegmental area (VTA) and then projected to *Nucleus Accumbens* (NAcc), *Striatum* and PFC. DA is stored in vesicles and released into the synaptic cleft where it may act binding dopaminergic receptors, or may be re-uptaken into dopaminergic neurons by the dopamine transporter (DAT) [22], or metabolized by Catecholamine-O-methyltrasferase (COMT) [23], [24]. While DAT is mainly expressed in the *Striatum*, COMT is abundantly expressed in pyramidal neurons of the PFC [23], [25], likely because of the scarcity of cortical DA transporters [26]. For this reason, a dysregulation of DAT and/or COMT activity may lead to a disruption of the dopaminergic system.

1.2 The endocannabinoid system

Cannabis is among the worldwide used psychoactive drugs, with an estimates 125-227 million consumers all over the world [27]. The most powerful component of cannabis is the Δ9-Tetrahydrocannabinol (Δ9-THC), which acts as agonist on cannabinoid receptors CBRs modulating several functions such as learning [28], memory [29], cerebral development [30], but also social and emotional memories [31] and inflammatory responses [32].

Actually, the most active receptor within the central nervous system (CNS) is the cannabinoid receptor 1 (CB1) [33]–[36], ubiquatary expressed in CNS [37]. Intriguingly, over the last few years, several studies displayed that CB1 receptor is present on astrocytes and their functioning in the astrocytes is particularly important in mediating cannabinoid-related effects [38], [39].

Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are endogenous ligands for CBRs, which are "on demand" synthesized at pre- synaptic level starting from phospholipid precursors [40]. Unlike other neurotransmitters, AEA and 2-AG did not activate after Ca^{2+} signaling, but because of their lipophilicity cross the cell- membrane and bind pre-synaptic CBRs [40]. However, AEA and 2-AG can act as retrograde neurotransmitters and regulating the release of "classical" neurotransmitters, including glutamate, GABA [41] and dopamine [42]. Indeed, AEA and 2-AG are able to act on a wide variety of neuronal populations by means of retrograde signaling, modulating their synaptic plasticity [34], [36], [43].

As mentioned above, a dysfunction in dopaminergic signaling is the most validated theory about schizophrenia etiology [9]. Nevertheless, dopaminergic dysregulation is insufficient to explain the non-psychotic symptoms of schizophrenia, which required alternative conceptual models of schizophrenia [44].

Several studies have demonstrated an endocannabinoid systems dysfunctions in patients affected by schizophrenia [45], [46]. In fact, elevated endocannabinoids levels have been detected in the blood and cerebrospinal fluid [45]–[47] of patients with schizophrenia, which are normalized with both antipsychotics and clinical remission [44], [45]. In addition, other studies have demonstrated a strong relationship between heavy cannabis consumption and higher risk to develop psychosis [48].

Nevertheless, not all cannabis consumers show psychosis, which means that other factors may be implicated in schizophrenia onset. Since genetic mutation were already proposed as main factor in developing psychiatric disorders, researchers investigated the role of COMT mutation in this pathology [17], [49], [50] in humans.

Despite the increased number of scientific reports, the mechanism by which gene x environmental interaction might cause schizophrenia is still unknown. Further research is needed to unravel the exact mechanism related to the interaction between genetic modifications and environmental factors in order to find new pharmacological strategies to ameliorate patients everyday life.

1.3 The endocannabinoid system and emotion

"Social cognition" is the ability to understand and process information about the other people, and it is strictly related to the ability to distinguish emotions in the other [51]. However, patients affected by psychiatric disorders, such as schizophrenia or autism, showed deficits in this set of abilities. The most used task to diagnose anomalies in social cognition is the facial emotion recognition task (FERT). In fact, several studies of functional magnetic resonance, performed during FERT, highlighted brain areas involved in social cognition, such as PFC. Despite this, mounting evidence from human studies [52]–[55] converged on indicating a strong involvement of the endocannabinoid system in FERT. Nevertheless, these studies showed some limitations [53], [55]. In fact, in some cases the sample size was too small [53], in other cases cannabis users reported alcohol use for more days per month and years than controls and, although the cannabis users were instructed to remain abstinent from drugs for at least 24 hours before testing, this was not tested [55]. For these reasons, rodents might help to clarify biological mechanisms and neural circuits involved in social cognition. However, there is no evidence that rodents may be able to discriminate individuals on the base of their emotional state. Although, previous studies identified the existence of a transmission of emotions from one rodent to a familiar observer, but the cognitive processes by which rodents discriminate conspecifics emotional states are not still understood. In order to investigate what are the systems involved in social cognition we designed a new powerful translational test to investigate emotion recognition in mice. We found that mice are able to discriminate negative and positive emotions evoked in unfamiliar conspecifics, provided first proof about the involvement of endocannabinoid system in modulation emotion recognition in mice.

1.4 Aims of the thesis

The overall goal of my doctoral work was to develop and asses new tools with translational valence to investigate how genetic mutations and/or environmental factors might contribute to develop psychiatric disorder. In order to ultimately investigate mechanisms behind psychiatric disorders, we decided to use genetically modified mouse models, which allow us to better control the impact of specific genetic and environmental factors. In particular, we specifically focused on these four subtopics:

- ADHD, schizophrenia and bipolar disorder are psychiatric diseases with a strong genetic component, which share dopaminergic alterations. DAT genetics might be implicated in all these disorders. However, the effects of DAT hypofunction especially in developmental trajectories have been poorly addressed. Thus, we comprehensively studied DAT hypofunctional mice (DAT +/-) from adolescence to adulthood to disentangle DAT-dependent alterations in the development of psychiatric-relevant phenotypes [56].
- Adolescence is a critical period for the development of higher-order cognitive functions. Unlike in humans, very limited tools are available to assess such cognitive abilities in adolescent rodents. We implemented a modified 5-Choice Serial Reaction Time Task (5CSRTT) to selectively measure attentiveness, impulsivity, broad monitoring, processing speed and distractibility in adolescent mice [57]
- The PFC is a crucial hub for the flexible modulation of recent memories (executive functions) as well as for the stable organization of remote memories. DA in the PFC is implicated in both these processes and genetic variants affecting its neurotransmission might control the unique balance between cognitive stability and flexibility present in each individual. Functional genetic variants in the COMT gene result in a different catabolism of dopamine in the PFC. However, despite the established role played by COMT genetic variation in executive functions, its impact on remote memory formation and recall is still poorly explored. We investigated how transgenic mice overexpressing the human COMT-Val gene (COMT-Val-tg) might affect recent and remote memories. Indeed, COMT genetic over activity produced a selective overdrive of the endocannabinoid system within the PFC which was associated with enhanced remote memories [4].
- Social emotion recognition abilities are also evident in non-human primates, as well as in dogs and sheep. Despite this, there is no evidence that mice, the most widely

used laboratory animals, might be able to discriminate individuals on the base of their emotional state. Indeed, we developed a new method to investigate emotion discrimination abilities in mice. Recent studies demonstrated the endocannabinoid system implication in psychiatric disorders. Starting from the evidence that astrocytes express cannabinoid receptors and synthesize endocannabinoids, we hypothesized a dysfunction in this kind of cell in 'social' deficits.

1.5 References

[1] American Psychiatric Association, *Diagnostic and statistical manual of mental disorders (5th ed.)*. .

[2] D. Hoops and C. Flores, "Making Dopamine Connections in Adolescence," *Trends Neurosci.*, vol. 40, no. 12, pp. 709–719, Dec. 2017.

[3] C. Regehr and V. R. Leblanc, "PTSD, Acute Stress, Performance and Decision-Making in Emergency Service Workers," 2017.

[4] D. Scheggia *et al.*, "Remote memories are enhanced by COMT activity through dysregulation of the endocannabinoid system in the prefrontal cortex," *Mol. Psychiatry*, vol. 23, no. 4, 2018.

[5] T. Halldorsdottir and E. B. Binder, "Gene × Environment Interactions: From Molecular Mechanisms to Behavior," *Annu. Rev. Psychol.*, vol. 68, no. 1, pp. 215–241, Jan. 2017.

[6] R. H. Wozniak, N. B. Leezenbaum, J. B. Northrup, K. L. West, and J. M. Iverson, "The development of autism spectrum disorders: variability and causal complexity," *Wiley Interdiscip. Rev. Cogn. Sci.*, vol. 8, no. 1–2, p. e1426, Jan. 2017.

[7] P. Moran *et al.*, "Gene × Environment Interactions in Schizophrenia : Evidence from Genetic Mouse Models," vol. 2016, 2016.

[8] Q. Yu *et al.*, "Dopamine and serotonin signaling during two sensitive developmental periods differentially impact adult aggressive and affective behaviors in mice," *Mol. Psychiatry*, vol. 19, no. 6, pp. 688–698, 2014.

[9] A. Carlsson, "Antipsychotic drugs, neurotransmitters, and schizophrenia," *Am. J. Psychiatry*, vol. 135, no. 2, pp. 165–173, Feb. 1978.

[10] F. Papaleo *et al.*, "Genetic Dissection of the Role of Catechol- O - Methyltransferase in Cognition and Stress Reactivity in Mice," vol. 28, no. 35, pp. 8709–8723, 2008.

[11] M. Mereu *et al.*, "Dopamine transporter (DAT) genetic hypofunction in mice produces alterations consistent with ADHD but not schizophrenia or bipolar disorder," *Neuropharmacology*, vol. 121, pp. 179–194, 2017.

[12] M. E. Sloan, C. W. Grant, J. L. Gowin, V. A. Ramchandani, and B. Le Foll, "Endocannabinoid signaling in psychiatric disorders: a review of positron emission tomography studies," *Acta Pharmacol. Sin.*, p. 1, Aug. 2018.

[13] I. Ibarra-Lecue *et al.*, "The endocannabinoid system in mental disorders: Evidence from human brain studies," 2018.

[14] E. Puighermanal, A. Busquets-Garcia, R. Maldonado, and A. Ozaita, "Cellular and intracellular mechanisms involved in the cognitive impairment of cannabinoids," *Philos. Trans. R. Soc. B Biol. Sci.*, vol. 367, no. 1607, pp. 3254–3263, 2012.

[15] E. H. Simpson, C. Kellendonk, and E. Kandel, "A Possible Role for the Striatum in the Pathogenesis of the Cognitive Symptoms of Schizophrenia," *Neuron*, vol. 65, no. 5, pp. 585–596, 2010.

[16] D. Parolaro, N. Realini, D. Vigano, C. Guidali, and T. Rubino, "The endocannabinoid system and psychiatric disorders," *Exp. Neurol.*, vol. 224, no. 1, pp. 3–14, 2010.

[17] A. Caspi *et al.*, "Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: Longitudinal evidence of a gene X environment interaction," *Biol. Psychiatry*, vol. 57, no. 10, pp. 1117–1127, 2005.

[18] J. Mcgrath, S. Saha, D. Chant, and J. Welham, "Schizophrenia : A Concise Overview of Incidence , Prevalence , and Mortality," vol. 30, pp. 67–76, 2008.

[19] L. H. Shen, M. H. Liao, and Y. C. Tseng, "Recent advances in imaging of dopaminergic neurons for evaluation of neuropsychiatric disorders," *J. Biomed. Biotechnol.*, vol. 2012, 2012.

[20] H. Takahashi, "PET neuroimaging of extrastriatal dopamine receptors and prefrontal cortex functions," *J. Physiol. Paris*, vol. 107, no. 6, pp. 503–509, 2013.

[21] R. Brisch *et al.*, "A morphometric analysis of the septal nuclei in schizophrenia and affective disorders: Reduced neuronal density in the lateral septal nucleus in bipolar disorder," *Eur. Arch. Psychiatry Clin. Neurosci.*, vol. 261, no. 1, pp. 47–58, 2011.

[22] "Giros et al., 1996.pdf." .

[23] M. Matsumoto *et al.*, "Catechol O-methyltransferase mRNA expression in human and rat brain: evidence for a role in cortical neuronal function.," *Neuroscience*, vol. 116, no. 1, pp. 127–137, 2003.

[24] B. S. Mcewen and N. York, "Catechol- O -methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior," vol. 95, no. August, pp. 9991–9996, 1998.

[25] J. A. Gogos *et al.*, "Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior," *Proc. Natl. Acad. Sci.*, vol. 95, no. 17, pp. 9991–9996, 1998.

[26] S. R. Sesack, V. A. Hawrylak, C. Matus, M. A. Guido, and A. I. Levey, "Dopamine axon varicosities in the prelimbic division of the rat prefrontal cortex exhibit sparse

immunoreactivity for the dopamine transporter.," *J. Neurosci.*, vol. 18, no. 7, pp. 2697–708, 1998.

[27] A. Busquets-Garcia *et al.*, "Pregnenolone blocks cannabinoid-induced acute psychotic-like states in mice," *Mol. Psychiatry*, vol. 22, no. 11, pp. 1594–1603, 2017.

[28] T. T. Y. Lee, M. N. Hill, and F. S. Lee, "Developmental regulation of fear learning and anxiety behavior by endocannabinoids," *Genes, Brain Behav.*, vol. 15, no. 1, pp. 108–124, 2016.

[29] M. Qin, Z. Zeidler, K. Moulton, L. Krych, Z. Xia, and C. B. Smith, "Endocannabinoid-mediated improvement on a test of aversive memory in a mouse model of fragile X syndrome," *Behav. Brain Res.*, vol. 291, pp. 164–171, 2015.

[30] I. G. Æ. J. P. Æ, T. Aguado, and Æ. M. Guzma, "The endocannabinoid system and the regulation of neural development : potential implications in psychiatric disorders," pp. 371–382, 2009.

[31] P. Campolongo and V. Trezza, "The endocannabinoid system: a key modulator of emotions and cognition.," *Front. Behav. Neurosci.*, vol. 6, no. November, p. 73, 2012.

[32] R. Tanasescu and C. S. Constantinescu, "Cannabinoids and the immune system: An overview," *Immunobiology*, vol. 215, no. 8, pp. 588–597, 2010.

[33] A. Ameri, "The effects of cannabinoids on the brain," *Prog. Neurobiol.*, vol. 58, no. 4, pp. 315–348, 1999.

[34] T. Ohno-Shosaku, T. Maejima, and M. Kano, "Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals," *Neuron*, vol. 29, no. 3, pp. 729–738, 2001.

[35] F. A. Pamplona and R. N. Takahashi, "Psychopharmacology of the endocannabinoids: far beyond anandamide," *J. Psychopharmacol.*, vol. 26, no. 1, pp. 7–22, Jun. 2011.

[36] E. Puighermanal, A. Busquets-garcia, and R. Maldonado, "Cellular and intracellular mechanisms involved in the cognitive impairment of cannabinoids," pp. 3254–3263, 2012.

[37] C. R. Lupica and A. C. Riegel, "Endocannabinoid release from midbrain dopamine neurons: A potential substrate for cannabinoid receptor antagonist treatment of addiction," *Neuropharmacology*, vol. 48, no. 8 SPEC. ISS., pp. 1105–1116, 2005.

[38] J. F. Oliveira da Cruz, L. M. Robin, F. Drago, G. Marsicano, and M. Metna-Laurent, "Astroglial type-1 cannabinoid receptor (CB1): A new player in the tripartite synapse," *Neuroscience*, vol. 323, pp. 35–42, 2016.

[39] M. Metna-Laurent and G. Marsicano, "Rising stars: Modulation of brain functions by

astroglial type-1 cannabinoid receptors," *Glia*, vol. 63, no. 3, pp. 353–364, 2015.

[40] V. Di Marzo *et al.*, "Formation and inactivation of endogenous cannabinoid anandamide in central neurons," *Nature*, vol. 372, p. 686, Dec. 1994.

[41] G. Marsicano and B. Lutz, "Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain," *Eur. J. Neurosci.*, vol. 11, no. 12, pp. 4213–4225, 1999.

[42] D. Piomelli, "The molecular logic of endocannabinoid signalling," *Nat. Rev. Neurosci.*, vol. 4, no. 11, pp. 873–884, 2003.

[43] R. I. Wilson, G. Kunos, and R. A. Nicoll, "Presynaptic specificity of endocannabinoid signaling in the hippocampus," *Neuron*, vol. 31, no. 3, pp. 453–462, 2001.

[44] J. P. Leite and L. S. Bueno-junior, "Cannabinoids and Vanilloids in Schizophrenia : Neurophysiological Evidence and Directions for Basic Research," vol. 8, no. June, pp. 1–27, 2017.

[45] A. Giuffrida *et al.*, "Cerebrospinal Anandamide Levels are Elevated in Acute Schizophrenia and are Inversely Correlated with Psychotic Symptoms," pp. 2108–2114, 2004.

[46] F. M. Leweke *et al.*, "Anandamide levels in cerebrospinal fluid of first-episode schizophrenic patients: Impact of cannabis use," *Schizophr. Res.*, vol. 94, no. 1–3, pp. 29–36, 2007.

[47] M. Hellmich and D. Koethe, "Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia," no. January, 2012.

[48] R. M. Murray *et al.*, "Cannabis-associated psychosis: Neural substrate and clinical impact," *Neuropharmacology*, vol. 124, pp. 89–104, 2017.

[49] C. Henquet *et al.*, "An Experimental Study of Catechol-O-Methyltransferase Val158Met Moderation of Δ-9-Tetrahydrocannabinol-Induced Effects on Psychosis and Cognition," *Neuropsychopharmacology*, vol. 31, no. 12, pp. 2748–2757, 2006.

[50] C. Henquet *et al.*, "Prospective cohort study of cannabis use, predisposition for psychosis, and psychotic symptoms in young people," *Bmj*, vol. 330, no. 7481, p. 11, 2005.

[51] S. Vanheule, R. Meganck, and M. Desmet, "Alexithymia, social detachment and cognitive processing," *Psychiatry Res.*, vol. 190, no. 1, pp. 49–51, 2011.

[52] A. Manuscript, "Emotional Images," vol. 26, no. 10, pp. 1289–1298, 2013.

[53] M. G. Bossong, H. H. van Hell, G. Jager, R. S. Kahn, N. F. Ramsey, and J. M. Jansma, "The endocannabinoid system and emotional processing: A pharmacological fMRI

study with {increment}9-tetrahydrocannabinol," *Eur. Neuropsychopharmacol.*, vol. 23, no. 12, pp. 1687–1697, 2013.

[54] B. Platt, S. Kamboj, C. J. A. Morgan, and H. V. Curran, "Processing dynamic facial affect in frequent cannabis-users: Evidence of deficits in the speed of identifying emotional expressions," *Drug Alcohol Depend.*, vol. 112, no. 1–2, pp. 27–32, 2010.

[55] C. Hindocha *et al.*, "Acute effects of delta-9-tetrahydrocannabinol, cannabidiol and their combination on facial emotion recognition: A randomised, double-blind, placebo-controlled study in cannabis users," *Eur. Neuropsychopharmacol.*, vol. 25, no. 3, pp. 325–334, 2015.

[56] M. Mereu *et al.*, "Dopamine transporter (DAT) genetic hypofunction in mice produces alterations consistent with ADHD but not schizophrenia or bipolar disorder," *Neuropharmacology*, vol. 121, 2017.

[57] M. Ciampoli, G. Contarini, M. Mereu, and F. Papaleo, "Attentional Control in Adolescent Mice Assessed with a Modified Five Choice Serial Reaction Time Task," *Sci. Rep.*, vol. 7, no. 1, 2017.

Chapter 2

Dopamine transporter (DAT) genetic hypofunction in mice produces alterations consistent with ADHD but not schizophrenia or bipolar disorder

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Abstract

ADHD, schizophrenia and bipolar disorder are psychiatric diseases with a strong genetic component which share dopaminergic alterations. Dopamine transporter (DAT) genetics might be potentially implicated in all these disorders. However, in contrast to DAT absence, the effects of DAT hypofunction especially in developmental trajectories have been scarcely addressed. Thus, we comprehensively studied DAT hypofunctional mice (DAT+/-) from adolescence to adulthood to disentangle DATdependent alterations in the development of psychiatric-relevant phenotypes. From pre-adolescence onward, DAT+/- displayed a hyperactive phenotype, while responses to external stimuli and sensorimotor gating abilities were unaltered. General cognitive impairments in adolescent DAT +/- were partially ameliorated during adulthood in males but not in females. Despite this, attentional and impulsivity deficits were evident in DAT+/- adult males. At the molecular level, DAT +/- mice showed a reduced expression of Homer1a in the prefrontal cortex, while other brain regions as well as Arc and Homer1b expression were mostly unaffected. Amphetamine treatments reverted DAT +/- hyperactivity and rescued cognitive deficits. Moreover, amphetamine shifted DAT-dependent Homer1a altered expression from prefrontal cortex to striatal regions. These behavioral and molecular phenotypes indicate that a genetic-driven DAT hypofunction alters neurodevelopmental trajectories consistent with ADHD, but not with schizophrenia and bipolar disorders.

2.1. Introduction

Dopamine dysfunction is believed to be significantly implicated in the pathophysiology of several psychiatric disorders, among these being schizophrenia (SZ), attention deficit hyperactivity disorder (ADHD), and bipolar disorder (BD)[1]–[4]. These are conceptualized also as diseases of aberrant synaptic function, possibly on a neurodevelopmental basis [2]. These psychiatric disorders all share a strong genetic component [5]–[7]. However, how dopamine-related genetic variations might differently affect neurodevelopment, giving rise to divergent abnormalities consistent with ADHD-, SZ- or BD-related dimensions is still not clear. Dopamine pathophysiology, especially in subcortical regions, is highly linked to the function of the dopamine transporter (DAT) [2], whose gene variants have been implicated to different degrees in the above disorders [6], [8].

Animal models of DAT disruption have mainly focused on mice with a complete absence of DAT. DAT null mutant (-/-) mice exhibit extreme phenotypes such as lack of ability to re-uptake dopamine from the synaptic cleft, growth retardation, anterior pituitary hypoplasia, dwarfism, early life mortality and exorbitant hyperactivity [9]–[11].

In agreement, DAT-/- have been ascribed as a bona-fide model for the DAT deficiency syndrome, also known as early Parkinson's disease)[12], [13]. In contrast, more subtle changes in DAT activity could be more suitable for understanding its contribution to phenotypes relevant to disorders such as ADHD, SZ, and BD, as suggested by human studies [14], [15]. Partial DAT hypofunctioning has been studied prevalently for locomotor responses and reactivity to psychostimulants such as cocaine and amphetamine, and only in adult mice (Supplementary Table1).

Thus, in order to gain insight into the impact of partial DAT genetic disruption on disorders such as ADHD, SZ and BD, we characterized DAT hypofunctioning mice (DAT+/-) at different ages. In particular, considering the developmental aspect of these disorders, we performed behavioral investigations in adolescent and adult animals to follow the trajectory of dopamine dysfunction in DAT+/- mice.

We also compared male and female DAT+/+ and +/- mice with and without exposure to amphetamine. Indeed, amphetamines may ameliorate symptoms in ADHD [16], conversely, these same drugs may precipitate or exacerbate psychotic symptoms in both BD [17] and SZ patients [18] Moreover, all these psychiatric disorders show sex-dependent differences of the correlated behavioral abnormalities [19], [20], [21]. Finally, in line with the hypothesis that dopamine dysregulation in SZ, BP and ADHD has been associated with the common final

pathway of an aberrant synaptic function influencing all dopamine and glutamate physiology [1], [22], we investigated in cortical and subcortical brain regions alterations of key transcripts of the postsynaptic density (Homer1a, Homer1b, and Arc). These genes have been demonstrated to be implicated in the pathophysiology and animal modeling of ADHD, SZ and BP [23]–[27].

2.2. Methods and materials

All procedures were approved by the Italian Ministry of Health (permit n.17 BIS/2014) and Animal Use Committee and were conducted in accordance with guidelines for the care and use of laboratory animals of the NIH and the European Community Council Directives. Original DAT-/- mice [10] were backcrossed with C57BL6J mice for at least 8 generations. The breeding scheme used to obtain the experimental mice involved mating DAT hypofunctioning (DAT+/-) male mice with C57BL6J (DAT +/+) females. DAT+/+ mice were used as female breeders in order to avoid altered maternal behavior. Only DAT+/+ and +/- littermates were used for all experiments. Mice were genotyped by PCR analysis of tail DNA. Mice were group-housed (two to four per cage) in a climate-controlled animal facility (22 ± 2 C) and maintained on a 12 h light/dark cycle (7am-7pm) with ad libitum access to food and water, unless specified in particular experiments. All experimental tests were conducted in male and female adolescent (PND 28e45) and adult (3e7 months old) mice during the light phase. Mice were handled by the experimenter on alternate days during the week preceding the test. Experimenters were blind to the genotype during testing.

2.2.1. Acoustic startle response (ASR) and prepulse inhibition (PPI)

Acoustic startle response (ASR) and prepulse inhibition (PPI) were measured using four SR-Lab System (San Diego Instruments) as previously described [27]–[29]. Startle experiments test sessions began by placing the mouse in the Plexiglas holding cylinder (5 cm diameter) for a 5 min acclimation period. After the acclimation period, each subject received 36 trials over the 9 min test session. There were six different sound levels (in decibels) presented: 70, 75, 80, 85, 90, and 120. Each stimulus was 40 ms and presented four times in pseudorandom order such that each sound level was presented within a block of six trials. The interval between trials was 10e20 s. The ASR was recorded for 65 ms (measuring the response every 1 ms) starting with the onset of the startle stimulus. The maximum startle amplitude recorded during the 65 ms sampling window was used as the dependent variable. The PPI is an attenuation of the startle response when the startle-eliciting stimulus (pulse), is preceded by a weaker sensory stimulus (prepulse). In this test, mice were presented with each of seven trial types across six blocks of trials for a total of 42 trials. Trial types were presented randomly within each block. The interval between trials was 10e20 s. One trial type measured the response to no stimulus (baseline movement), and another presented the startle stimulus alone (startle), which was a 40 ms, 120 dB sound. The other five were acoustic prepulse plus acoustic startle stimulus trials.

Prepulse tones were 20ms at 70, 75, 80, 85, and 90 dB, presented 100 ms before the startle stimulus (120 dB). The maximum startle amplitude was the dependent variable. A background level of 70 dB white noise was maintained over the duration of the test session.

2.2.2. Locomotor activity (LMA) and sensitization to psychostimulants

The experimental apparatus consisted of four open field arenas (42 x 42 x 30 cm), illuminated by overhead white lighting (25 ± 5 lux). To quantify exploratory and locomotor activities a video tracking system (ANYMAZE©) was used during 1 h of test. Parameters analyzed were total distance travelled (m) and percentage of time in the internal zone. One week after basal assessment, mice were treated with amphetamine HCl (1.5 mg/kg i.p) immediately before the LMA test and the test was repeated for 5 consecutive days.

2.2.3. Temporal order object recognition (TOR) task

The test was performed as previously described [27], [30]. Mice were tested in an experimental apparatus consisting of an opaque open field box (42 x 42 x 30 cm) with even, overhead white lighting (25 ± 5 lux). Each session was video-recorded using an overhead camera from ANY-maze (Stoelting Co.). Each mouse was monitored for its locomotor activity in the empty open field boxes for 1 h. The next day, in the TOR test, the subjects' ability to differentiate between two objects presented at different intervals was assessed. The objects presented were rectangular boxes (3 x 3 x 6 cm), or two laboratory flasks (4 x 6 cm), each either black or white and too heavy for the animals to displace. The objects were placed in two corners of the open field apparatus, 8 cm from the sidewalls. This task comprised of two sample phases and one test trial. In each sample phase, the subjects were allowed to explore two copies of an identical object for a total of 5 min. Different objects were used for sample phases 1 and 2, with a 1-h delay between the two sample phases. The test trial (5- min duration) was performed 3 h after sample phase 2. During the test trial, a third copy of the objects from both sample phase 1 and sample phase 2 were used. Time spent exploring each object was subsequently scored from the ANY-maze videos as the number of seconds when each subject was facing the object and 1 cm away. If temporal order memory is intact, subjects will spend more time exploring the object from sample 1 (i.e., the object presented less recently compared with the object from sample 2 (i.e., the object minimum of 2 s exploration in the sample or test phases were excluded from the analysis. Discrimination between the objects was calculated using a discrimination ratio that takes into account individual differences in the total amount of exploration. In particular, the discrimination ratio was calculated as the difference in time spent by each subject exploring

the objects from sample phase 1 compared with the objects from sample phase 2 divided by the total time spent exploring both objects during the test period.

2.2.4 Five choice serial reaction time task (5CSRTT)

The classical 5CSRTT has been used to measure different aspects of attentional control in rodents [31], with relevance to numerous clinical disorders such as ADHD [32], [33], SZ [34] and BD [35]. The first part of the task was performed as previously described [28], while the implemented modified parts as well as the SARAT paradigm was performed as extensively described in [36](Huang et al., 2017).

2.2.4.1. Apparatus

Training and testing were conducted in operant chambers (Med Associates, St. Albans, VT, USA), housed in sound-attenuating and ventilated boxes. Each operant chamber contained a 5 nose-poke hole wall outfitted with a LED stimulus light for each hole. Two additional LED cue lights (red and green) were installed above each of the 5 nose-poke holes. Nose-poke was detected by an infrared beam. On the wall the 5-hole array there was a food magazine and a head entry detector where food reinforcement (14 mg pellets of the 5 TUL purified rodent tablet, Test Diet®) was delivered by a pellet dispenser. A water dispenser on the latter wall allowed the mice to have full access to water throughout the test. A house light was located 7 cm above the food magazine. The operant chambers were connected to a Smart Control Panel and interfaced to a Windows computer equipped with a MED-PC IV software (Med Associates, St. Albans, USA).

2.2.4.2. Habituation

During the handling sessions, mice were weighed to obtain a baseline of their ad libitum body weight. Food restriction was imposed on adult mice during the duration of the experiment in order to maintain at least 90% of body weight. All animals received 1 g pellet after each daily session. Mice were weighed and then placed into the operant chambers each morning at 9.30 a.m.

2.2.4.3. Training protocol

Each day, adult mice were presented with one session. When head entry to retrieve the free reinforcement pellet was detected, the first trial began with an inter-trial interval (ITI). Any nose-pokes during the ITI was recorded as premature responses and resulted in a time-out

(default 5s) with the house-light turned on. At the end of the time-out, the house-light turned off and the ITI restarted. Any nose-pokes during the time-out reset the time-out. At the end of the ITI, the program randomly selected a stimulus location (1 out of 5 stimulus lights) and turned on the corresponding stimulus light. The stimulus light remained on for the stimulus duration (SD) value set. The animal has limited hold (LH) time to nose-poke into the lit hole. If the animal nose-poked into the correct lit hole before the LH time runs out, this was recorded as a correct response, the first response to stimulus was recorded, the stimulus light was turned off if not turned off earlier, the correct response latency was recorded and the pellet issued. If the animal responded to one of the nose-poke holes that was not lit, this was recorded as an incorrect response, the first response to stimulus was recorded, the stimulus light was turned off if not turned off earlier, the incorrect response latency recorded, and the house-light turned on for the time-out period. At the end of the time-out, the house-light was turned back off and the ITI started. Any nose-poke during the timeout reset the time-out. If the animal did not nose-poke into any hole before the limited hold time runs out, this was recorded as an omission error, the first response to stimulus was recorded as 0, the stimulus light turned off if not turned off earlier, the correct/incorrect response latency was recorded as 0 (this value was not included in the calculation of correct/incorrect response latencies), and the house-light turned back off and the ITI started. Any nose pokes during the time-out reset the time-out. Training consisted of 6 stages. To precede to each subsequent stage, mice were required to reach the criterion for 2 consecutive sessions. Each stage was more challenging than the last, with the SD and LH period decreasing while other criteria (such as the required number of correct trials) become more demanding. Each 30-min session has a maximum limit of 100 trials. The following measures were recorded to assess task performance as showed in the results section:

- Correct responses: Number of correct responses divided by total number of trials, multiplied by 100.
- Correct Latency (average latency to a correct response): Total time from onset of light stimulus to the performance of a correct response divided by number of correct responses.
- Choice accuracy: Number of correct responses divided by the sum of number of correct and incorrect responses, multiplied by 100.
- Incorrect responses: Number of incorrect responses divided by total number of trials, multiplied by 100.
- Omissions: Number of omissions divided by total number of trials, multiplied by 100.

- Reward Latency (average latency to collect a food reward): total time from the performance of a correct response to the retrieval of the food reward from the food magazine divided by the number of correct responses.
- Premature responses: Number of premature responses divided by sum of correct, incorrect, premature, perseverative and timeout responses (total number of responses), multiplied by 100.
- Perseverative responses: Number of perseverative responses divided by total number of responses, multiplied by 100.

2.2.5. Number of time-out responses

2.2.5.1. Stabilization (basic task)

Upon reaching the final training stage, mice were subjected to one week of testing at this stage in order to achieve a stable performance.

2.2.5.2. Spatial attentional resource allocation task (SARAT)

The week after, mice were tested on the SARAT protocol. This consisted of 3 different trial types randomly presented. The trial type 1 (normal trial) was the same as in the stabilization stage where the stimulus light was turned on randomly in 1 of the 5 nose-poke holes. The second type of trial (Cued 1 trial) was the same as the normal trial type with the addition of a red cue light appearing over the correct nose-poke hole from 1 s before to 1 s after the stimulus light duration (i.e. number of cues= 1). The third type of trial (Cued 5 trial) was the same as the normal trial type with the addition of a red cue light appearing over each of the 5 nose-poke holes from 1 s before to 1 s after the stimulus cue duration (i.e. number of cues= 5). Any nose-pokes made during the red cue light was lit, but before the stimulus light was presented, was considered a premature response and was not rewarded
resulting in a time-out period. Each trial type was presented equally in a random fashion throughout each session. Mice were exposed to 1 week to this SARAT protocol presented in 2 out of 7 testing days (Monday and Thursday) while on the other 5 days (Saturday, Sunday, Tuesday, Wednesday, Friday), a normal stabilization stage was run.

2.2.5.3. Sub-chronic treatment with low doses of amphetamine

After these different test manipulations, mice were administered daily with low doses of amphetamine (0.375 mg/kg/10ml i.p.) and immediately tested on the normal stabilization trial type for 5 consecutive days. After that, the last day immediately after the amphetamine injection mice were tested on the SARAT protocol. The amphetamine dose was selected based on the lowest one previously used in the 5CSRTT in mice [28] in order to avoid affecting locomotor activity.

2.2.6. In situ hybridization

Animals treated with vehicle or amphetamine (1.5 mg/kg, i.p.) were killed by decapitation 90 min after the last injection. Brains were removed, quickly frozen on powdered dry ice and stored at -70 °C prior to sectioning. Serial coronal sections of 12 mm were cut on a cryostat at -18 °C through the forebrain at the level of the prefrontal cortex (PFC) and the middle-rostral striatum, using the mouse brain atlas by Paxinos and Watson as an anatomical reference. Sections were thaw-mounted on to gelatin-coated slides, and stored at 70 C for subsequent analysis. Probes used for radioactive in situ hybridization were oligodeoxyribonucleotides complementary to sequences of target genes mRNAs (MWG Biotech, Firenze). Details for all probes are listed in Supplementary Table 1. All probes were designed from Gen-Bank sequences and checked with BLAST in order to avoid cross hybridization. For each probe, a 50 ml labeling reaction mix was prepared on ice using DEPC-treated water, 1X tailing buffer, 7.5 pmol/ml of oligodeoxyribonucleotide, 125 units of terminal deoxynucleotidyl transferase (TdT) and 100 mCi 35S-dATP. The mix was incubated for 20 min at 37 °C. Unincorporated nucleotides were separated from radiolabeled DNA using ProbeQuant G-50 Micro Columns (Amersham- GE Healthcare Biosciences, Milano, Italy). Sections were processed for radioactive in situ hybridization according to previously published protocols. All solutions were prepared with sterile double distilled water. The sections were fixed in 4% formaldehyde in 0.12MPBS (pH 7.4), quickly rinsed three times with PBS, and placed in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl, pH 8.0, for 10 min. Next, the sections were dehydrated in 70%, 80%, 95% and 100% ethanol, delipidated in chloroform for 5 min, rinsed again in 100% and 95% ethanol and air-dried. Sections were hybridized with 0.4e0.6106 cpm of radiolabeled oligonucleotide in buffer containing 50% formamide, 600mMNaCl, 80 mMTris-HCl (pH 7.5), 4 mM EDTA, 0.1% pyrophosphate, 0.2 mg/ml heparin sulfate, and 10% dextran sulfate. Slides were covered with coverslips and incubated at 37 °C in a humidified chamber for 22e24 h. After hybridization the coverslips were removed in SSC and the sections

were washed 4 -15 min in 2x SSC/50% formamide at 43e44 °C, followed by two 30-min washes with 1X SSC at room temperature. The slides were rapidly rinsed in distilled water and then in 70% ethanol. The sections were dried and exposed to Kodak-Biomax MR autoradiographic film (Sigma). A slide containing a scale of 16 known amounts of 14C standards (ARC-146C, American Radiolabeled Chemical) was co-exposed with the samples. The autoradiographic films were exposed in a time range of 14e30 days. The optimal time of exposure was chosen to maximize signal-to-noise ratio but to prevent optical density from approaching the limits of saturation. Quantitation of the autoradiographic signal was performed using a computerized image analysis system by ImageJ software (v. 1.46v, http://rsb.info.nih.gov/ij/). All hybridized sections used for comparative statistical analysis were exposed on the same sheet of X-ray film. Signal intensity analysis was carried out on digitized autoradiograms measuring mean optical density within outlined regions of interest (ROIs) as shown in Fig. 4A. Measurements of optical density within ROIs were converted into "relative dpm" using a calibration curve based on the standard scale co-exposed to the sections. 14C standard values from 4 through 12 were previously cross-calibrated to 35S brain paste standards. In order to obtain a calibration curve for each X-ray film, a "best fit" 3rd degree polynomial was used. For each animal, measurements from 3 adjacent sections were averaged and the final data reported in relative dpm as mean ± S.E.M. The in situ hybridization procedure (sectioning, hybridization and quantification) was performed blinded with coded frozen brains.

2.2.7. Statistical analysis

Three and four-way ANOVAs (Analysis of Variance) were used to analyze the contribution of the categorization factors taken into account (genotype, sex, developmental stage, repeated measure) on the outcome of the dependent variable (behaviors and gene expressions). Newmane Keul's post-hoc test was used for making comparisons between groups when the overall ANOVA showed statistical significant differences for the main factors or their interactions. We also performed Student's t-test in each sex subgroup in order to analyze differences in gene expression levels in

male and female animals separately. The accepted value for significance was $p < 0.05$. The statistical analyses were performed using JMP 9.0.1 and Statistica 11 (StatSoft) software.

2.3. Results

2.3.1. DAT genetic hypo-function did not affect startle reactivity and sensorimotor gating abilities in adolescent and adult mice

PPI is a sensorimotor gating measure that is highly conserved across mammalian species, and can be studied experimentally in mice [29]. PPI deficits are evident in patients with SZ [37], [38] and BD [39], while for ADHD current evidence are negative [38], [40].

A detailed analysis on responses to several acoustic startle stimuli and reaction thresholds revealed no DAT genotype effect in adolescent male ($F_{1,31}$= 0.02; p = 0.88; Fig. 1A) and female mice ($F_{1,36}$= 0.22; p= 0.63; Fig.1B), as well as in adult male ($F_{1,32}$ = 0.28; p= 0.60; Fig.1C) and female mice ($F_{1,23}$= 0.0018; p= 0.96; Fig.1D). Similarly, the PPI of a 120 dB acoustic startle stimulus showed no DAT-dependent effects in all tested groups ($F_{1,134}$ = 0.082; p= 0.77, Fig.1E e H). In all groups, PPI progressively increased with higher prepulse intensities ($p < 0.0001$). Again, no DAT-dependent differences were found in the startle amplitude measured (Supplementary Fig. 1). This is in contrast to DAT-/- mice which have been consistently reported to show startle and PPI deficits [41], [42] (see Supplementary Table 1). Overall, these results demonstrate that DAT hypofunctioning throughout the animal's life did not affect startle and PPI measures, discrepant to PPI deficits found in SZ and BD, but consistent with evidence reporting that these abilities are mostly intact in patients with ADHD.

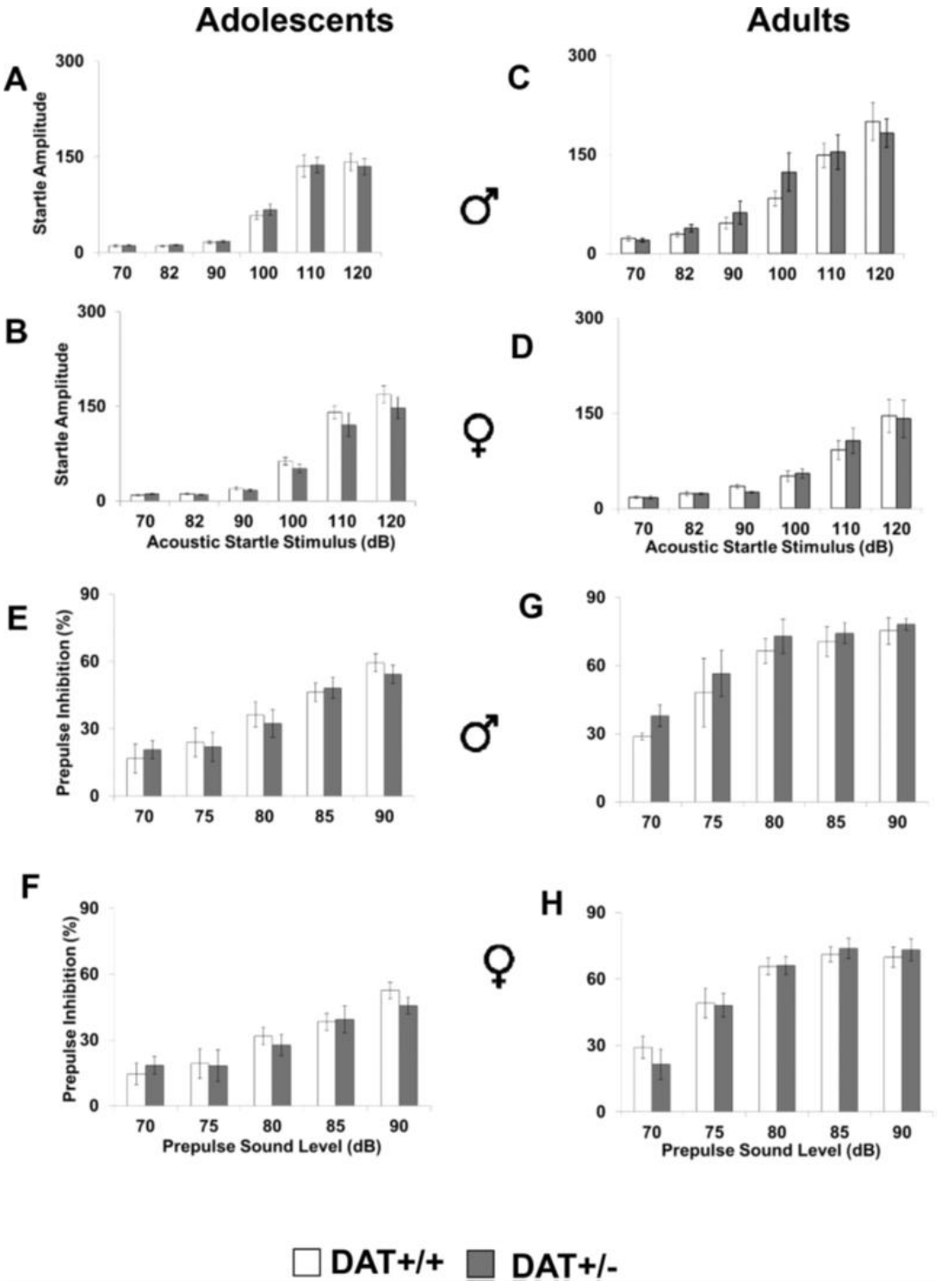


Figure 1. Acoustic startle reaction amplitude (in arbitrary units) displayed by DAT+/+ and DAT+/- (A) adolescent males (+/+ = 15; +/-= 18), (B) adolescent females (+/+= 26; +/-= 12), (C) adult males (+/+= 21; +/-= 13), and (D) adult females (+/+= 15; +/-= 10) after the presentation of 70, 82, 90, 100, 110 and 120 acoustic startle stimuli. Prepulse inhibition (in percentage) of the 120 dB acoustic startle response displayed by (E) adolescent males (+/+= 15; +/-= 21), (F) adolescent females (+/+= 24; +/-= 15), (G) adult males (+/+= 17; +/-= 15), and (H) adult females (+/+= 15; +/-= 12) after the presentation of 70, 75, 80, 85, 90 dB prepulse sound stimuli. Values represent mean ± SEM throughout all Figures.

2.3.2. DAT genetic hypofunction produces a hyperactive phenotype from adolescence to adulthood

Hyperactivity is markedly evident in ADHD as an inherent diagnostic factor and often a pervasive symptom dimension among clinical manifestations [43], while for SZ and BD levels of activity may be not substantially affected [44]. The term hyperactivity generally refers to excessive motor activity and in patients with ADHD might be qualitatively different between individuals and might vary depending on patient's age and sex [45]. A strong DAT genotype effect in the distance travelled in an empty open field arena was evident ($F_{1,129}$= 33.82, $p < 0.0001$; Fig. 2A e D). This effect was independent from the sex of the subjects or their developmental stage as no DAT-by-sex, DAT-by-developmental stage or DAT-by-sex-by-developmental stage interactions were present ($F_{1, 129}$= 33.82, $p > 0.53$). In particular, DAT+/- mice were consistently hyperactive compared to their +/+ littermates ($p < 0.0005$; Fig. 2). Moreover, in contrast to +/+ mice, DAT+/- did not show a normal habituation to the novelty of the open field arena ($p < 0.05$). No DAT-dependent differences were evident for other parameters such as time spent in the internal zone compared to the external one (Supplementary Fig. 2). Despite being significant, this hyperactive phenotype was much weaker than in DAT-/- mice [10] (see Supplementary Table 1). Thus, previous statistically negative findings in DAT+/- [10] might derive by the overwhelming DAT-/- phenotype and/or by less sensitive tracking technology. Finally, general health assessment in DAT +/+ and +/- mice demonstrated no DAT-dependent differences, and no interactions with sex or developmental stage in body weight, physical aspect and life expectancy (Supplementary Table 3). Also this was in contrast with DAT-/- mice which show growth retardation, dwarfism, and very poor survival [46]. Overall, these data show that partial DAT genetic disruption results in a consistent hyperactive phenotype since adolescence (Fig. 2A e B) that persists throughout the lifespan. This is consistent with hyperactive phenotypes present in patients diagnosed with ADHD.

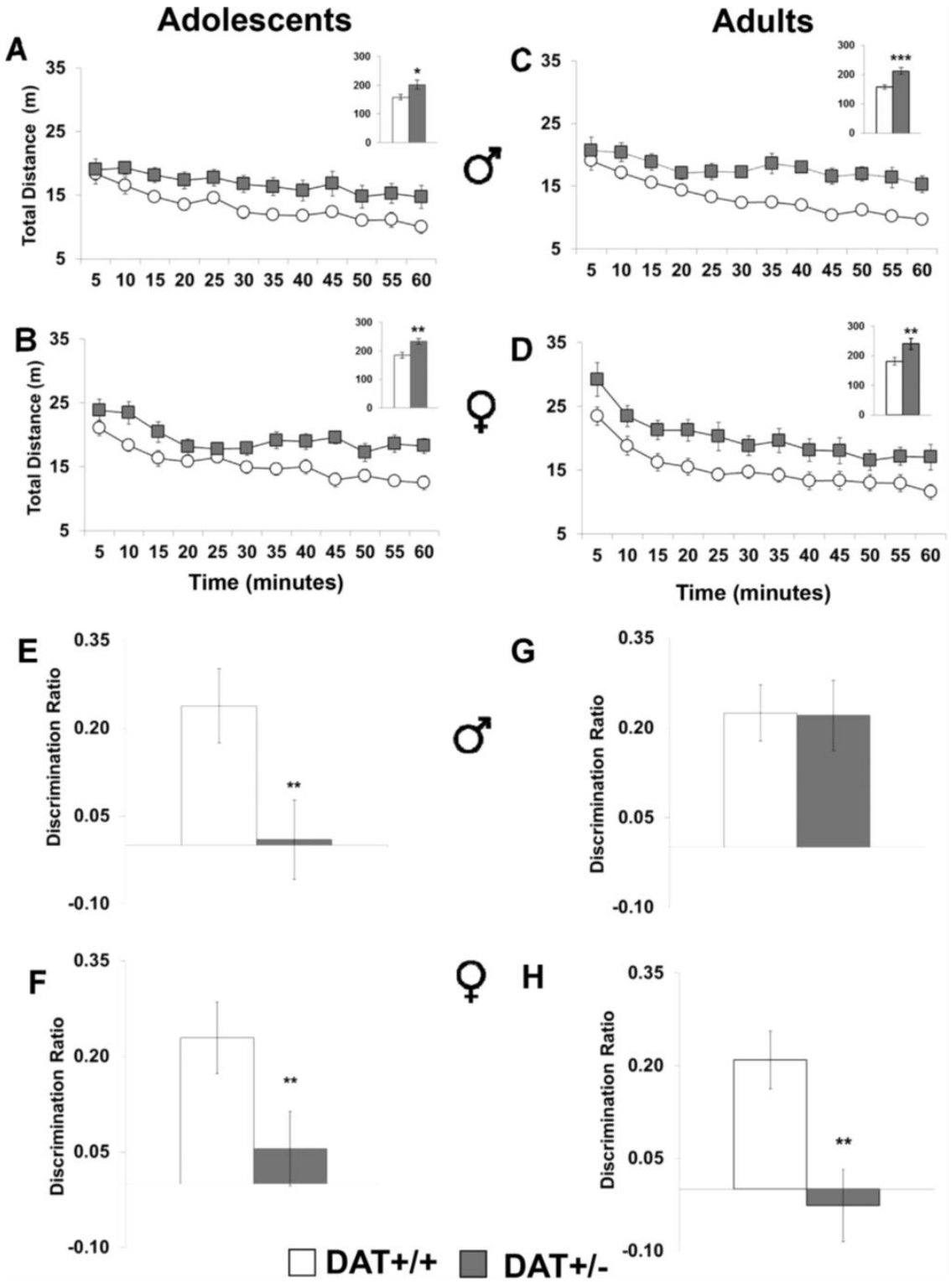


Figure 2. Spontaneous locomotor activity displayed by DAT+/+ and DAT+/- (A) adolescent males (+/+= 18; +/-= 19), (B) adolescent females (+/+= 26; +/-= 15), (C) adult males (+/+= 18; +/-= 11), and (D) adult females (+/+= 16; +/-=13). *p<0.05, **p<0.005, ***p < 0.0005 versus þ/þ littermates. Discrimination ratio displayed by DAT+/+ and DAT+/- (E) adolescent males (+/+=18; +/-= 14), (F) adolescent females (+/+= 21; +/-= 14), (G) adult males (+/+= 28; +/-= 8) and (H) adult females (+/+/=12; +/-= 8) during the 5-min temporal order object recognition test. **p<0.005 versus +/+ littermates.

2.3.3. DAT genetic hypofunction disrupts recency memory abilities more during adolescence than in adulthood

Cognitive deficits have been suggested as core symptoms of SZ (Elvevåg and Goldberg, 2000), ADHD [47] and BD [48]. Thus as a first screening test we tested DAT hypofunctional mice in a temporal order object recognition task that has a broad implication of the medial prefrontal cortex (mPFC), hippocampus and perirhinal cortex (PRH)[49]. Temporal order object discrimination was disrupted in both adolescent males ($F_{1, 30}$ = 5.98, p= 0.020; Fig. 2E) and females ($F_{1, 27}$ = 4.43, p = 0.045; Fig. 2F). In contrast, only adult DAT+/- female mice showed this cognitive impairment ($F_{1, 18}$ = 6.04, p = 0.03; Fig. 2H), while DAT+/- males were not affected ($F_{1, 18}$ = 0.02; p = 0.95; Fig. 2G). No significant differences among groups were evident in the total time spent exploring both objects during sample and test phases in both adolescent (male: $F_{1, 30}$ = 0.01, p = 0.90, females: $F_{1, 27}$ = 0.40, p = 0.53; Supplementary Figs. 3A e B) and adult mice (males: $F_{1,37}$ = 0.78, p = 0.38, females: F1,18 = 0.40, p = 0.53; Supplementary Figs. 3C e D). Thus, DAT genetic reduction did not alter motivation, curiosity, motor, olfactory, tactile, or visual functions that might affect object recognition. No previous study assessed DAT involvement in recency memory in this kind of task. However, in the novel object recognition task, assessing different cognitive functions and relying on different brain areas [27], [49], adult DAT-/-, +/- and knock-in mice show impairments in the ability to recognize a familiar object [42], [50]–[53] (see Supplementary Table 1). Thus, our data indicate that DAT hypofunctioning produced general cognitive deficits during adolescence that seemed to diminish in male mice in adulthood.

2.3.4. DAT genetic hypofunction produces selective inattentive and impulsive phenotypes in adulthood

Based on evidence suggesting an ADHD-like profile in DAT+/- mice (Fig. 2) and a less general cognitive impairment in DAT+/- adult males (Fig. 2G), we aimed to dissect more subtle and ADHD-relevant cognitive impairments in adult DAT+/- male mice. To achieve this, DAT+/+ and +/- adult males were tested in the 5CSRTT which has been designed and validated to assess ADHD-relevant cognitive deficits at the preclinical level [36], [54]. A DAT genotype effect was found for parameters such as choice accuracy ($F_{1, 17}$ = 7.23; p= 0.015), incorrect responses ($F_{1,17}$ = 5.88; p = 0.027), and premature responses (F1,17= 10.39; p = 0.006). In particular, compared to +/+ littermates, DAT +/- mice displayed a decreased level of choice accuracy ($p < 0.005$; Fig. 3C), and they made more incorrect ($p < 0.05$; Fig. 3D) and more premature responses ($p < 0.005$; Fig. 3G).

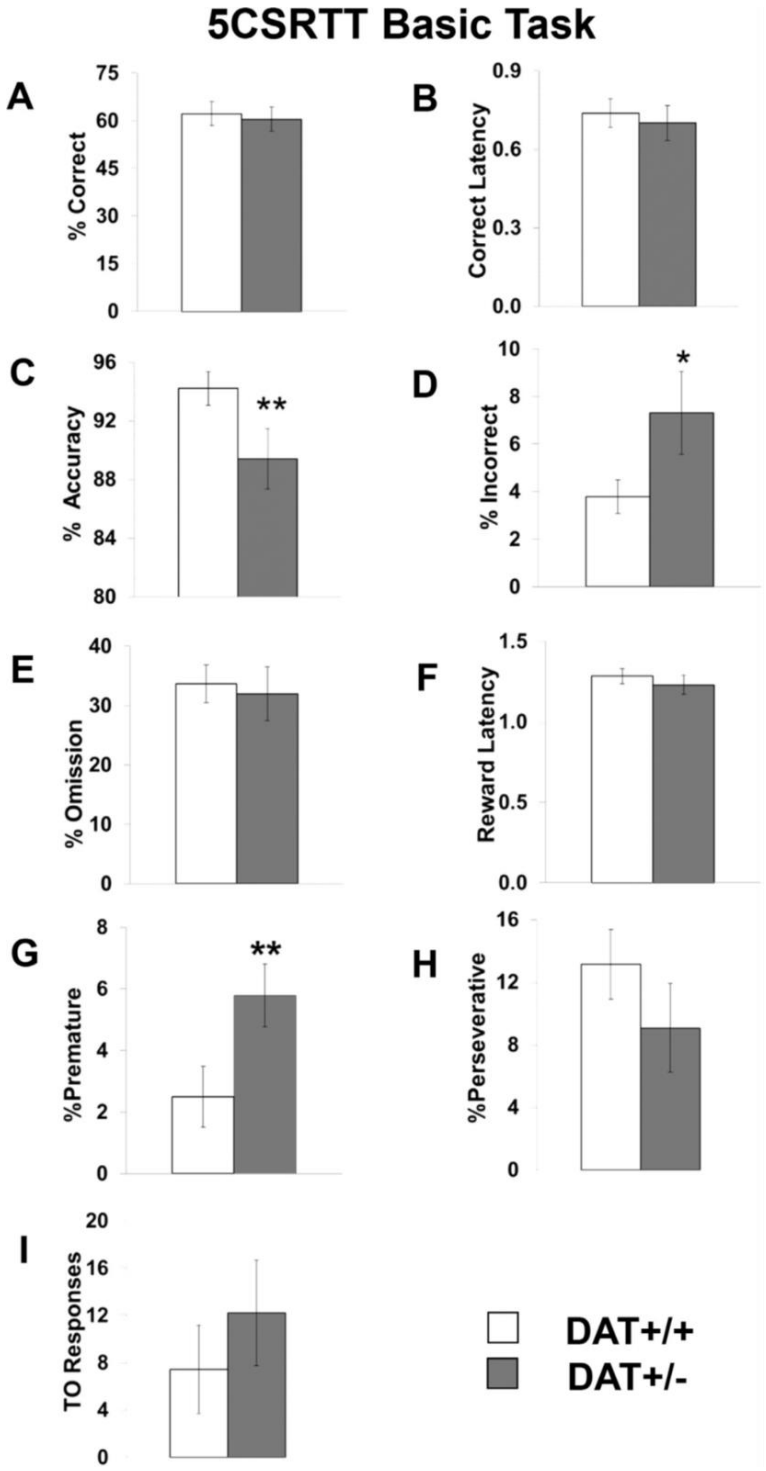


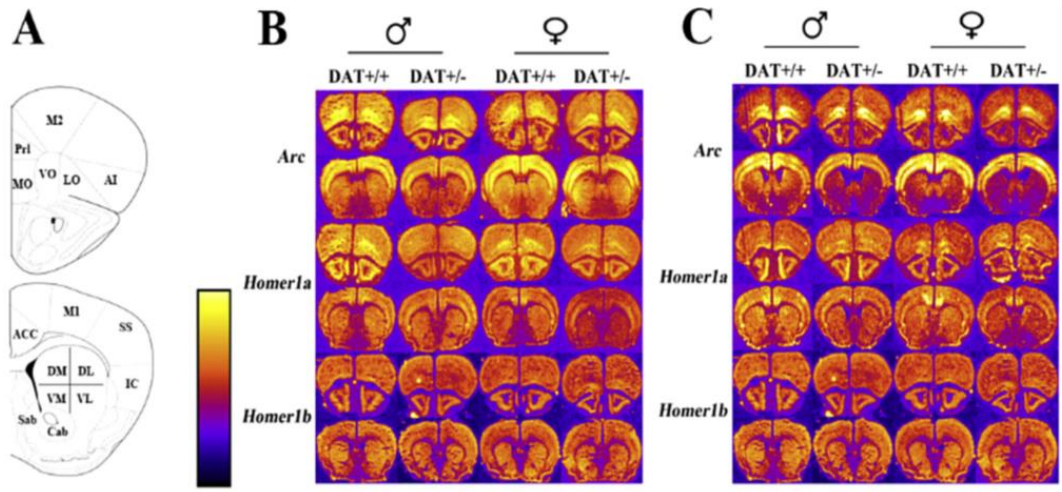
Figure 3. Different parameters measured in the 5CSRTT displayed by adult male DAT+/+ (9) and DAT+/- (11) mice, during one week of stabilization at stage 6 after animals reached the training criteria. (A) Percentage of correct responses; (B) latency (in seconds) to a correct response; (C) percentage of choice accuracy; (D) percentage of incorrect responses; (E) percentage of omitted responses; (F) latency (in seconds) to collect a food reward; (G) percentage of premature responses; (H) percentage of perseverative responses; (I) number of time out responses. *p<0.05, **p<0.005 versus þ/þ littermates.

These deficits were selective as no DAT genotype effect was evident for all other parameters measured including correct responses ($F_{1,17}$ = 0.11; p = 0.74; Fig. 3A), latency to make a correct response ($F_{1,17}$ = 0.39; p = 0.54; Fig. 3B), omissions ($F_{1,17}$ = 0.048; p =b0.83; Fig. 3E), reward latency ($F_{1,17}$ =0.38; p = 0.54; Fig. 3F), perseverative responses ($F_{1,15}$ = 2.98; p = 0.10; Fig. 3H), and time out responses ($F_{1,17}$ = 2.25; p = 0.15; Fig. 3I). To our knowledge, DAT mutant mice have not been previously assessed in this task. Moreover, because of their extreme physical and hyperactive phenotypes, no meaningful data could be derived by DAT-/- mice. These current data demonstrate that DAT hypofunctioning produced selective impairments in indices of attentional and impulsive control, consistent with core behavioral alterations observed in patients with ADHD.

2.3.5. DAT genetic hypofunction decreases Homer 1a expression in cortical executive function-related regions while sparing Arc and Homer 1b

The short Homer protein Homer1a is transcriptionally induced only upon neuronal cell stimulation [23], [55]–[57]. Notably, specific silencing of Homer1a reportedly produces a hyperactive phenotype, and deficits in attentional and learning abilities reminiscent of ADHD-related behaviors [58] . Moreover, the PFC expression of Homer1a gene and protein is disrupted in male spontaneous hypertensive rats (SHR), the most frequently used animal model of ADHD (Hong et al., 2009). Gene expression was analyzed by ANOVA. In case of significant ANOVA, group pairs were compared by the Newman Keul's post hoc test. For clarity, groups have been subdivided per genders in graphical rendering of gene expression levels. A DAT genotype effect on Homer1a mRNA levels was found in PFC regions particularly related to executive functions (ACC: $F_{3,12}$ = 4.5, p = 0.02; MO: $F_{3,11}$= 10.7, p = 0.001; VO: $F_{3,11}$=18 04, p= 0.0001; LO: $F_{3,11}$= 10.5, p= 0.001; Fig. 4A e D and Supplementary Figs. 5A and D). Specifically, DAT +/- mice displayed lower levels of Homer1a mRNA compared to +/+ littermates (ACC p= 0.003, MO p= 0.0004, VO p= 0.0001, LO p= 0.0002). These effects were consistent in both male and female animals. No DAT-dependent differences in Homer1a expression were found in lateral regions of the caudate-putamen, nucleus accumbens and in cortical regions related to motor and sensory functions (p > 0.05; Fig. 4 and Supplementary Figs. 5BeE). Alternative splicing of the Homer1 gene leads to the long isoform Homer1b, which is a constitutively expressed splice isoform without any activity-dependent regulation. Within dendritic spines, long Homer isoforms tetrameric complexes, which are required for

synaptic localization of the post-synaptic proteins Shank and PSD-95 and provide a binding platform for other synaptic proteins [59].



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	Naive			Subchronic amphetamine		
	Arc	*Homer1a*	*Homer1b*	*Arc*	*Homer1a*	*Homer1b*
CORTICAL EXECUTIVE FUNCTIONS RELATED ROIS						
ACC	↓	↓		↓	↓	
MO		↓				
VO	↓	↓		↓	↓	↓
LO		↓				
PrL						
STRIATUM ROIS						
DM		↓		↓	↓	
DL					↓	
VL				↓	↓	
VM		↓		↓	↓	
Cab					↓	
Sab					↓	
CORTICAL MOTOR AND SENSORY FUNCTIONS RELATED ROIS						
M2						
M1		↓			↓	
SS				↓	↓	
IC				↓	↓	
AI						

Figure 4. (A) Graphical depiction of the regions of Interest (ROIs), where gene expression has been analyzed. (B) Representative autoradiograms of Arc, Homer1a and Homer1b mRNA expression throughout cortical and striatal subregions in naive DAT +/+ and DAT +/- male and female littermates. (C) Representative autoradiograms of Arc, Homer1a and Homer1b mRNA expression throughout cortical and striatal subregions in amphetamine-treated DAT +/+ and DAT +/- male and female littermates. (D) Summary table showing the DAT genotype differences of Arc, Homer1a and Homer1b mRNA expression in naive and amphetamine-treated DAT +/+ and DAT +/- mice. Y significant decreased expression versus +/+ littermates. ACC: anterior cingulate cortex; MO: medial orbital cortex; VO: ventral orbital cortex; LO: lateral orbital cortex; PrL: prelimbic cortex; DM: dorsomedial caudate putamen; DL: dorsolateral caudate-putamen; VM: ventromedial caudate-putamen; VL: ventrolateral caudate-putamen; CAb: core of the nucleus accumbens; SAb: shell of the nucleus accumbens; M2: secondary motor cortex; M1: motor cortex; SS: somatosensory cortex; IC: insular cortex; AI: agranular insular cortex.

DAT hypofunction did not alter expression of the constitutive Homer1b gene throughout the brain including PFC regions ($p > 0.05$), striatal ($p > 0.05$), and other cortical motor and sensory function-related ROIs ($p > 0.05$) (Fig. 4, Supplementary Fig. 6). Activity-regulated cytoskeletal-associated (Arc) postsynaptic signaling complexes are converging point of SZ-relevant genetic variants. Arc genetic disruption has been associated with SZ [60], [61] and rodent correlates of SZ symptoms [27]. Moreover, reduced expression of Arc mRNA has been detected in the PFC of individuals with SZ [62]. DAT genotype effects on Arc expression were evident only in the ACC and VO ($F_{3,9}= 4.1$; $p= 0.04$ and $F_{3,9}= 5.1$; $p= 0.02$, respectively). In particular, mRNA levels in DAT +/- mice were significantly lower than in +/+ littermates (ACC $p= 0.033$, VO $p= 0.008$; Fig. 4 and Supplementary Fig. 7D). Comparison analyses revealed that these effects were mostly driven by female DAT+/- mice but not present in males (Supplementary Figs. 7A and D). No DAT-dependent effects were evident for Arc expression in both striatal ($p > 0.05$) and cortical motor and sensory functions-related ROIs. Overall, data from molecular imaging of gene expression in DAT +/- mice were consistent with the hypothesis that the DAT +/- genotype may be relevant to ADHD, but not to SZ and BD phenotypes. Indeed, the molecular alterations found in this genotype were strongly divergent, both quantitatively and topographically, from those reported in clinical and preclinical studies on SZ and BD.

2.3.6. Amphetamine rescues inattentive and impulsive phenotypes produced by DAT genetic hypofunction

Amphetamines are the first-line pharmacological treatments in the management of ADHD, since they improve symptomatic domains such as inattentiveness and impulsivity [16]. Thus, we sub-chronically treated with amphetamine DAT +/+ and +/- adult male mice and tested them in the different protocols of the 5CSRTT. In contrast to what was found in drug naïve conditions (Fig. 3), DAT +/- treated with amphetamine did not show deficits in parameters such as choice accuracy ($F_{1,15}$= 0.39; p= 0.54; Fig. 5C), incorrect ($F_{1,15}$= 0.30; p= 0.59; Fig. 5D) and premature responses ($F_{1,15}$= 0.006; p= 0.94; Fig. 5G). As for previous tests, no DAT-dependent effects were evident for all other parameters. These data indicate that treatment with amphetamine was sufficient to abolish DAT-dependent alterations in inattentive and impulsive cognitive domains.

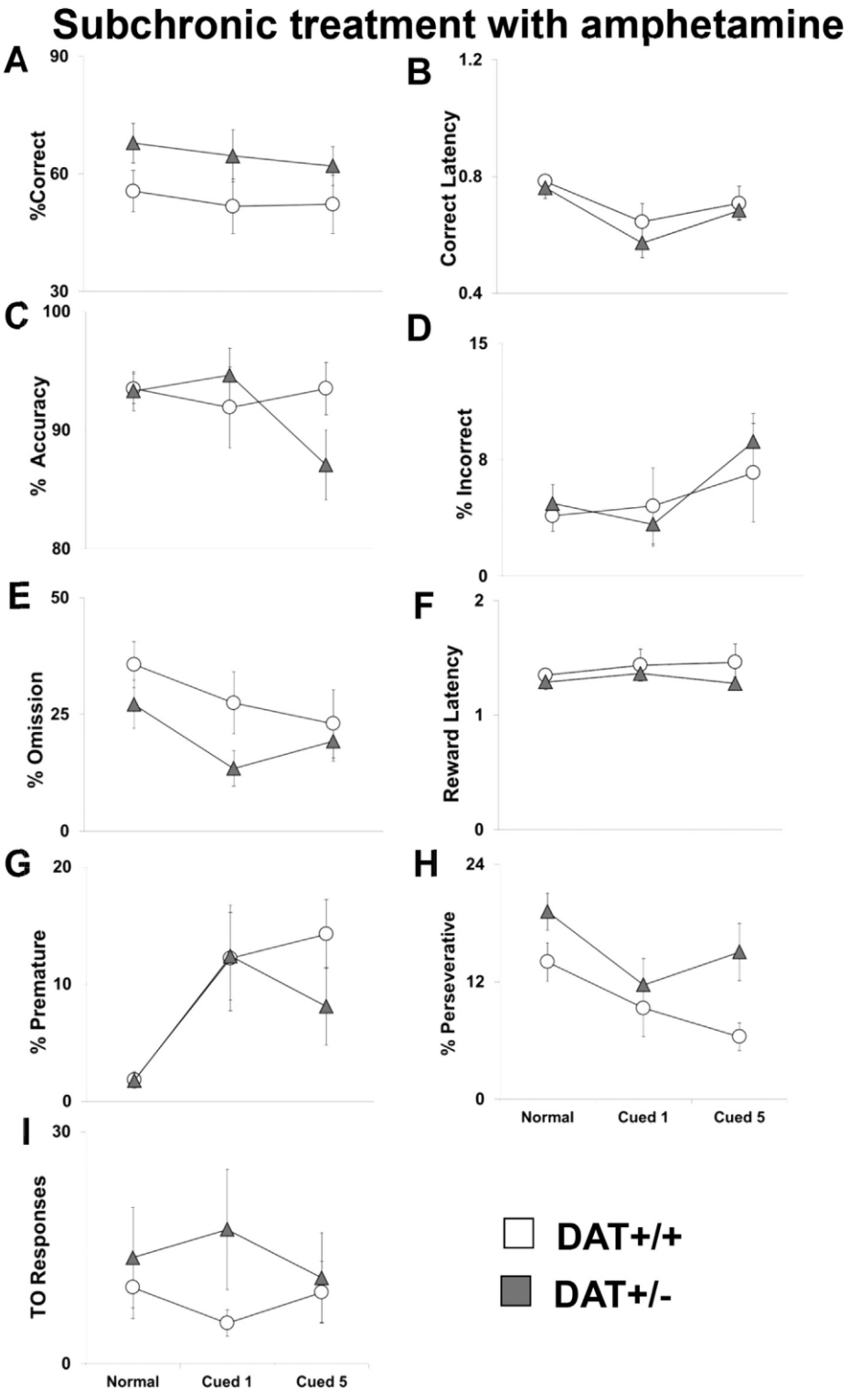

Figure 5. Different parameters measured in the 5CSRTT displayed by adult male DAT+/+ (9) and DAT+/- (11) mice, following the daily exposure to amphetamine treatments. (A). Percentage of correct responses; (B) latency (in seconds) to a correct response; (C) percentage of choice accuracy; (D) percentage of incorrect responses; (E) percentage of omitted responses; (F) latency (in seconds) to collect a food reward; (G) percentage of premature responses; (H) percentage of perseverative responses; (I) number of time out responses.

2.3.7. Amphetamine decreases DAT-dependent hyperactivity in adults in both sexes, but only in males in adolescence

Amphetamine exacerbates psychotic experiences in patients with SZ, can be psychogenic in normal human subjects, and its locomotor responses are used in rodents as correlates of SZ-like positive and BD mania-like symptoms [63], [64]. In contrast, treatment with amphetamines ameliorates hyperactive phenotypes in patients diagnosed with ADHD [65]. Amphetamine treatments for 5 consecutive days revealed a DAT genotype-by-sex-by-developmental stage-by treatment day interaction effect ($F_{4,260}$= 2.35,p= 0.05; Fig. 6). In particular, as previously shown in adult DAT -/- males [11], adult DAT +/- males and females did not show the expected behavioral sensitization to amphetamine treatment as in DAT +/+ littermates ($p < 0.005$; Fig. 6 C e D). A similar effect was evident in adolescent DAT +/- males ($p < 0.005$; Fig. 6A). In contrast, adolescent DAT +/- females responded to the amphetamine challenges as their +/+ littermates ($p = 0.85$; Fig. 6B). Overall, these findings indicate that DAT genetic hypofunctioning produced a paradoxical decreased locomotor response to amphetamine, which was absent only in adolescent females.

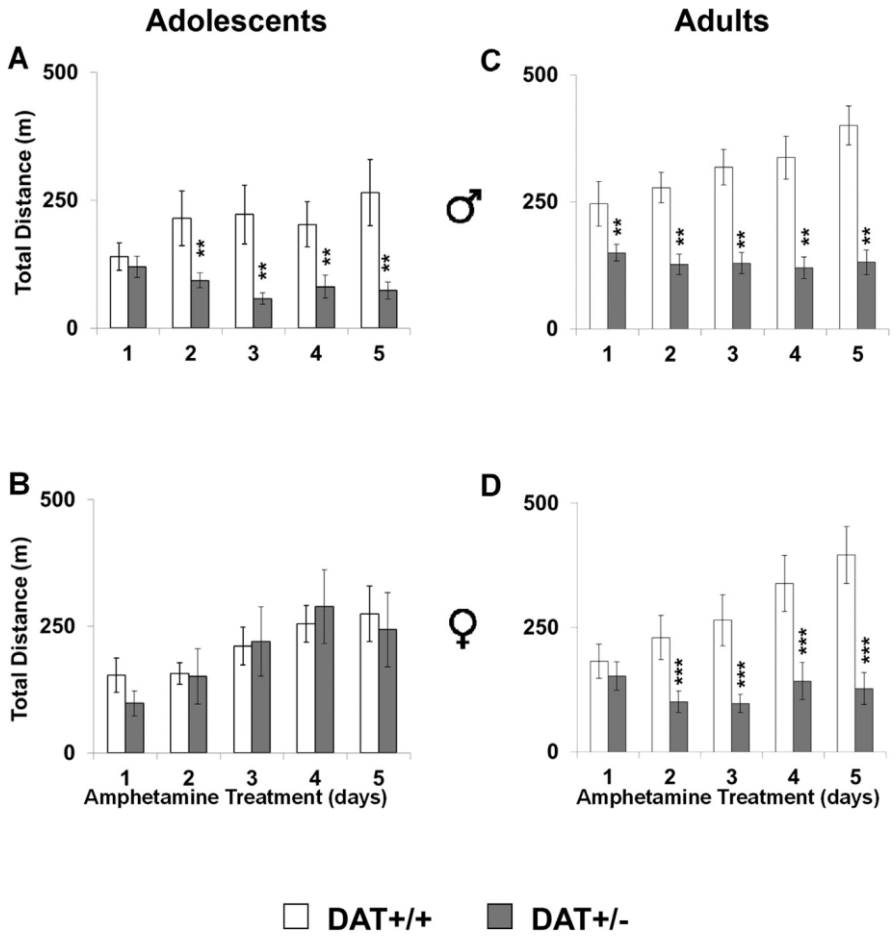

Figure 6. Locomotor activity measure after 5 consecutive days of amphetamine treatments displayed by DAT +/+ and DAT +/- (A) adolescent males (+/+= 5; +/-= 7), (B) adolescent females (+/+= 8; +/-= 7), (C) adult males (+/+ = 10; +/-= 13), and (D) adult females (þ/þ ¼ 13; þ/_ ¼ 11). **p<0.005, ***p < 0.0005 versus þ/þ littermates at the same time point.

2.3.8. Amphetamine ameliorates the DAT-dependent altered pattern of expression of Homer1a in the PFC, while extended it at striatal levels

Based on our behavioral findings that amphetamine treatment ameliorates ADHD-relevant behavioral abnormalities in DAT hypofunctional mice, we next investigated if amphetamine treatment might also modulate DAT-dependent molecular alterations. In the amphetamine-treated groups, significant differences in Homer1a expression between DAT +/- and DAT +/+ mice were found in fewer executive function-related cortical regions than in drug naïve animals (only ACC: $F_{3,\ 10}$= 10.9, p= 0.0017; MO: $F_{3,9}$= 9.4, p= 0.003; VO: $F_{3,10}$= 34.5, p= 0.0001). Moreover, the prominent effect of the DAT genotype on gene expression observed in naïve animals was lost (Fig. 4, Supplementary Fig. 5A). Amphetamine treated DAT +/- and DAT +/+ mice almost did not differ in the levels of PFC Homer1a gene expression, and this result

was more pronounced in the male sub-group ($p > 0.05$; Supplementary Fig. 8A). In contrast to drug-naïve mice, in amphetamine-treated mice, several DAT-dependent significant differences appeared in all striatal regions (DM: F3,10= 36.5, p ¼ 0.0001; DL: $F_{3,10}$= 33.4, p= 0.0001; VL: $F_{3,9}$= 81.4, p= 0.0001; VM: $F_{3,10}$ = 35, p= 0.0001; Cab: $F_{3,9}$= 34.5, p= 0.0001; $F_{3,9}$= 15.6, p= 0.0006; Fig. 4, Supplementary Fig. 8 C,F). In particular, DAT +/- displayed significantly lower levels of Homer1a mRNA compared to +/+ mice (DM p= 0.0003, DL p= 0.0008, VL p= 0.0002, VM p= 0.0004, Cab p= 0.001, Sab p= 0.001). Planned comparisons revealed these effects to be largely driven by DAT +/- males, although similar effects were evident in females as well (Supplementary Fig. 8F). Homer1a expression in cortical regions related to motor and sensory functions was significantly affected in amphetamine-treated mice ($p < 0.05$), while in drug-naïve mice this was not the case (Fig. 4, Supplementary Figs. 5 and 8). As for drug-naïve mice, no DAT-dependent effects were evident in Homer1b expression in amphetamine-treated mice in PFC ($p > 0.05$; Fig. 4, Supplementary Fig. 9 A, D), striatal ($p > 0.05$) (Fig. 4, Supplementary Fig. 9 C, F), and motor and sensory functions-related ROIs ($p > 0.05$) (Fig. 4, Supplementary Figs. 9B and E). The pattern of Arc expression in amphetamine-treated mice closely resembled that observed in naive mice in the same cortical regions (ACC: $F_{3, 12}$ = 3.63, p= 0.04; VO: $F_{3, 12}$= 5.34, p= 0.014; Fig. 4; Supplementary Figs. 10B and E). Indeed, also after amphetamine treatment a significant genotype effect was observed on cortical Arc mRNA expression (ACC p= 0.0007, VO p= 0.002), with DAT +/- mice expressing lower gene levels compared to controls. Conversely, in contrast to drug-naïve mice, in the amphetamine treated groups Arc mRNA levels were significantly different through almost all caudate-putamen regions analyzed (DM: $F_{3,10=}$ 16.8, p= 0.0003; VL: $F_{3,11}$= 9.9, p= 0.001; VM: $F_{3,12}$= 80, p= 0.003). A genotype effect was observed in the above-mentioned regions (DM p ¼ 0.0001, VL p= 0.0005, VM p= 0.0007), and in particular gene levels were higher in DAT +/+ mice compared to DAT +/- mice (Fig. 4, Supplementary Figs. 10C and F). As concerns cortical regions related to motor and sensory functions, gene expression assessed by two-way ANOVA showed significant differences only in SS and IC of amphetamine-treated groups ($p < 0.05$) (Fig. 4, Supplementary Figs. 10B and E; Supplementary Table 2). Overall, amphetamine treatment reverted in large part the altered expression of Homer1a in the PFC of DAT +/- mice. On the other hand, amphetamine reduced the expression of both Arc and Homer1a in a DAT-dependent way mainly in striatal regions. These results parallel previous findings in which ADHD-like behaviors induced by Homer1a down-regulation were reverted by Homer1a up-regulation after methylphenidate administration [58].

2.4. Discussion

This study demonstrates that genetic variations resulting in DAT hypofunction produce several core behavioral alterations analogous to those reported in patients with ADHD, but not SZ or BD. These behavioral abnormalities were associated with a selectively reduced expression of Homer1a in cortical executive function related regions. Amphetamine ameliorated both DAT-dependent behavioral and molecular alterations. DAT+/- mice were characterized by ADHD-relevant phenotypes including persistent hyperactivity, cognitive alterations in attentional- and impulsive-control with intact sensorimotor gating abilities, as well as behavioral amelioration after treatment with amphetamine. In order to overcome previous weaknesses in DAT related literature and to get closer to the clinical setting, we specifically addressed two different time points: adolescence and adulthood. Like in ADHD, these behavioral abnormalities were evident throughout these developmental periods, while in SZ and BD onset of full-blown symptoms usually occurs during young adulthood [66], [67]. Consistent with DAT +/- phenotypes, patients with ADHD may show, starting from childhood, hyperactivity that persists during adulthood [43], [68]. In SZ patients, increased motor activity is not a trait of the disease, and when present is a more frequent agitation that is substantially different from hyperactivity. Moreover, an apparent opposite condition can be detected in SZ patients with predominating negative symptoms. In BD levels of activity are usually fluctuating, with episodic mood-congruent hypo/hyperactivity [44], [69]. Cognitive deficits, especially of the inattentive type, are another core and enduring feature of ADHD [68], [70], even if childhood ADHD persists into adulthood only in approximately one-half of patients [69]. Similarly, DAT +/- mice showed cognitive deficits more pronounced in attentional functioning. Indeed, DAT +/- male mice had a selective reduction in choice accuracy in the 5CSRTT, which is considered the main index of attention, thought to bypass non-specific influences such as motivational factors [31]. Similarly, clinical studies in adult ADHD patients demonstrated impaired focused and sustained attention [71], [72]. In contrast, patients with SZ have relatively spared visuospatial attentional cuing abilities [73], [74], while presenting more pronounced deficits in broad monitoring as measured with SARAT tests [75]. These same SZ relevant cognitive deficits in an equivalent new murine SARAT protocol [36] were completely absent in DAT +/- mice. Impulsivity indexes are used to describe different ADHD subtypes (e.g. hyperactive-impulsive, predominantly inattentive and combined type) [76]. DAT+/- mice showed consistent high levels of impulsive behaviors since premature responses remained high during the different test challenges. Premature responses are thought to reflect a failure of inhibitory

response control, appearing when preparatory response mechanisms are disrupted [77]. This is in further agreement with elevated levels of impulsivity present in ADHD subjects as measured by a variety of tasks [78]. Finally, available studies in children [79], [80] and adults [38], [40], [81] diagnosed with ADHD suggest no major alterations in PPI measures in this pathology, even if these studies are not yet conclusive. Indeed, it should be noted that these evidence are still mostly underpowered in relationship to sample sizes and might present potentially confounding factors such as comorbidity with other psychiatric disorders (e.g Tourette's syndrome) or pharmacological treatments. In contrast, sensorimotor gating deficits are consistently found in patients with SZ [82], [83] and BD [39] throughout the lifespan. Our findings disentangle DAT-dependent alterations with remarkable similarity to what have been used to define and diagnose ADHD.

Molecular outcomes further support DAT +/- as a model strongly related to ADHD but not SZ or BD. Indeed, previous evidence pointed to a reduction of Homer1a mRNA and protein levels in the PFC of SHR, which is one of the most widely used animal models of ADHD [25]. Furthermore, selective decreased expression of Homer1a by intra-cerebroventricular injection of a miRNA virus in rats resulted in increased locomotor activity and attentional disabilities [84] reminiscent of ADHD-like and DAT ± phenotypes. Moreover, viral restoration of Homer1a, but not Homer1c, into the PFC of homer1-/- mice rescued their persistent hyperactive phenotype while not influencing their PPI deficits [26]. Finally, pharmacological animal models SZ reported an up-regulation of Homer1a expression in fronto-cortical and striatal areas [85], [86]. Combined with previous evidence, our findings indicate that decreased Homer1a expression in the PFC may be relevant to ADHD but not SZ. In contrast, and again supporting DAT selective relevance for ADHD, but not SZ, is the lesser impact of DAT hypofunction in Arc expression. Indeed, Arc genetic disruption is gaining increasing relevance for the neuropathophysiology of SZ [27], [60]–[62], [87], [88]. Taken together, DAT hypofunctioning appears to implicate a PFC specific alteration of synaptic plasticity processes, above all those involving Homer1a that may be strongly relevant to ADHD. Stimulants such as amphetamine and methylphenidate are currently the most common treatment for ADHD [16], [74]. In particular, stimulant medications in ADHD patients ameliorate their hyperactive phenotype, improve attentional focus and alleviate impulsive behaviors. In contrast, these drugs may precipitate or exacerbate psychotic symptoms in both BD [17] and SZ patients [18]. In line with ADHD, DAT +/- mouse hyperactivity, attentional deficits and impulsive behavior were all ameliorated by administration of low doses of amphetamine. Low doses of psychostimulants, comparable to those used in ADHD patients, preferentially increase

catecholamine neurotransmission within the PFC enhancing cognitive functions [89]–[91]. Here, DAT dependent alterations of Homer1a in the PFC were normalized by amphetamine. Analogously, methylphenidate administration in male SHRs up-regulated Homer1a expression in their PFC [25]. Also, Homer1a silencing by RNA interference induced ADHD-like behaviors in rats that were reverted by methylphenidate administration, presumably via the up-regulation of Homer1a expression in the PFC [58]. Moreover, over-expression of Homer1a improved attention and cognitive processes in rats [26]. Thus, amphetamine modulation of Homer1a expression in the PFC could mirror the molecular adaptations underlying the potential beneficial effects of low-dose psychostimulant administration in ADHD patients. Considering the role of Homer1a in the regulation of dendritic spine function and synaptic plasticity, as well as Homer1a responsivity to dopamine perturbation, these findings could represent, at least in part, a molecular correlate of psychostimulant mechanism of action in condition of striatal mild hyperdopaminergia. Previous studies on DAT functioning have mostly used mice completely lacking DAT (see Supplementary Table 1). While being an optimal mouse model for early Parkinson's disease [12], [13], [92], DAT-/- present characteristics that might make them less informative for psychiatric pathologies such as ADHD, SZ or BD. For example, DAT-/- mice exhibit dwarfism, have growth problems, and must be fed with enriched diet to avoid premature death [46] which, by modulating their metabolic activity and microbiota, might influence behavior [93]. Furthermore, DAT-/- mice have to be obtained from DAT +/- or -/- mothers. DAT genetic modifications change maternal behavior [10] which, in turn, could influence behavior of the offspring. In contrast to DAT +/- , DAT-/- mice show strong PPI deficits [41], [94], [95]. This is again in agreement with PPI deficits in Parkinson patients [96] but not with ADHD [40], [79]–[81], [97], [98]. Finally, although DAT -/- mice show increased locomotor activity, this phenotype is so extreme that it renders problematic any other kind of behavioral assessment. Investigation of DAT +/- mice in the current study avoided all these issues. In conclusion, we report that genetic-driven hypofunction of DAT alter postnatal developmental trajectories consistent with ADHD-, but not SZ- and BD-relevant behavioral and molecular phenotypes. Particularly, taking into account different critical developmental periods, genetic mutations and the sex of a subject might help to implement early and personalized treatment in order to prevent or limit ADHD abnormalities.

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2.6. Conflict of interest

The authors declare no conflict of interest.

2.7. Acknowledgments

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2.8. References

[1] P. Fusar-Poli and A. Meyer-Lindenberg, “Striatal presynaptic dopamine in schizophrenia, part i: Meta-analysis of dopamine active transporter (DAT) density,” *Schizophr. Bull.*, vol. 39, no. 1, pp. 22–32, 2013.

[2] R. Gowrishankar, M. K. Hahn, and R. D. Blakely, “Good riddance to dopamine: Roles for the dopamine transporter in synaptic function and dopamine-associated brain disorders,” *Neurochem. Int.*, vol. 73, no. 1, pp. 42–48, 2014.

[3] J. K. Pinsonneault *et al.*, “Dopamine Transporter Gene Variant Affecting Expression in Human Brain is Associated with Bipolar Disorder,” *Neuropsychopharmacology*, vol. 36, no. 8, pp. 1644–1655, 2011.

[4] J. J. Weinstein, M. O. Chohan, M. Slifstein, L. S. Kegeles, H. Moore, and A. Abi-Dargham, “Pathway-specific dopamine abnormalities in schizophrenia,” *Biol. Psychiatry*, 2016.

[5] S. V. Faraone *et al.*, “Molecular genetics of attention-deficit/hyperactivity disorder,” *Biological Psychiatry*, vol. 57, no. 11. pp. 1313–1323, 2005.

[6] T. a Greenwood, N. J. Schork, E. Eskin, and J. R. Kelsoe, “Identification of additional variants within the human dopamine transporter gene provides further evidence for an association with bipolar disorder in two independent samples,” *Mol Psychiatry*, vol. 11, p. 115,125-133, 2006.

[7] P. M. Moran, C. M. P. O’Tuathaigh, F. Papaleo, and J. L. Waddington, “Dopaminergic function in relation to genes associated with risk for schizophrenia: Translational mutant mouse models,” *Prog. Brain Res.*, vol. 211, pp. 79–112, 2014.

[8] S. V Faraone, T. J. Spencer, B. K. Madras, Y. Zhang-James, and J. Biederman, “Functional effects of dopamine transporter gene genotypes on in vivo dopamine transporter functioning: a meta-analysis.,” *Mol. Psychiatry*, vol. 19, no. 8, pp. 880–9, 2014.

[9] S. R. Jones, R. R. Gainetdinov, M. Jaber, B. Giros, R. M. Wightman, and M. G. Caron, “Profound neuronal plasticity in response to inactivation of the dopamine transporter.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 95, no. 7, pp. 4029–34, 1998.

[10] B. Giros, M. Jaber, S. R. Jones, R. M. Wightman, and M. G. Caron, “Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter,” *Nature*, vol. 379, no. 6566, pp. 606–612, 1996.

[11] C. Spielewoy, C. Roubert, M. Hamon, M. Nosten-Bertrand, C. Betancur, and B. Giros, “Behavioural disturbances associated with hyperdopaminergia in dopamine-transporter

knockout mice.," *Behav. Pharmacol.*, vol. 11, no. 3–4, pp. 279–290, 2000.

[12] M. A. Kurian *et al.*, "Clinical and molecular characterisation of hereditary dopamine transporter deficiency syndrome: An observational cohort and experimental study," *Lancet Neurol.*, vol. 10, no. 1, pp. 54–62, 2011.

[13] J. Ng *et al.*, "Dopamine transporter deficiency syndrome: Phenotypic spectrum from infancy to adulthood," *Brain*, vol. 137, no. 4, pp. 1107–1119, 2014.

[14] S. Fuke, S. Suo, N. Takahashi, H. Koike, N. Sasagawa, and S. Ishiura, "The VNTR polymorphism of the human dopamine transporter (DAT1) gene affects gene expression," *Pharmacogenomics J.*, vol. 1, no. 2, pp. 152–156, 2001.

[15] M. a Mergy *et al.*, "The rare DAT coding variant Val559 perturbs DA neuron function, changes behavior, and alters in vivo responses to psychostimulants.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 44, pp. E4779-88, 2014.

[16] E. Chan, J. M. Fogler, and P. G. Hammerness, "Treatment of Attention-Deficit/Hyperactivity Disorder in Adolescents: A Systematic Review.," *JAMA*, vol. 315, no. 18, pp. 1997–2008, 2016.

[17] C. Koehler-Troy, M. Strober, and R. Malenbaum, "Methylphenidate-induced mania in a prepubertal child," *J. Clin. Psychiatry*, vol. 47, no. 11, pp. 566–567, 1986.

[18] M. Toda and A. Abi-Dargham, "Dopamine hypothesis of schizophrenia: Making sense of it all," *Current Psychiatry Reports*, vol. 9, no. 4. pp. 329–336, 2007.

[19] J. Biederman *et al.*, "Influence of gender on attention deficit hyperactivity disorder in children referred to a psychiatric clinic.," *Am. J. Psychiatry*, vol. 159, no. 1, pp. 36–42, 2002.

[20] I. Kawa *et al.*, "Gender differences in bipolar disorder: Age of onset, course, comorbidity, and symptom presentation," *Bipolar Disord.*, vol. 7, no. 2, pp. 119–125, 2005.

[21] S. Sannino *et al.*, "COMT Genetic Reduction Produces Sexually Divergent Effects on Cortical Anatomy and Working Memory in Mice and Humans.," *Cereb. cortex*, no. September, pp. 2529–2541, 2014.

[22] J. S. Rao, M. Kellom, E. A. Reese, S. I. Rapoport, and H. W. Kim, "Dysregulated glutamate and dopamine transporters in postmortem frontal cortex from bipolar and schizophrenic patients," *J. Affect. Disord.*, vol. 136, no. 1–2, pp. 63–71, 2012.

[23] A. De Bartolomeis, C. Tomasetti, and F. Iasevoli, "Update on the Mechanism of Action of Aripiprazole: Translational Insights into Antipsychotic Strategies beyond Dopamine Receptor Antagonism," *CNS Drugs*, vol. 29, no. 9, pp. 773–799, 2015.

[24] Q. Hong *et al.*, "Homer expression in the hippocampus of an animal model of attention-deficit/hyperactivity disorder," *Mol. Med. Rep.*, vol. 4, no. 4, pp. 705–712, 2011.

[25] Q. Hong *et al.*, “Prefrontal cortex Homer expression in an animal model of attention-deficit/hyperactivity disorder,” *J. Neurol. Sci.*, vol. 287, no. 1–2, pp. 205–211, 2009.

[26] K. D. Lominac *et al.*, “Distinct roles for different Homer1 isoforms in behaviors and associated prefrontal cortex function,” *J. Neurosci. Off. J. Soc. Neurosci.*, vol. 25, no. 50, pp. 11586–11594, 2005.

[27] F. Managò *et al.*, “Genetic Disruption of Arc/Arg3.1 in Mice Causes Alterations in Dopamine and Neurobehavioral Phenotypes Related to Schizophrenia,” *Cell Rep.*, vol. 16, no. 8, pp. 2116–2128, 2016.

[28] F. Papaleo, L. Erickson, G. Liu, J. Chen, and D. R. Weinberger, “Effects of sex and COMT genotype on environmentally modulated cognitive control in mice,” pp. 2–7, 2012.

[29] F. Papaleo *et al.*, “Genetic dissection of the role of catechol-O-methyltransferase in cognition and stress reactivity in mice.,” *J. Neurosci.*, vol. 28, no. 35, pp. 8709–23, 2008.

[30] H. Huang *et al.*, “Chronic and acute intranasal oxytocin produce divergent social effects in mice.,” *Neuropsychopharmacology*, vol. 39, no. 5, pp. 1102–14, 2014.

[31] T. W. Robbins, “The 5-choice serial reaction time task: Behavioural pharmacology and functional neurochemistry,” *Psychopharmacology*, vol. 163, no. 3–4. pp. 362–380, 2002.

[32] A. Barbelivien, S. Ruotsalainen, and J. Sirvio, “Metabolic alterations in the prefrontal and cingulate cortices are related to behavioral deficits in a rodent model of attention-deficit hyperactivity disorder,” *Cereb Cortex*, vol. 11, no. 11, pp. 1056–1063, 2001.

[33] R. Navarra *et al.*, “Effects of atomoxetine and methylphenidate on attention and impulsivity in the 5-choice serial reaction time test,” *Prog. Neuro-Psychopharmacology Biol. Psychiatry*, vol. 32, no. 1, pp. 34–41, 2008.

[34] Y. Chudasama and T. W. Robbins, “Dopaminergic modulation of visual attention and working memory in the rodent prefrontal cortex.,” *Neuropsychopharmacology*, vol. 29, no. 9, pp. 1628–1636, 2004.

[35] J. T. Baker *et al.*, “Disruption of cortical association networks in schizophrenia and psychotic bipolar disorder.,” *JAMA psychiatry*, vol. 71, no. 2, pp. 109–18, 2014.

[36] H. Huang *et al.*, “A schizophrenia relevant 5-Choice Serial Reaction Time Task for mice assessing broad monitoring, distractibility and impulsivity,” *Psychopharmacology (Berl).*, vol. 234, no. 13, pp. 2047–2062, 2017.

[37] D. L. Braff, N. R. Swerdlow, and M. A. Geyer, “Symptom correlates of prepulse inhibition deficits in male schizophrenic patients,” *Am. J. Psychiatry*, vol. 156, no. 4, pp. 596–602, 1999.

[38] S. Kohl, K. Heekeren, J. Klosterkötter, and J. Kuhn, “Prepulse inhibition in

psychiatric disorders--apart from schizophrenia.," *J. Psychiatr. Res.*, vol. 47, no. 4, pp. 445–52, 2013.

[39] W. Perry, A. Minassian, D. Feifel, and D. L. Braff, "Sensorimotor gating deficits in bipolar disorder patients with acute psychotic mania," *Biol. Psychiatry*, vol. 50, no. 6, pp. 418–424, 2001.

[40] D. Feifel, A. Minassian, and W. Perry, "Prepulse inhibition of startle in adults with ADHD," *J. Psychiatr. Res.*, vol. 43, no. 4, pp. 484–489, 2009.

[41] R. J. Ralph, M. P. Paulus, F. Fumagalli, M. G. Caron, and M. A. Geyer, "Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists.," *J. Neurosci.*, vol. 21, no. 1, pp. 305–13, 2001.

[42] P. Wong, C. C. R. Chang, C. E. Marx, M. G. Caron, W. C. Wetsel, and X. Zhang, "Pregnenolone Rescues Schizophrenia-Like Behavior in Dopamine Transporter Knockout Mice," *PLoS One*, vol. 7, no. 12, pp. 1–9, 2012.

[43] G. Masi *et al.*, "Attention-deficit hyperactivity disorder - Bipolar comorbidity in children and adolescents," *Bipolar Disord.*, vol. 8, no. 4, pp. 373–381, 2006.

[44] C. Marangoni, L. De Chiara, and G. L. Faedda, "Bipolar Disorder and ADHD: Comorbidity and Diagnostic Distinctions," *Curr. Psychiatry Rep.*, vol. 17, no. 8, 2015.

[45] American Psychiatric Association, *Diagnostic and Statistical Manual of Mental Disorders DSM-V*. 2013.

[46] R. Boss?? *et al.*, "Anterior pituitary hypoplasia and dwarfism in mice lacking the dopamine transporter," *Neuron*, vol. 19, no. 1, pp. 127–138, 1997.

[47] R. A. Barkley, "Behavioral inhibition, sustained attention, and executive functions: Constructing a unifying theory of ADHD.," *Psychol. Bull.*, vol. 121, no. 1, pp. 65–94, 1997.

[48] K. E. Burdick, R. J. Braga, J. F. Goldberg, and A. K. Malhotra, "Cognitive dysfunction in bipolar disorder: Future place of pharmacotherapy," *CNS Drugs*, vol. 21, no. 12. pp. 971–981, 2007.

[49] G. R. I. Barker and E. C. Warburton, "When Is the Hippocampus Involved in Recognition Memory?," *J. Neurosci.*, vol. 31, no. 29, pp. 10721–10731, 2011.

[50] A. S. C. França, L. Muratori, G. C. Nascimento, C. M. Pereira, S. Ribeiro, and B. Lobão-Soares, "Object recognition impairment and rescue by a dopamine D2 antagonist in hyperdopaminergic mice," *Behav. Brain Res.*, vol. 308, pp. 211–216, 2016.

[51] F. Napolitano *et al.*, "Role of Aberrant Striatal Dopamine D1 Receptor/cAMP/Protein Kinase A/DARPP32 Signaling in the Paradoxical Calming Effect of Amphetamine," *J.*

Neurosci., vol. 30, no. 33, pp. 11043–11056, 2010.

[52] P. Wong, Y. Sze, C. C. R. Chang, J. Lee, and X. Zhang, "Pregnenolone sulfate normalizes schizophrenia-like behaviors in dopamine transporter knockout mice through the AKT/GSK3β pathway.," *Transl. Psychiatry*, vol. 5, no. 3, p. e528, 2015.

[53] X. Zhuang *et al.*, "Hyperactivity and impaired response habituation in hyperdopaminergic mice.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 98, no. 4, pp. 1982–7, 2001.

[54] A. Bari, J. W. Dalley, and T. W. Robbins, "The application of the 5-choice serial reaction time task for the assessment of visual attentional processes and impulse control in rats.," *Nat. Protoc.*, vol. 3, no. 5, pp. 759–767, 2008.

[55] D. Bottai *et al.*, "Synaptic activity-induced conversion of intronic to exonic sequence in Homer 1 immediate early gene expression," *J Neurosci*, vol. 22, no. 1, pp. 167–175, 2002.

[56] A. de Bartolomeis and F. Iasevoli, "The Homer family and the signal transduction system at glutamatergic postsynaptic density: potential role in behavior and pharmacotherapy.," *Psychopharmacol. Bull.*, vol. 37, no. 3, pp. 51–83, 2003.

[57] A. Vazdarjanova, B. L. McNaughton, C. a Barnes, P. F. Worley, and J. F. Guzowski, "Experience-dependent coincident expression of the effector immediate-early genes arc and Homer 1a in hippocampal and neocortical neuronal networks.," *J. Neurosci.*, vol. 22, no. 23, pp. 10067–10071, 2002.

[58] L. Yang *et al.*, "The role of Homer 1a in increasing locomotor activity and non-selective attention, and impairing learning and memory abilities," *Brain Res.*, vol. 1515, pp. 39–47, 2013.

[59] M. K. Hayashi, H. M. Ames, and Y. Hayashi, "Tetrameric hub structure of postsynaptic scaffolding protein homer," *J. Neurosci. Off. J. Soc. Neurosci.*, vol. 26, no. 33, pp. 8492–8501, 2006.

[60] T. M. Hu, C. H. Chen, Y. A. Chuang, S. H. Hsu, and M. C. Cheng, "Resequencing of early growth response 2 (EGR2) gene revealed a recurrent patient-specific mutation in schizophrenia," *Psychiatry Res.*, vol. 228, no. 3, pp. 958–960, 2015.

[61] M. J. Huentelman *et al.*, "Association of SNPs in EGR3 and ARC with schizophrenia supports a biological pathway for schizophrenia risk," *PLoS One*, vol. 10, no. 10, 2015.

[62] A. L. Guillozet-Bongaarts *et al.*, "Altered gene expression in the dorsolateral prefrontal cortex of individuals with schizophrenia.," *Mol. Psychiatry*, vol. 19, no. 4, pp. 478–85, 2014.

[63] V. E. Cosgrove, J. R. Kelsoe, and T. Suppes, "Toward a valid animal model of bipolar disorder: How the research domain criteria help bridge the clinical-basic science divide,"

Biological Psychiatry, vol. 79, no. 1. pp. 62–70, 2016.

[64] M. Laruelle and A. Abi-Dargham, "Dopamine as the wind of the psychotic fire: new evidence from brain imaging studies," *J. Psychopharmacol.*, vol. 13, no. 4, pp. 358–371, 1999.

[65] C. Gillberg *et al.*, "Long-term stimulant treatment of children with attention-deficit hyperactivity disorder symptoms. A randomized, double-blind, placebo-controlled trial.," *Arch. Gen. Psychiatry*, vol. 54, no. 9, pp. 857–864, 1997.

[66] J. Egeland, "Differentiating attention deficit in adult ADHD and schizophrenia," *Arch. Clin. Neuropsychol.*, vol. 22, no. 6, pp. 763–771, 2007.

[67] I. Grande, M. Berk, B. Birmaher, and E. Vieta, "Bipolar disorder.," *Lancet (London, England)*, vol. 387, no. 10027, pp. 1561–1572, 2015.

[68] G. Bush, "Attention-Deficit/Hyperactivity Disorder and Attention Networks," *Neuropsychopharmacology*, vol. 35, no. 1, pp. 278–300, 2010.

[69] K. A. Gleason, S. G. Birnbaum, A. Shukla, and S. Ghose, "Susceptibility of the adolescent brain to cannabinoids: long-term hippocampal effects and relevance to schizophrenia.," *Transl. Psychiatry*, vol. 2, p. e199, 2012.

[70] M. Weiss, C. Murray, and G. Weiss, "Adults with attention-deficit/hyperactivity disorder: current concepts.," *J. Psychiatr. Pract.*, vol. 8, no. 2, pp. 99–111, 2002.

[71] R. Gallagher and J. Blader, "The diagnosis and neuropsychological assessment of adult attention deficit/hyperactivity disorder. Scientific study and practical guidelines.," *Ann. N. Y. Acad. Sci.*, pp. 148–171, 2001.

[72] M. Weiss and C. Murray, "Assessment and management of attention-deficit hyperactivity disorder in adults.," *CMAJ*, vol. 168, no. 6, pp. 715–22, 2003.

[73] J. M. Gold, B. Hahn, G. P. Strauss, and J. A. Waltz, "Turning it upside down: Areas of preserved cognitive function in schizophrenia," *Neuropsychol. Rev.*, vol. 19, no. 3, pp. 294–311, 2009.

[74] T. J. Spencer *et al.*, "Effect of psychostimulants on brain structure and function in ADHD: A qualitative literature review of magnetic resonance imaging-based neuroimaging studies," *J. Clin. Psychiatry*, vol. 74, no. 9, pp. 902–917, 2013.

[75] B. Hahn *et al.*, "Visuospatial attention in schizophrenia: Deficits in broad monitoring.," *J. Abnorm. Psychol.*, vol. 121, no. 1, pp. 119–128, 2012.

[76] J. T. Nigg, "Response Inhibition and Disruptive Behaviors: Toward a Multiprocess Conception of Etiological Heterogeneity for ADHD Combined Type and Conduct Disorder Early-Onset Type," in *Annals of the New York Academy of Sciences*, 2003, vol. 1008, pp.

170–182.

[77] Y. Chudasama and T. W. Robbins, “Dissociable contributions of the orbitofrontal and infralimbic cortex to pavlovian autoshaping and discrimination reversal learning: further evidence for the functional heterogeneity of the rodent frontal cortex.,” *J. Neurosci.*, vol. 23, no. 25, pp. 8771–8780, 2003.

[78] M. V Solanto, “Neuropsychopharmacological mechanisms of stimulant drug action in attention-deficit hyperactivity disorder: a review and integration.,” *Behav. Brain Res.*, vol. 94, no. 1, pp. 127–52, 1998.

[79] F. X. Castellanos, E. J. Fine, D. Kaysen, W. L. Marsh, J. L. Rapoport, and M. Hallett, “Sensorimotor gating in boys with Tourette’s syndrome and ADHD: Preliminary results,” *Biol. Psychiatry*, vol. 39, no. 1, pp. 33–41, 1996.

[80] E. M. Ornitz *et al.*, “Prepulse inhibition of startle and the neurobiology of primary nocturnal enuresis,” *Biol. Psychiatry*, vol. 45, no. 11, pp. 1455–1466, 1999.

[81] M. C. Hanlon, F. Karayanidis, and U. Schall, “Intact sensorimotor gating in adult attention deficit hyperactivity disorder,” *Int. J. Neuropsychopharmacol.*, vol. 12, no. 5, pp. 701–707, 2009.

[82] D. L. Braff, M. A. Geyer, and N. R. Swerdlow, “Human studies of prepulse inhibition of startle: Normal subjects, patient groups, and pharmacological studies,” *Psychopharmacology*, vol. 156, no. 2–3. pp. 234–258, 2001.

[83] D. L. Braff, C. Grillon, and M. A. Geyer, “Gating and habituation of the startle reflex in schizophrenic patients. Special Issue: : Gating and habituation of the startle reflex in schizophrenic patients,” *Arch. Gen. Psychiatry.*, pp. 206–215, 1992.

[84] Q. Hong *et al.*, “Increased locomotor activity and non-selective attention and impaired learning ability in SD rats after lentiviral vector-mediated RNA interference of Homer 1a in the brain,” *Int. J. Med. Sci.*, vol. 10, no. 1, pp. 90–102, 2012.

[85] S. M. Cochran, M. Fujimura, B. J. Morris, and J. A. Pratt, “Acute and delayed effects of phencyclidine upon mRNA levels of markers of glutamatergic and GABAergic neurotransmitter function in the rat brain,” *Synapse*, vol. 46, no. 3, pp. 206–214, 2002.

[86] F. Iasevoli, A. Ambesi-Impiombato, G. Fiore, F. Panariello, G. Muscettola, and A. de Bartolomeis, “Pattern of acute induction of Homer1a gene is preserved after chronic treatment with first- and second-generation antipsychotics: effect of short-term drug discontinuation and comparison with Homer1a-interacting genes.,” *J. Psychopharmacol.*, vol. 25, no. 7, pp. 875–87, 2011.

[87] M. Fromer *et al.*, “De novo mutations in schizophrenia implicate synaptic networks,”

Nature, vol. 506, no. 7487, pp. 179–184, 2014.

[88] S. M. Purcell *et al.*, "A polygenic burden of rare disruptive mutations in schizophrenia," *Nature*, vol. 506, no. 7487, pp. 185–190, 2014.

[89] K. C. Berridge, "The debate over dopamine's role in reward: The case for incentive salience," *Psychopharmacology*, vol. 191, no. 3. pp. 391–431, 2007.

[90] D. E. Greydanus, M. A. Sloane, and M. D. Rappley, "Psychopharmacology of ADHD in adolescents. [Review] [137 refs]," *Adolesc. Med. State Art Rev.*, vol. 13, no. 3, pp. 599–624, 2002.

[91] D. S. Segal and R. Kuczenski, "Human methamphetamine pharmacokinetics simulated in the rat: single daily intravenous administration reveals elements of sensitization and tolerance.," *Neuropsychopharmacology*, vol. 31, pp. 941–955, 2006.

[92] T. D. Sotnikova, J. M. Beaulieu, L. S. Barak, W. C. Wetsel, M. G. Caron, and R. R. Gainetdinov, "Dopamine-independent locomotor actions of amphetamines in a novel acute mouse model of parkinson disease," *PLoS Biol.*, vol. 3, no. 8, 2005.

[93] T. G. Dinan and J. F. Cryan, "The impact of gut microbiota on brain and behaviour: implications for psychiatry.," *Curr. Opin. Clin. Nutr. Metab. Care*, vol. 18, no. 6, pp. 552–8, 2015.

[94] M. Yamashita *et al.*, "Norepinephrine transporter blockade can normalize the prepulse inhibition deficits found in dopamine transporter knockout mice.," *Neuropsychopharmacology*, vol. 31, no. 10, pp. 2132–2139, 2006.

[95] M. Yamashita *et al.*, "Impaired cliff avoidance reaction in dopamine transporter knockout mice," *Psychopharmacology (Berl).*, vol. 227, no. 4, pp. 741–749, 2013.

[96] L. Gee *et al.*, "The Influence of Bilateral Subthalamic Nucleus Deep Brain Stimulation on Impulsivity and Prepulse Inhibition in Parkinson's Disease Patients," *Stereotact. Funct. Neurosurg.*, vol. 93, no. 4, pp. 265–270, 2015.

[97] A. Conzelmann *et al.*, "Early attentional deficits in an attention-to-prepulse paradigm in ADHD adults.," *J. Abnorm. Psychol.*, vol. 119, no. 3, pp. 594–603, 2010.

[98] E. M. Ornitz, G. L. Hanna, and J. de Traversay, "Prestimulation-induced startle modulation in attention-deficit hyperactivity disorder and nocturnal enuresis.," *Psychophysiology*, vol. 29, no. 4, pp. 437–451, 1992.

2.9. Supplementary Tables

2.9.1 Table 1

	Mouse Model					
Behavioral Tasks	**DAT-/-**	**DAT+/-**	**DAT-KD**	**DAT-CI**	**DAT-OE**	**Current Data (DAT +/-)**
Locomotor Activity (LMA)[1,2,3,4,5,6,7,8,12,18,23,24]	↑	=	↑	↑	=	↑
Psychostimulants sensitization (LMA)[9,10,11,13]	↓	↓	/	=	/	↓
Spatial Memory (Morris water Maze; Y-Maze; T-Maze)[14,25]	↓	/	/	/	/	/
General Learning impairments (Automated H-maze; Elevated Plus Maze)[15,24]	↓	=	/	/	/	/
Familiarity to the Object (Novel Object Recognition)[16,17,18,19,20]	↓	↓	↓	↓	/	/
Recency memory (temporal order object recognition)	/	/	/	/	/	↓
Attention (5-CSRTT)	/	/	/	/	/	↓
Broad Monitoring (5-CSRTT)	/	/	/	/	/	=
Impulsivity (5-CSRTT)	/	/	/	/	/	↑
Risky Behavior (Iowa Gambling Task)[21]	/	/	↑	/	/	/
Cocaine (5 and 10 mg/kg) induced Conditioned Place Preference (CPP) [2,4]	=	=	=	/	/	/
Methylphenidate (2-20 mg/kg) induced CPP [2,22]	=	=	/	↓	/	/
Amphetamine (5 mg/kg) induced CPP [26]	↑	/	/	/	/	/
Sensorimotor gating (Prepulse Inhibition (PPI))[3,16]	↓	=	=	=	/	=

Supplementary Table 1. Behavioral tasks in dopamine transporter (DAT) mutant mice. Summary table indicating previous studies in DAT mutant mice according to the following acronyms: DAT-/-= DAT knockout homozygous; DAT+/- = DAT knockout heterozygous; DAT KD= DAT knockdown; DAT-CI= DAT Cocaine Insensitive; DAT-OE= DAT overexpressing. The symbols indicate behavioral changes during the task used:

↑ signiticant behavioral increase compared to WT mice.

↓ significant behavioral decrease or impairment compared to WT.

= no behavioral differences compared to WT.

/ no data available.

2.9.1.1 Supplementary Table 1 references

[1] Giros B, Jaber M, Jones SR, Wightman RM, Caron MG. 1996. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature. 379:606–612.

[2] Sora I, Wichems C, Takahashi N, Li XF, Zeng Z, Revay R, Lesch KP, Murphy DL, Uhl GR. 1998. Cocaine reward models: conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. Proc Natl Acad Sci U S A. 95:7699–7704.

[3] Ralph RJ, Paulus MP, Fumagalli F, Caron MG, Geyer MA. 2001. Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists. J Neurosci. 21:305–313.

[4] Tilley MR, Cagniard B, Zhuang X, Han DD, Tiao N, Gu HH. 2007. Cocaine reward and locomotion stimulation in mice with reduced dopamine transporter expression. BMC Neurosci. 8:42.

[5] Ralph-Williams RJ, Paulus MP, Zhuang X, Hen R, Geyer MA. 2003. Valproate attenuates hyperactive and perseverative behaviors in mutant mice with a dysregulated dopamine system. Biol Psychiatry. 53:352–359.

[6] van Enkhuizen J, Geyer MA, Kooistra K, Young JW. 2013. Chronic valproate attenuates some, but not all, facets of mania-like behaviour in mice. Int J Neuropsychopharmacol. 16:1021–1031.

[7] Van Enkhuizen J, Geyer MA, Halberstadt AL, Zhuang X, Young JW. 2014. Dopamine depletion attenuates some behavioral abnormalities in a hyperdopaminergic mouse model of bipolar disorder. J Affect Disord. 155:247–254.

[8] Salahpour A, Ramsey AJ, Medvedev IO, Kile B, Sotnikova TD, Holmstrand E, Ghisi V, Nicholls PJ, Wong L, Murphy K, Sesack SR, Wightman RM, Gainetdinov RR, Caron MG. 2008. Increased amphetamine-induced hyperactivity and reward in mice overexpressing the dopamine transporter. Proc Natl Acad Sci U S A. 105:4405–4410.

[9] Spielewoy C, Biala G, Roubert C, Hamon M, Betancur C, Giros B. 2002. Hypolocomotor effects of acute and daily d-amphetamine in mice lacking the dopamine transporter. Psychopharmacology (Berl). 159:2–9.

[10] Morice E, Denis C, Giros B, Nosten-Bertrand M. 2010. Evidence of long-term expression of behavioral sensitization to both cocaine and ethanol in dopamine transporter knockout mice. Psychopharmacology (Berl). 208:57–66.

[11] Martin BJ, Naughton BJ, Thirtamara-Rajamani K, Yoon DJ, Han DD, Devries AC, Gu HH. 2011. Dopamine transporter inhibition is necessary for cocaine-induced increases in dendritic spine density in the nucleus accumbens. Synapse. 65:490–496.
[12] Chen YC, Lai WS. 2010. Behavioural phenotyping of dopamine transporter knockdown mice using local small interference RNA. Behav Brain Res. 214:475–479.
[13] O'Neill B, Gu HH. 2013. Amphetamine-induced locomotion in a hyperdopaminergic ADHD mouse model depends on genetic background. Pharmacol Biochem Behav. 103:455–459.
[14] Weiss S, Nosten-Bertrand M, McIntosh JM, Giros B, Martres M-P. 2007. Nicotine improves cognitive deficits of dopamine transporter knockout mice without long-term tolerance. Neuropsychopharmacology. 32:2465–2478.
[15] Del'Guidice T, Lemasson M, Etiévant A, Manta S, Magno LA V, Escoffier G, Roman FS, Beaulieu JM. 2014. Dissociations between cognitive and motor effects of psychostimulants and atomoxetine in hyperactive DAT-KO mice. Psychopharmacology (Berl). 231:109–122.
[16] Wong P, Chang CCR, Marx CE, Caron MG, Wetsel WC, Zhang X. 2012. Pregnenolone Rescues Schizophrenia-Like Behavior in Dopamine Transporter Knockout Mice. PLoS One.
[17] Wong P, Sze Y, Chang CCR, Lee J, Zhang X. 2015. Pregnenolone sulfate normalizes schizophrenia-like behaviors in dopamine transporter knockout mice through the AKT/GSK3β pathway. Transl Psychiatry. 5:e528.
[18] França ASC, Muratori L, Nascimento GC, Pereira CM, Ribeiro S, Lobão-Soares B. 2016. Object recognition impairment and rescue by a dopamine D2 antagonist in hyperdopaminergic mice. Behav Brain Res. 308:211–216.
[19] Zhuang X, Oosting RS, Jones SR, Gainetdinov RR, Miller GW, Caron MG, Hen R. 2001. Hyperactivity and impaired response habituation in hyperdopaminergic mice. Proc Natl Acad Sci U S A. 98:1982–1987.
[20] Napolitano F, Bonito-Oliva A, Federici M, Carta M, Errico F, Magara S, Martella G, Nistico R, Centonze D, Pisani A, Gu HH, Mercuri NB, Usiello A. 2010. Role of Aberrant Striatal Dopamine D1 Receptor/cAMP/Protein Kinase A/DARPP32 Signaling in the Paradoxical Calming Effect of Amphetamine. J Neurosci. 30:11043–11056.
[21] Young JW, van Enkhuizen J, Winstanley C a, Geyer M a. 2011. Increased risk-taking behavior in dopamine transporter knockdown mice: further support for a mouse model of mania. J Psychopharmacol. 25:934–943.

[22] Tilley MR, Gu HH. 2008. The Effects of Methylphenidate on Knockin Mice with a Methylphenidate-Resistant Dopamine Transporter. J Pharmacaology Exp Ther. 327:554–560.
[23] Fox MA, Panessiti MG, Hall FS, Uhl GR, Murphy DL. An evaluation of the serotonin system and perseverative, compulsive, stereotypical, and hyperactive behaviors in dopamine transporter (DAT) knockout mice. Psychopharmacology (Berl). 2013 Jun;227(4):685-95.
[24] Carpenter AC, Saborido TP, Stanwood GD. Development of hyperactivity and anxiety responses in dopamine transporter-deficient mice. Dev Neurosci. 2012;34(2-3):250-7.
[25] Li B, Arime Y, Hall FS, Uhl GR, Sora I. Impaired spatial working memory and decreased frontal cortex BDNF protein level in dopamine transporter knockout mice. Eur J Pharmacol. 2010 Feb 25;628(1-3):104-7
[26] Budygin EA, Brodie MS, Sotnikova TD, Mateo Y, John CE, Cyr M, Gainetdinov RR, Jones SR. Dissociation of rewarding and dopamine transporter-mediated properties of amphetamine. Proc Natl Acad Sci U S A. 2004 May 18;101(20):7781-6.

2.9.2 Table 2

Probe	cDNA length (bp)	cDNA position	mRNA	Gen-Bank#
Homer1a	48	1691–1738	*Homer1a*	AF093257.1
Homer1b	48	1714–1761	*Homer1b*	AB019479.1
Arc	48	278-325	*Arc*	NM_018790.3

Supplementary Table 2. Probes used for radioactive in situ hybridization were oligodeoxyribonucleotides complementary to bases sequence of target genes mRNAs (MWG Biotech, Firenze). All probes were designed from Gen-Bank sequences and checked with BLAST in order to avoid cross-hybridization.

2.9.3 Table 3

	DAT+/+	DAT+/-	DAT+/+	DAT+/-
General Health	**P28-P45**		**P90-P120**	
Body Weight (g)	12 ±1.0	12.0 ±1.0	25.0±1.0	25.0 ±1.0
Physical Abnormalities	0	0	0	0
Life expectancy (months)	>12	>12	>12	>12

♂

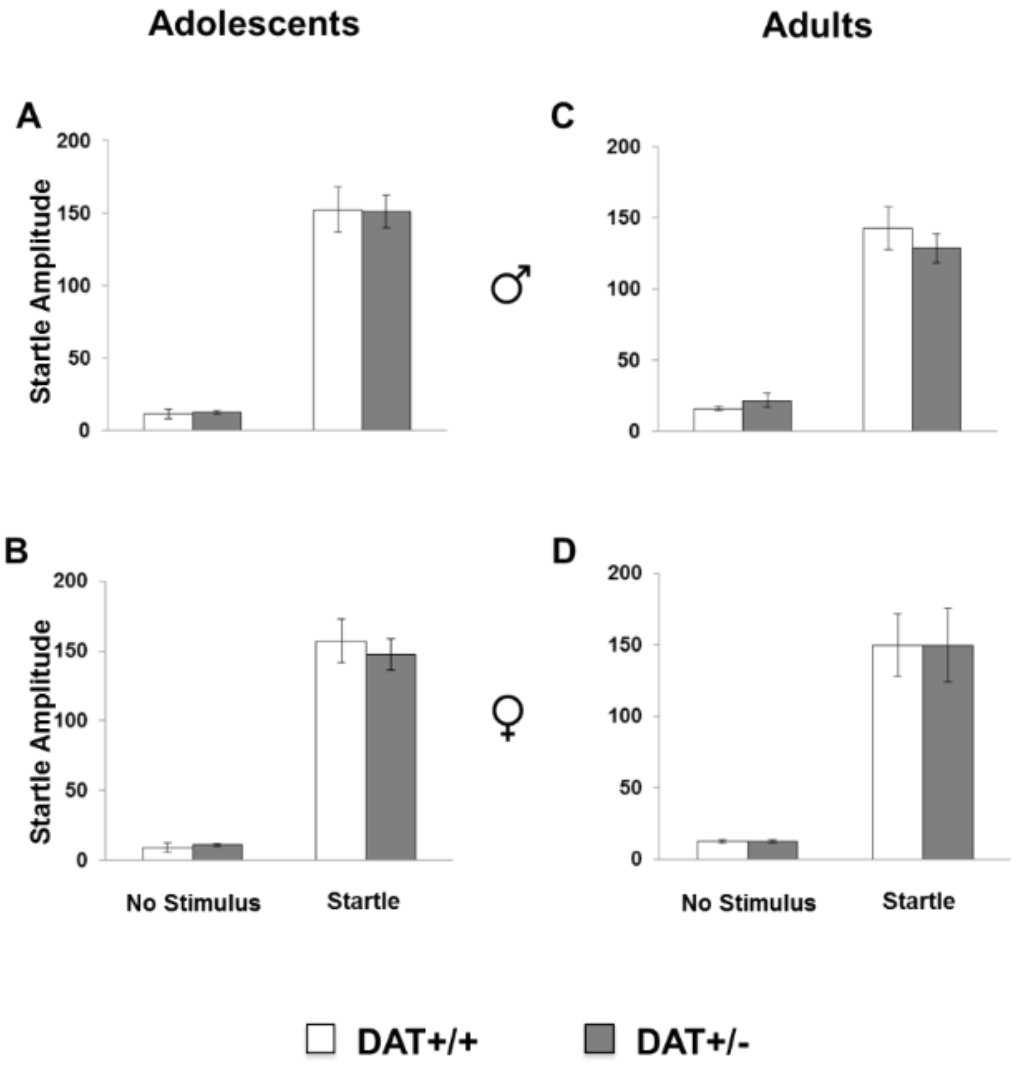
	DAT+/+	DAT+/-	DAT+/+	DAT+/-
General Health	**P28-P45**		**P90-P120**	
Body Weight (g)	12 ±1.0	12.0 ±1.0	20.0 ±1.0	20.0 ±1.0
Physical Abnormalities	0	0	0	0
Life expectancy (months)	>12	>12	>12	>12

♀

Supplementary table 3. General Health assessment for DAT+/+ and DAT +/-: body weight (g) was checked at different developmental time points from adolescence (P28-45) until adulthood (P90-120); Physical Abnormalities and life expectancy were evaluated to detect any physical artifacts that could confound the interpretation of phenotypes on more complex behavioral tasks.

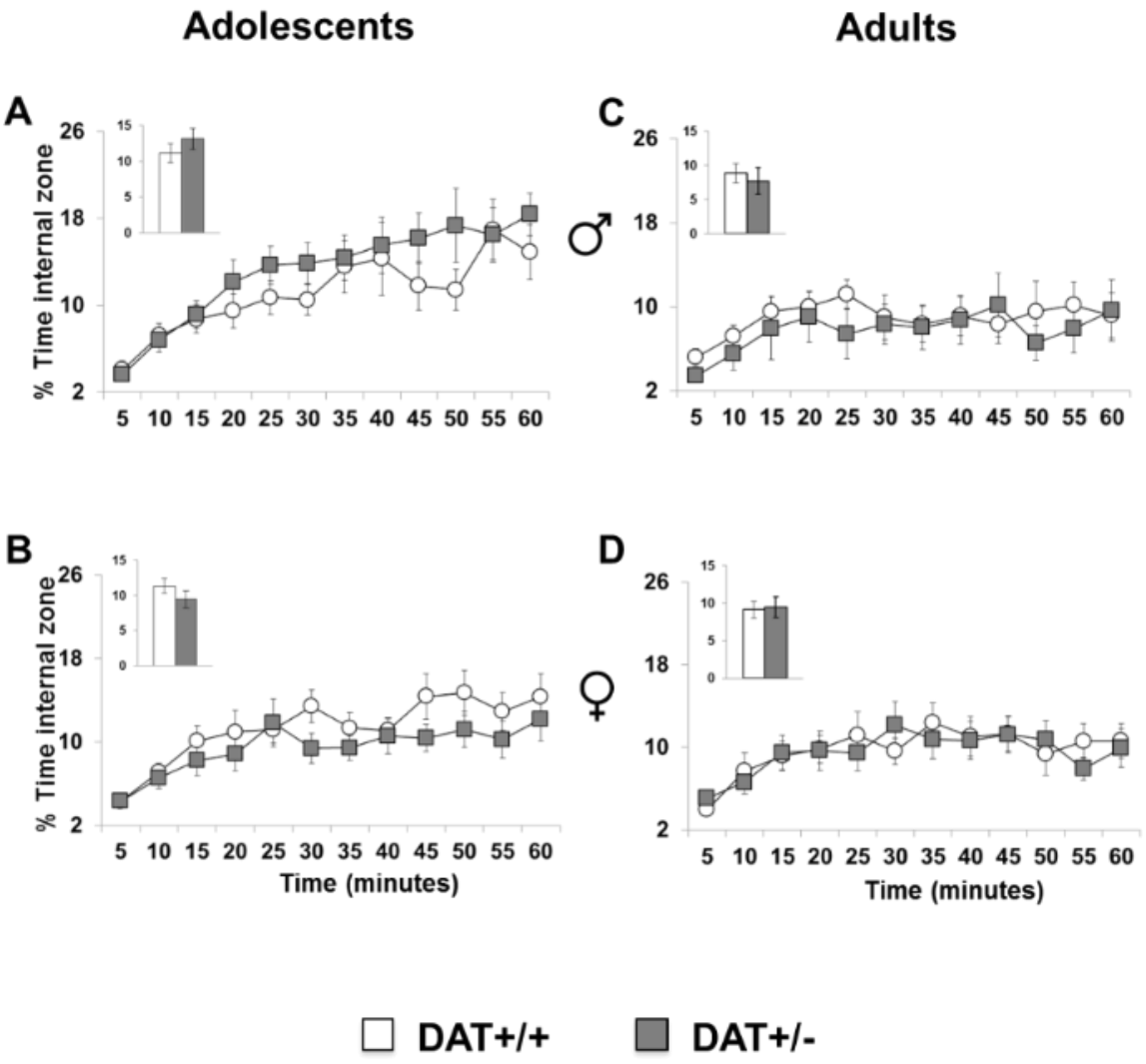
2.10. Supplemetary figures

2.10.1. Supplementary figure 1



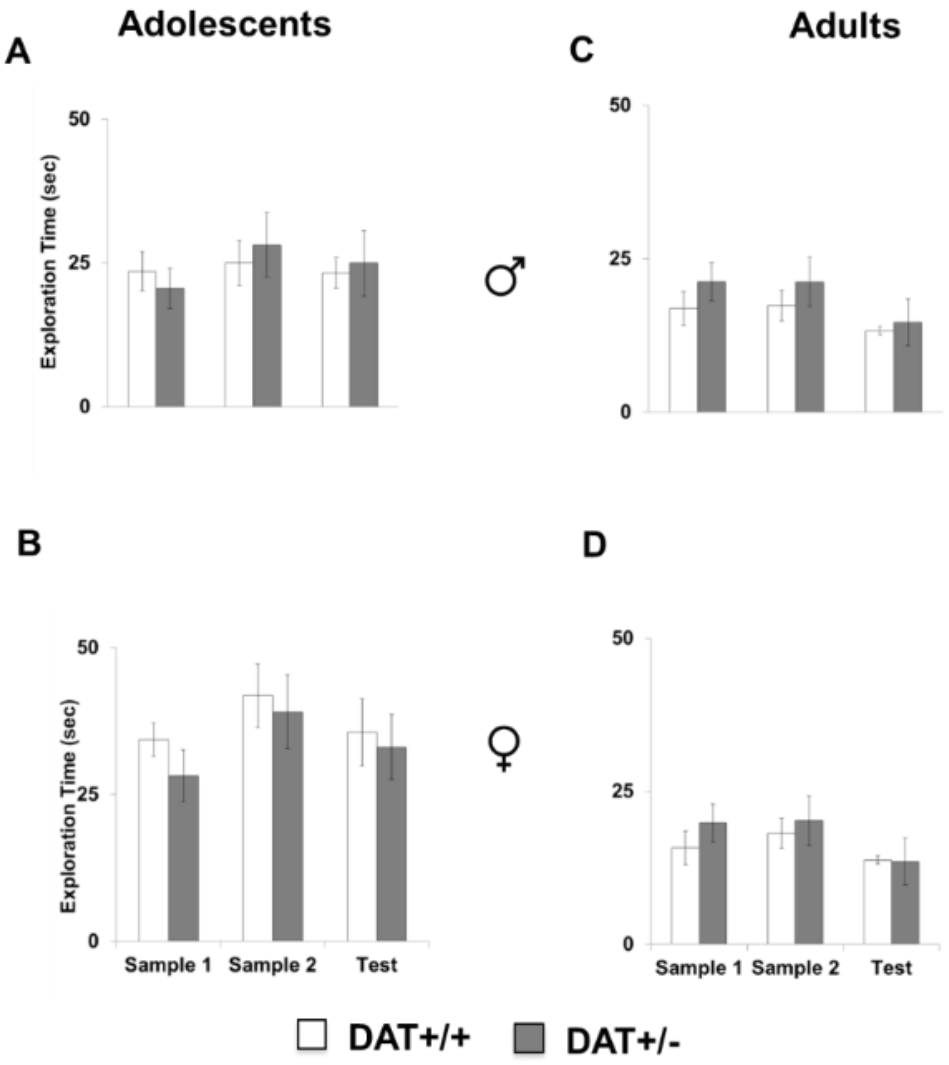
Supplementary Figure 1. Startle reaction amplitude displayed in DAT+/+ and DAT+/- mice without stimuli and after presentation of 120 db acoustic startle stimuli **a, b, c, d**; in order adolescent male(DAT+/+n=15 DAT+/-n=18) (**a**), female(DAT+/+n=26; DAT+/-n=12) (**b**) mice and adult male(DAT+/+n=21; DAT+/-n=13) (**c**) and female(DAT+/+n=15; DAT+/-n=10) (**d**) mice.

2.10.2. Supplementary figure 2



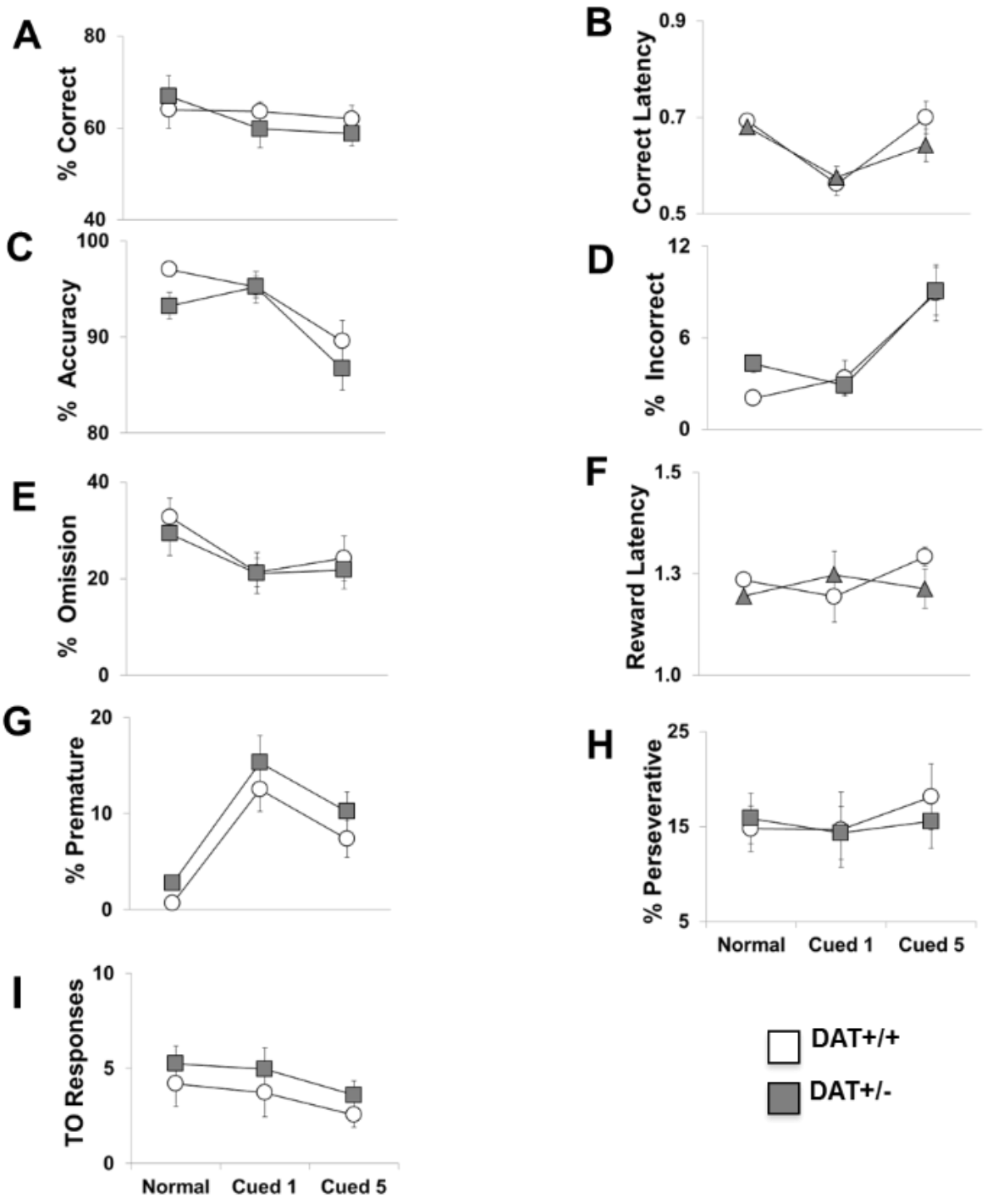
Supplementary Figure 2. Time spent in the internal zone displayed by DAT+/+ and DAT +/-: adolescent male (DAT+/+n=18; DAT+/-n=19) (a) and female (DAT+/+n=26; DAT+/-n=15) (b), adult male (DAT+/+n=18; DAT+/-n=11) (c) and female(DAT+/+n=16; DAT+/-n=13) (d) mice.

2.10.3. Supplementary figure 3



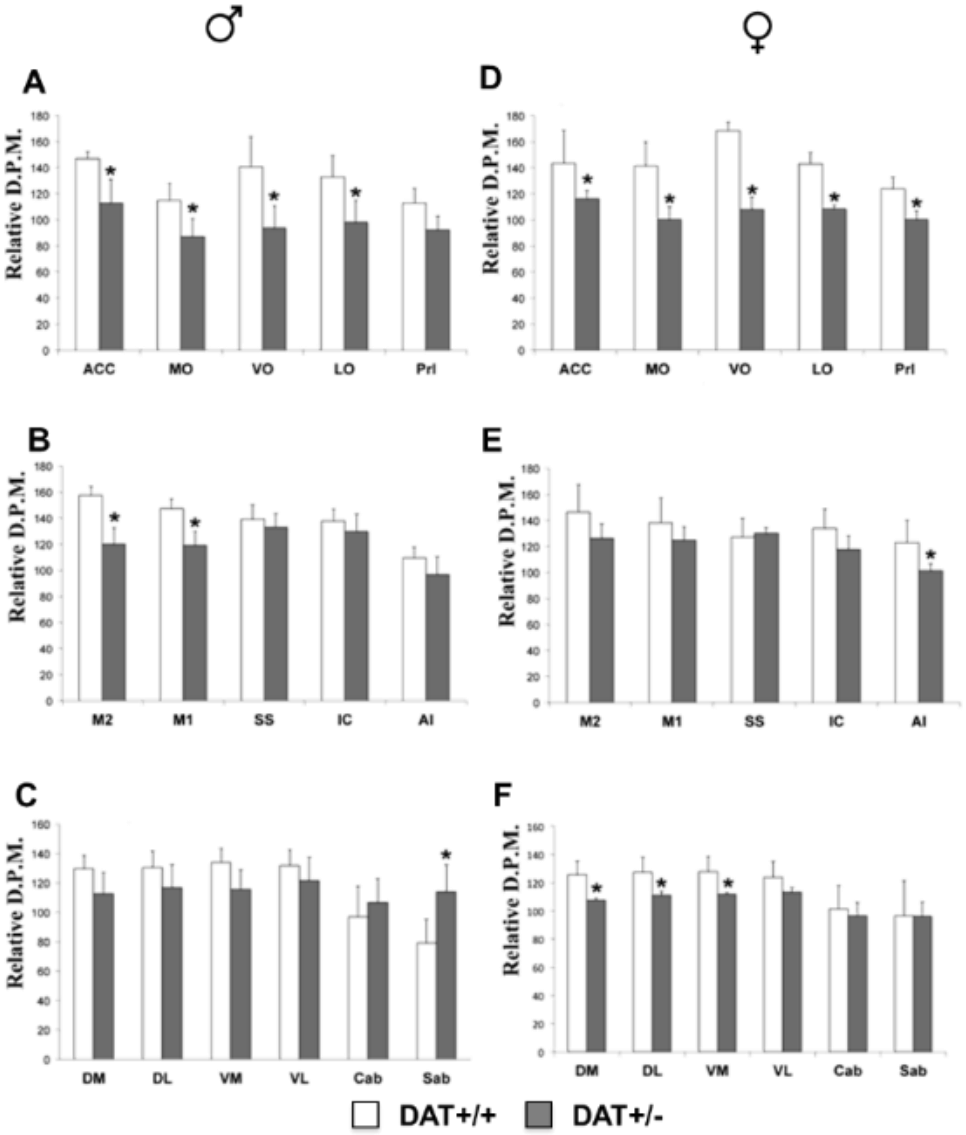
Supplementary Figure 3. Time spent exploring two identical objects displayed by DAT+/+ and DAT +/- mice during 5 min acquisition session of the temporal order object recognition test. a,b,c,d. Exploration time in seconds (sec) during the sample and test phases of the temporal order object recognition test displayed by adolescent male (a) and female (b) and adult male (c) and female (d) mice.

2.10.4. Supplementary figure 4



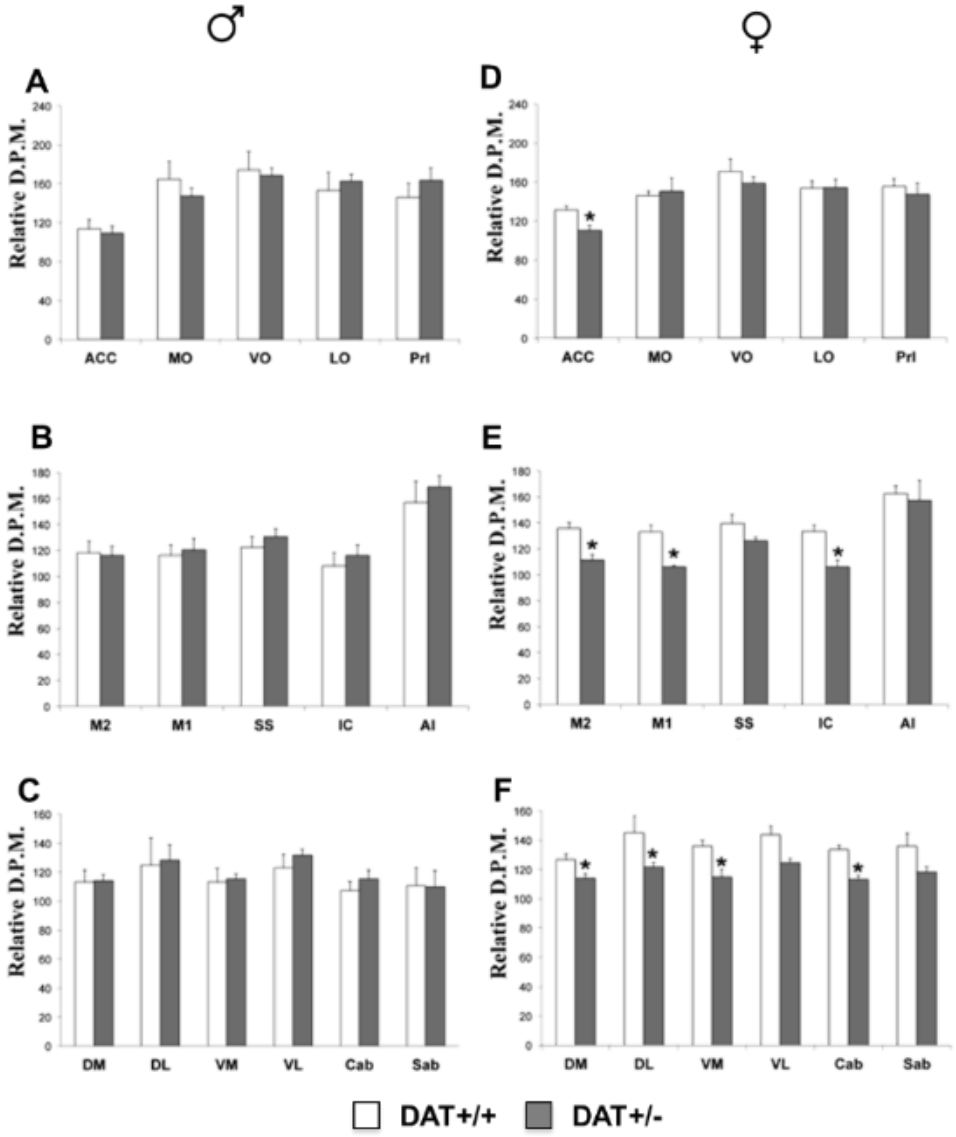
Supplementary Figure 4. Different parameters measured in the novel 5CSRTT Spatial Attentional Resource Allocation Task (SARAT) protocol displayed by adult male DAT+/+ (9) and DAT+/- (11) mice. (A) Percentage of correct responses; (B) latency (in seconds) to a correct response; (C) percentage of choice accuracy; (D) percentage of incorrect responses; (E) percentage of omitted responses; (F) latency (in seconds) to collect a food reward; (G) percentage of premature responses; (H) percentage of perseverative responses; (I) number of time out responses.

2.10.5. Supplementary figure 5



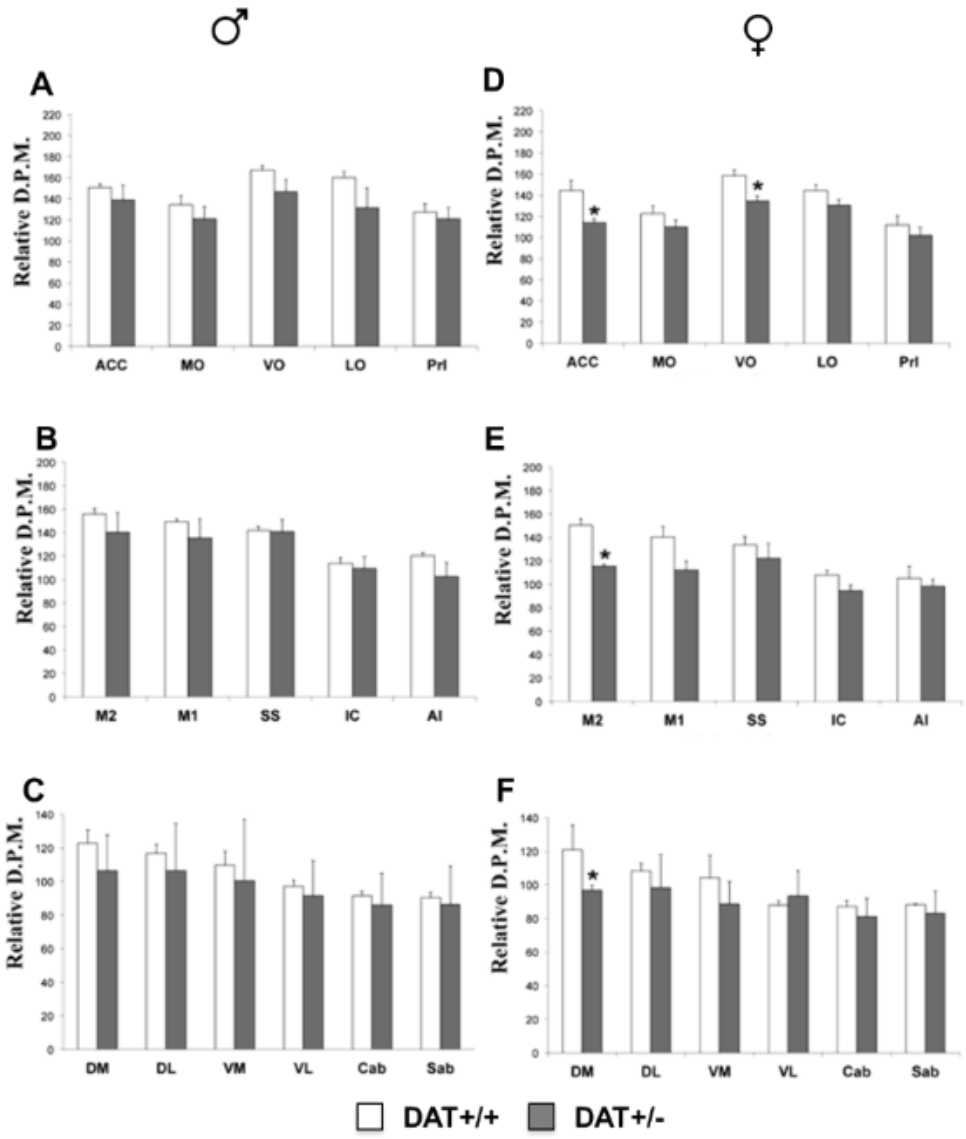
Supplementary figure 5. Levels of Homer1a mRNA expression (expressed in relative dpm) for each Region of Interest (ROI, see Figure 5 for a depiction) displayed by DAT+/+ and +/- mice without any drug treatment. Histogram bars illustrate means ± SEM. *p<0,05 versus DAT+/+ at the same ROI.

2.10.6. Supplementary figure 6



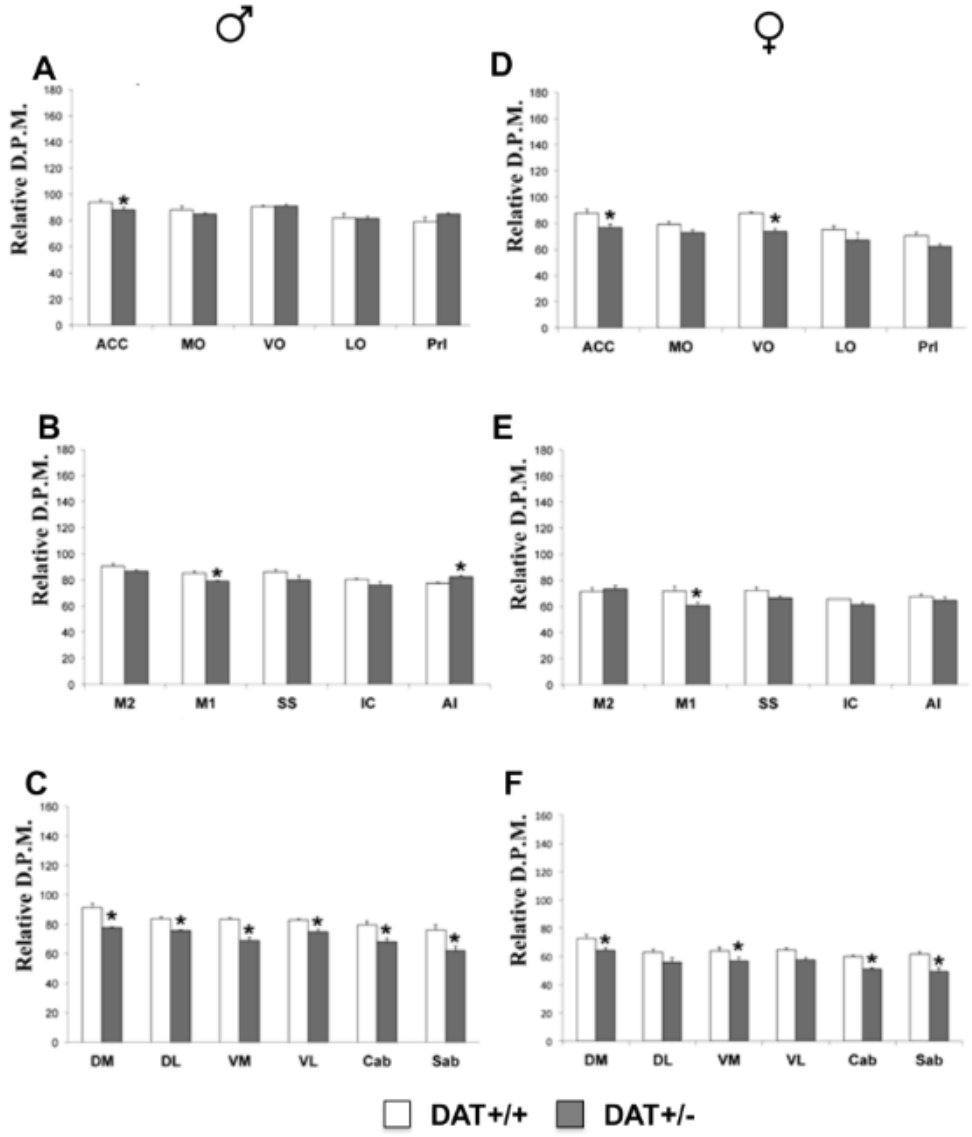
Supplementary figure 6. Levels of Homer1b mRNA expression (expressed in relative dpm) for each Region of Interest (ROI, see Figure 5 for a depiction) displayed by DAT+/+ and +/- mice without any drug treatment. Histogram bars illustrate means ± SEM. *p<0,05 versus DAT+/+ at the same ROI.

2.10.7. Supplementary figure 7



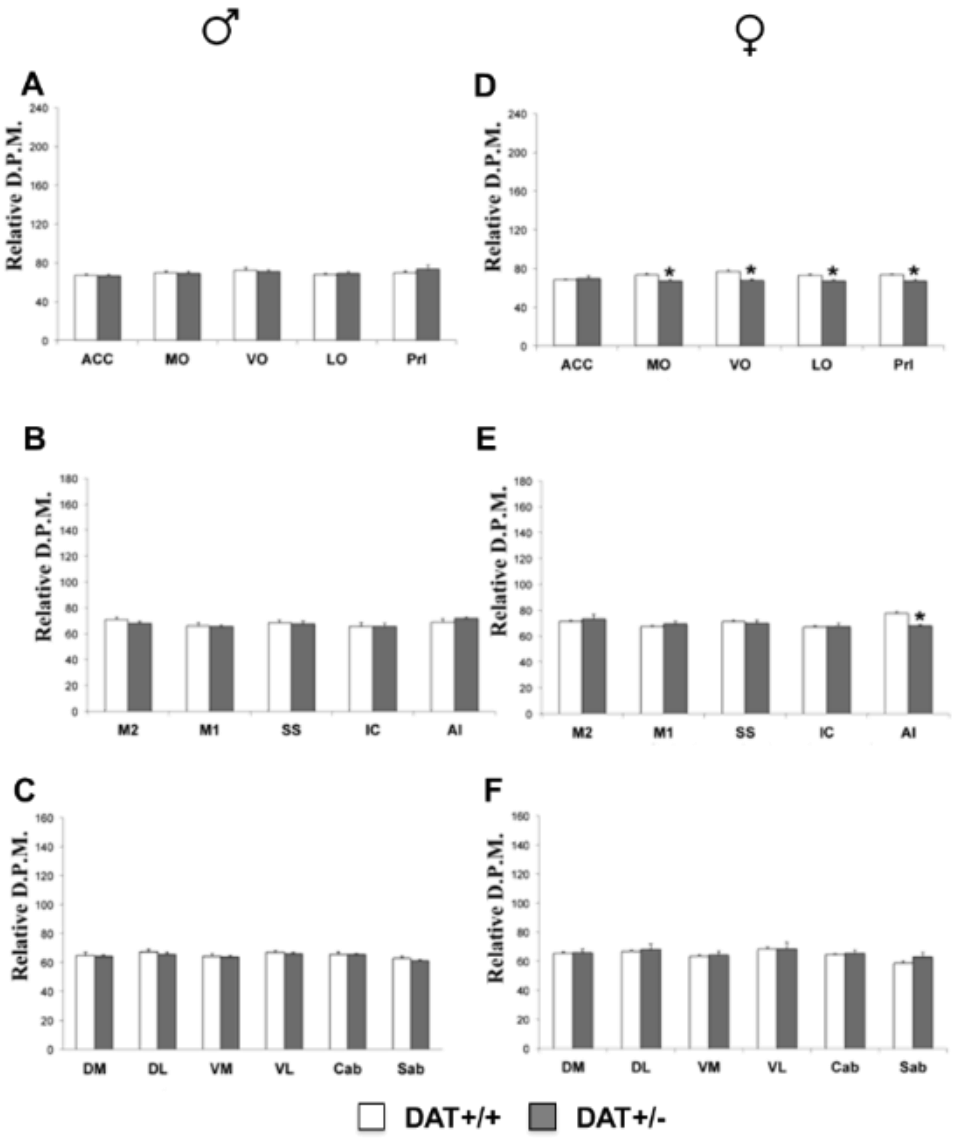
Supplementary figure 7. Levels of Arc mRNA expression (expressed in relative dpm) for each Region of Interest (ROI, see Figure 5 for a depiction) displayed by DAT+/+ and +/- mice without any drug treatment. Histogram bars illustrate means ± SEM. *p<0,05 versus DAT+/+ at the same ROI.

2.10.8. Supplementary figure 8



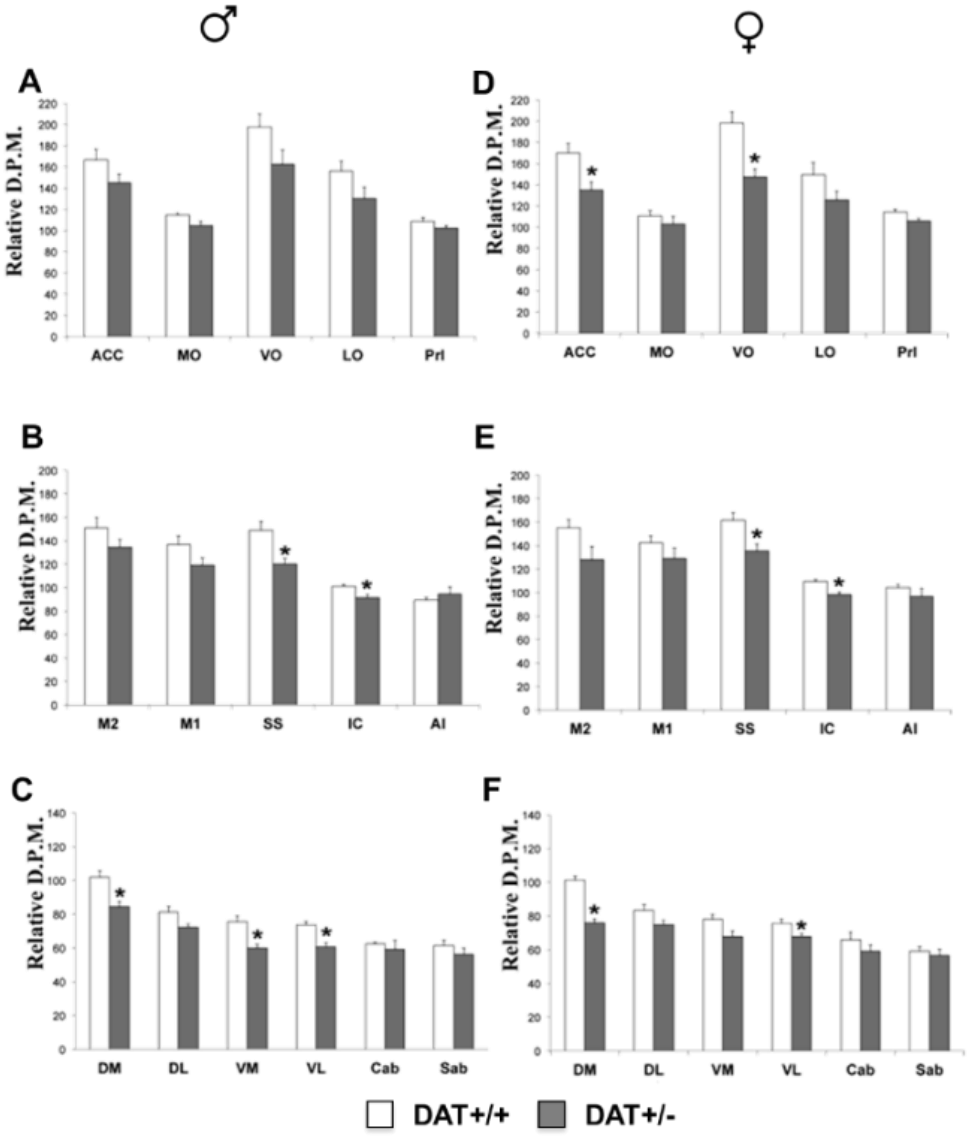
Supplementary figure 8. Levels of Homer1a mRNA expression (expressed in relative dpm) for each Region of Interest (ROI, see Figure 5 for a depiction) displayed by DAT+/+ and +/- mice subchronically treated with amphetamine. Histogram bars illustrate means ± SEM. *p<0,05 versus DAT+/+ at the same ROI.

2.10.9. Supplementary figure 9



Supplementary figure 9. Levels of Homer1b mRNA expression (expressed in relative dpm) for each Region of Interest (ROI, see Figure 5 for a depiction) displayed by DAT+/+ and +/- mice subchronically treated with amphetamine. Histogram bars illustrate means ± SEM. *p<0,05 versus DAT+/+ at the same ROI.

2.10.10. Supplementary figure



Supplementary figure 10. Levels of Arc mRNA expression (expressed in relative dpm) for each Region of Interest (ROI, see Figure 5 for a depiction) displayed by DAT+/+ and +/- mice subchronically treated with amphetamine. Histogram bars illustrate means ± SEM. *p<0,05 versus DAT+/+ at the same ROI.

Chapter 3

Attentional Control in Adolescent Mice Assessed with a Modified Five Choice Serial Reaction Time Task

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Abstract

Adolescence is a critical period for the development of higher-order cognitive functions. Unlike in humans, very limited tools are available to assess such cognitive abilities in adolescent rodents. We implemented a modified 5-Choice Serial Reaction Time Task (5CSRTT) to selectively measure attentiveness, impulsivity, broad monitoring, processing speed and distractibility in adolescent mice. 21-day old C57BL/6J mice reliably acquired this task with no sex-dependent differences in 10–12 days. A protocol previously used in adults was less effective to assess impulsiveness in adolescents, but revealed increased vulnerability in females. Next, we distinctively assessed selective, divided and broad monitoring attention modeling the human Spatial Attentional Resource Allocation Task (SARAT). Finally, we measured susceptibility to distractions using non-predictive cues that selectively disrupted attention. These paradigms were also applied to two genetically modified lines: the dopamine transporter (DAT) and catechol-O-methyltransferase (COMT) heterozygous. Adolescent DAT hypofunctioning mice showed attentional deficits and higher impulsivity as found in adults. In contrast to adults, adolescent COMT hypo-functioning mice showed decreased impulsivity and attentional resilience to distractors. These paradigms open new avenues to study the establishment of higher-order cognitive functions in mice, as well as an effective tool for drug-testing and genetic screenings focusedon adolescence.

3.1. Introduction

Adolescence is a critical transitional period of development from infancy to adulthood in which neurochemical and hormonal brain maturational processes extensively shape mammalian behaviors [1]–[3]. In particular, higher order cognitive functions drastically develop and mature during this time period[4]. Indeed, significant improvements in cognitive functioning are evident throughout late childhood and adolescence, with the most dramatic progress occurring in the development of attentional control, processing speed, decision making, planning, and response inhibition [5]–[7]. The development of attentional control is crucial because this ability might strongly influence all other cognitive domains[7], [8]. Attentional control abilities start to emerge in childhood [9], [10], but their full maturation peaks during adolescence [11]. Moreover, the speed of attentional control, its accuracy, inhibitory control towards irrelevant stimuli and the ability to disengage from one focus to another greatly improve throughout adolescence. Additionally, it has been observed that adolescents are more prone to risk taking behavior and impulsiveness, compared to infants and adults [12], [13]. Notably, adolescents with poorer attentional regulation have worse health, earn less money and commit more crimes during adulthood [14]. To trace the development of the above mentioned abilities from infancy, through adolescence, to adulthood, the serial reaction time task and other similar tasks have been extensively used in human studies [15]–[17]. Animal models are a useful tool to identify molecular and circuital processes potentially underlying the neurobiological basis of the maturational changes observed in human adolescence. The most drastic changes in terms of neuronal architecture and function have been identified within the prefrontal cortical areas (PFC) [18]–[20]. For example, in the PFC, adolescent rodents show prolonged neuronal pruning [21], a drastic maturation of the glutamatergic, dopaminergic and GABAergic systems [21]–[23] and a shift in the balance between mesocortical and mesolimbic systems [24]. Similarly, human neuroimaging studies suggest that adolescence is characterized by changes in patterns of brain activation, including increased activation in ventral PFC regions [25]–[27] and exaggerated accumbens activity related to rewarding outcomes compared to children or adults [28], [29]. However, despite several elegant studies dissecting the changes in brain circuits and molecular footprints in animal models, very limited behavioral tools that reliably assess higher order cognitive functions are available for adolescent rodents. Thus, there is still a significant gap between the extensive and complex human literature on cognitive development and the scarce equivalent tools in rodents. Behavioral paradigms able to selectively dissect different forms of attentional control during rodent adolescence could help

clarify the dynamic changes creditably observed at the molecular level, drawing better parallelisms with human studies. Finally, because adolescence is considered to be a period of higher vulnerability and increased risk of onset for several psychiatric disorders [30], appropriate cognitive tasks for rodents could help to discern the impact of genetic and environmental factors. Here we validated a modified version of the 5-Choice Serial Reaction Time Task (5CSRTT) for adolescent mice (Fig. 1a). Available tasks to assess higher-order cognitive functions have been designed and tested only in adult mice and rats [31]–[34]. This is mostly due to the long periods required for training, which are incompatible with the very short duration of rodent adolescence. Similarly to another recently modified 5CSRTT [35], our task is acquired by adolescent mice in about 12 days only, in the context of no food restriction regimens. Additionally, our task is performed minimizing single-housing, since adolescence is considered to be a delicate period for the development of social skills. Moreover, this new task did not require any additional cage other than the 5CSRTT apparatus. The novel automatic paradigms implemented are effective in differentially measuring multiple attentional functions such as selective and divided attention, broad monitoring, vulnerability to distractors, impulsivity, speed of processing and motivation in adolescent mice. This was validated in both males and females as well as in two different genetically modified mouse lines (i.e. DAT and COMT), highlighting substantial divergences in performance between adolescents and adults. Combined with the advanced techniques currently available to study the impact of molecular-, circuital-, cell- and genetic-specific factors in mice, this new behavioral tool will help improve our understanding of adolescence.

3.2. Materials and Methods

3.2.1. Mice

All procedures were approved by the Italian Ministry of Health (permit n. 230/2009-B) and local Animal Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH and the European Community Council Directives. The time period defined as "adolescence" is individually variable, but it generally corresponds to the onset of puberty (from about 9–12 to 15–17 years old in humans; from about 28 to 45 days old in rodents [1], [36]). We used in-house bred mice within the range of 21–45 days old C57BL/6J (a total of 19 males and 16 females), or genetically modified (12 DAT+/+, 12 DAT+/−, 7 COMT+/+ and 8 COMT+/−) littermates. Every other generation new C57BL/6J breeders bought from Charles River were used for the C57BL/6J colony, while the lines of genetically modified mice were backcrossed with C57BL/6J for at least 10 generations. The COMT and DAT colonies were the same as described in refs 48 and 70. The breeding scheme used to obtain the genetically modified mice involved mating a +/− heterozygous male with C57BL/6J females, in order to avoid altered maternal behavior. Experimenters were blind to genotype during testing. Mice were weaned at 21 or 26 postnatal day (PND), separated for sex and housed 2–4 per cage. Mice were housed in a climate-controlled animal facility (22 ± 2 °C) and maintained on a 12-hour light/dark cycle (light on: 7 am–7 pm). All behavioral tests were conducted during the dark phase of the cycle.

3.2.2. Apparatus

12 operant chambers (Med Associates, St. Albans, VT, USA), housed in sound-attenuating boxes each containing a fan for ventilation and constant background noise were used (schematics in Fig. 1a). Two strings of LED lights (one providing warm light and one providing cool light) were installed onto the ceiling of each of the sound-attenuating boxes controlled by a timer so that the 12-hour light/dark cycle was regulated (9 Lux when on). Each operant chamber contains, on 1 wall, 5 nose-poke holes (1 cm in diameter) that were each outfitted with a recessed stimulus light. Two additional LED pre-cue lights (red and green) were installed above each of the 5 nose-poke holes. An infrared beam transecting the aperture of each hole detected nose-pokes. Placed on the wall opposite to the 5-hole array, was a food magazine with an infrared beam and a head entry detector, where a pellet dispenser (ENV-203-14P) delivered food reinforcement in the form of a reward pellet (14 mg 5TUL Purified rodent tablet, TestDiet). Such reward pellets are designed to be a complete diet for the animals. A

water dispenser into each operant chamber ensured full access to water throughout the training/test sessions. A house-light was located 7 cm above the food magazine. The operant chambers were connected to a Smart Control Panel and interfaced to a Windows computer equipped with a MED-PC IV software (Med Associates, St. Albans, VT, USA).

3.2.3 Experimental design

3.2.3.1 Habituation

We tested different habituation protocols in order to check whether the weaning timing or food regimen could influence the task performance. In particular, the weaning was done or at 21 or 26 PND. From PND 21 to PND 23 mice were daily exposed to 1-min handling session, given ten 14-mg pellets of the 5TUL diet and weighted. Training was started or at 24 or 27 PND. When in the testing cage, mice received food in the form of pellets (5TUL Purified rodent tablet, Test Diet). Water was always ad libitum. With the "day-time food ad libitum" regime, mice received their normal food ad libitum when in the regular holding cage. In contrast, with the "day-time food restriction" regime, mice were not given access to the food when in their holding cage unless losing weight, in which case extra food was provided during the day in order to keep the mice at their normal body weight curve of adolescent growth. Such food regimens were kept throughout the entire test.

3.2.3.2 Training protocol

Throughout training and testing, mice were daily placed into the operant chambers in the evening between 5 and 5:30 pm and taken out of the chambers the following morning between 10 and 10:30 am to be placed back into their regular holding cages (grouped house as weaned). Each night (between 7 pm and 7 am), mice were presented with three testing sessions semi-randomly and automatically presented (with a variable delay between sessions of 2–5 hours). Mice were weighed every day in the morning immediately after being taken out of the apparatus. A free reinforcement pellet was delivered at the start of each testing session. When a head entry was detected, the first trial began with an inter-trial interval (ITI). Any nose-poke during the ITI was recorded as premature response resulting in a time-out period with the house-light turned on. At the end of the time-out, the house-light was turned back off and the ITI restarted. Any nose-poke during the time-out reset the time-out period. At the end of the ITI, the program randomly selected a stimulus location (1 out of 5 stimulus lights) and turned on the corresponding stimulus light. The stimulus light remained on for the stimulus duration (SD) value set. The animal had limited hold time (LH) to nose-poke into the lit hole. A nose-

poke into the lit hole during the LH, was recorded as a correct response, the stimulus light turned off if not turned off earlier and a food pellet was delivered in the opposite-wall food magazine. A nose-poke into any of the other apertures was recorded as an incorrect response. Errors resulted in the initiation of a 5-sec time out (TO) phase, during which the house light switched on and all holes were unresponsive. A lack of response within the LH period, was deemed as omission and resulted in a time-out and no reward. Premature responses (occurring in the ITI before presentation of the trigger light stimulus) also led to a time-out without reward and to a resetting of the trial. A perseverative response was scored when mice continued to poke in the same response hole when it no longer stood for a correct choice. Time from the onset of the light stimulus to the performance of a correct nose-poke response and from the correct response to the retrieval of the food reward from the magazine were recorded as correct latency and reward latency, respectively. Training consisted of 6 stages. To proceed to each subsequent stage, mice were required to reach the criterion for 2 consecutive sessions. Each stage was more challenging than the last, with the SD and LH period decreasing while other criteria become more demanding (see below). Sessions ended after 30 minutes or 100 trials, whichever comes first. Criteria to reach each subsequent stage:

1. Stage 1 to 2: SD = 20 s; LH = 30 s; ITI = 2 s.

Criteria: ≥20 correct trials; ≥20% correct.

2. Stage 2 to 3: SD = 10 s; LH = 30 s; ITI = 2 s.

Criteria: ≥30 correct trials; ≥30% correct.

3. Stage 3 to 4: SD = 8 s; LH = 20 s; ITI = 5 s.

Criteria: ≥40 correct trials; ≥80% accuracy; ≤60% omission.

4. Stage 4 to 5: SD = 4 s; LH = 10 s; ITI = 5 s.

Criteria: ≥40 correct trials; ≥80% accuracy; ≤60% omission.

5. Stage 5 to 6: SD = 2 s; LH = 7 s; ITI = 5 s.

Criteria: ≥45 correct trials; ≥80% accuracy; ≤60% omission.

6. Stage 6: SD = 1 s; LH = 7 s; ITI = 5 s

Upon reaching Stage 6, mice were subjected to an extra day of testing at Stage 6. After that, mice were tested with three different test protocols with in between a day of Stage 6 as explained below and in the timeline (Fig. 1a). The following measures were recorded to assess task performance as previously described [33], [34]. Accuracy: number of correct responses divided by sum of number of correct and incorrect responses, multiplied by 100.

- Correct responses: number of correct responses divided by total number of trials, multiplied by 100.

- Omissions: number of omissions divided by total number of trials, multiplied by 100.
- Premature responses: number of premature responses divided by sum of correct, incorrect, premature, perseverative and time-out responses (total number of responses), multiplied by 100.
- Perseverative responses: number of perseverative responses divided by total number of responses, multiplied by 100.
- Time-out responses: number of time-out responses divided by total number of responses, multiplied by 100.
- Correct latency: total time from onset of light stimulus to the performance of a correct response divided by number of correct responses.
- Reward latency: total time from the performance of a correct response to the retrieval of the food reward from the food magazine divided by number of correct responses.

3.2.3.3 5–7 ITI challenge

During the 3 sessions of the night, randomly, in a 20% of the trials the ITI was increased from 5 to 7 seconds. This implicated that mice must withhold an additional 2 seconds both before the appearance of the stimulus light and before making their correct choice. The SD and LH remain unchanged.

3.2.3.4 Spatial Attentional Resource Allocation Task (SARAT)

Two versions of the SARAT test were performed: SARAT v1 and v2 (Fig. 2 for a representative scheme). In SARAT v1 in each of the 3 sessions during the night, three different trial types were randomly presented: Cued 0, as stage 6. Cued 1, as stage 6 but with the addition of a red cue light appearing over the correct nose-poke hole from 1 s before to 1 s after the normal stage 6 yellow stimulus light. Cued 5, as stage 6 but with the addition of a red cue light appearing over each nose poke hole from 1 s before to 1 s after the stage 6 yellow stimulus light. Also in the SARAT v2 three different types of trials were randomly presented. Cued 1 trial as for SARAT v1. The Cued 3 trial was the same as the standard trial type with the addition of 1 red pre-cue light appearing over the correct nose-poke hole and 2 pre-cue red lights appearing over the 2 nose-poke holes adjacent the correct nose-poke hole from 1 s prior to 1 s after the stimulus light duration. The third type of trial was the Cued 5 trial as for SARAT v1. Each trial type was presented an equal number of times in a random fashion throughout each session.

3.2.3.5 Distractor test

In this manipulation, two versions of the Distractor test were performed: distractor v1 and v2 (Fig. 2 for a representative scheme). In v1, two different trial types were randomly presented. Cued 1 (80% of the time) like for the SARAT test. The Distractor (Dist) trial (20% of the time) was identical to the Cued 1 with the addition of three green cue lights flashing from 1 second before to 1 second after the normal stage 6 yellow stimulus light. In Distractor v2, the Cued 0 trial (presented 80% of the time within a session) was the standard trial type as in Stage 6. The Distractor trial occurred 20% of the time and was the same as the Cued 0 trial with the addition of a flashing green pre-cue light over the nose-poke holes number 1, 3, and 5. In Distractor v2, no predictive pre-cue red lights were used. The green pre-cue light over the nose-poke holes were turned on from 1 s prior to 1 s after the stimulus light duration. Any nose-poke that occurred while the red/green pre-cue lights were lit, but before the normal stimulus light was presented, was considered a premature response and was not rewarded, resulting in a time-out.

3.2.4. Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM) throughout. One or two-way analyses of variance (ANOVAs) with sex (male or female) or genotype (+/+ or +/−) as between subjects factors and trial type as the within-subject repeated measure was used to analyze each single parameter measured (body weight, % Correct, % Accuracy, % Omission, % Time out, % Premature, % Perseverative, Correct latency and Reward latency). Newman-Keul's post-hoc test with multiple comparisons corrections was used for making comparisons between groups when the overall ANOVA showed statistical significant differences for the main factors or interactions. Student's t-test was used to compare the days needed to reach the criteria between males and females, the % Perseverative between DAT+/+ and +/−, the % Premature between COMT+/+ and +/−. The accepted value for significance was $p < 0.05$. All statistical analyses were performed using the Statistica version 12 software (Statistica, StatSoft, Inc.).

3.3. Results

Adolescent mice readily acquired the modified 5CSRTT. In order to keep the duration of the task within the very short period of mice "adolescence" (i.e. ≈25–50 days old), the first challenge we had to face was to shorten the long training which is usually required for the classical 5CSRTT for adults [37]. The implementation of three testing sessions randomly presented during the night phase successfully decreased the time needed to acquire the task. Indeed, about 85% of mice were able to acquire the task in an average of about 12 days (Fig. 1b,c). There was no sex-dependent effect on the number of days needed to reach the final stage ($F_{1, 27} = 2.1$, $p = 0.15$; Fig. 1b). Notably, at the end of the training phase, mice were still in the middle portion of "adolescence" (≈32 days old). This was achieved maintaining a normal adolescent body weight-growing curve (Fig. 1d,e). If ad libitum access to food was kept in the home cage during the light phase of the day, mice typically lost weight the mornings that followed the first 3 nights of testing ($F_{21, 546} = 3.5$, $p < 0.0005$), but all mice quickly recovered gradually growing throughout the test ($p < 0.0001$; Fig. 1d). In contrast, restricting the access to food during the light phase of the day, as is usually done in adults [34- 37], abolished the initial morning body weight loss ($F_{21, 546} = 134.070$, $p < 0.0005$; Fig. 1e). Nonetheless, under the two food regime conditions, mice performance did not vary in all parameters described below, a part from the omissions (66.8 ± 2.0 or 59.7 ± 2.0 for day-time food ad libitum or restriction, respectively; $p = 0.01$). This demonstrates that the three test sessions per night were sufficient to keep the normal growing curve of adolescent mice, while ensuring a quick acquisition of the task and maintaining a good level of performance.

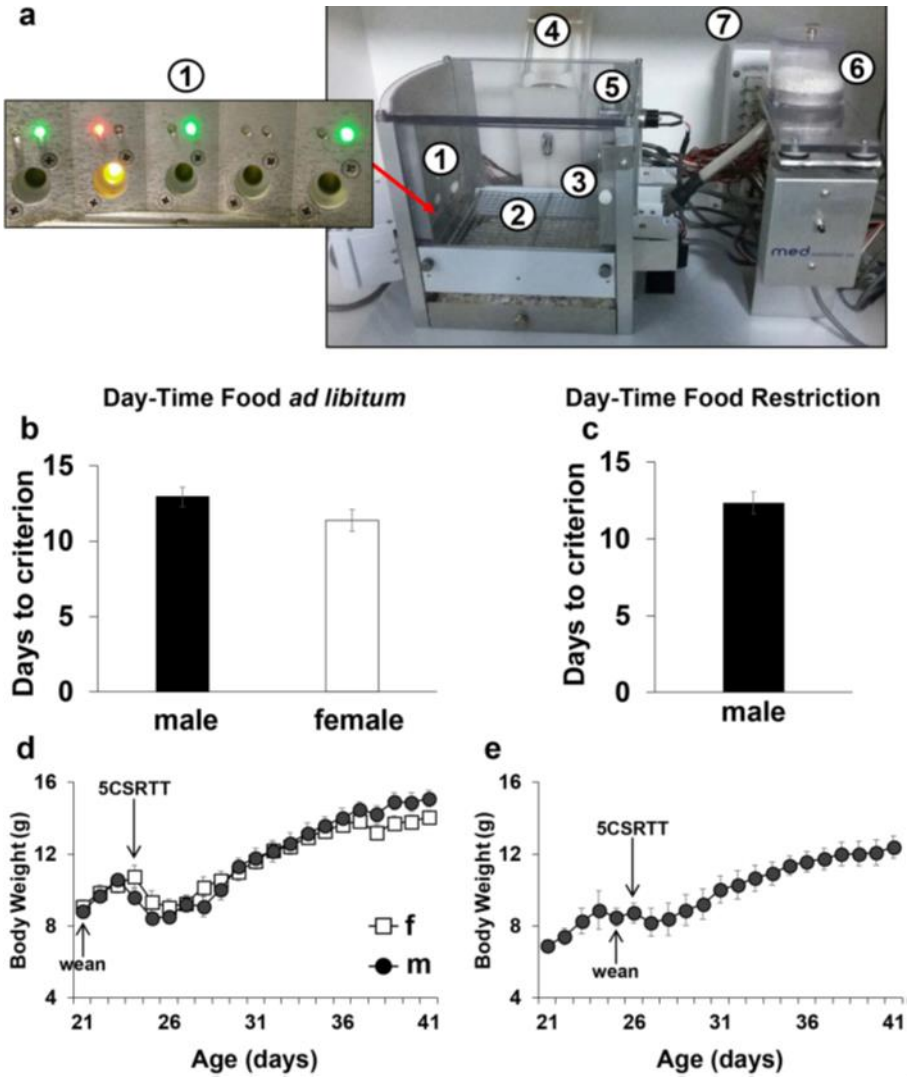

Figure 1. (**a**) The modified 5CSRTT apparatus: (1) modified 5 nose-poke holes wall, each outfitted with a recessed LED stimulus light and 2 additional LED cue lights (red and green) above each of the 5 nose-poke holes. (2) A stainless steel grid floor modified for the use in adolescent mice. (3) Food magazine on the wall opposite to the 5-hole array. (4) Water dispenser. (5) House-light. (6) Food pellet dispenser. (7) Smart Control Panel. (All the standard components were obtained from Med Associates, St. Albans, VT, USA). (**b**) Number of days taken by C57BL/6J male and female adolescent mice kept under food *ad libitum* condition during the light phase of the day to reach Stage 6 criteria. (**c**) Number of days taken by C57BL/6J male adolescent mice kept under food restriction condition during the light phase of the day to reach Stage 6 criteria. (**d**) Morning body weight measurements (in grams) of C57BL/6J male and female adolescent mice kept under food *ad libitum* condition during the light phase of the day. Ns = 15 males and 14 females. (**e**) Morning body weight measurements (in grams) of C57BL/6J male adolescent mice kept under food restriction condition during the light phase of the day. Ns = 6 males. Values represent mean ± SEM in all Figures.

3.3.1. The 5–7 second inter-trial interval (ITI) shift is not effective in triggering premature responses in male C57BL/6J adolescent mice.

Upon reaching the training criteria with the basic stage of the 5CSRTT, adolescent mice were exposed to different paradigms with different trial manipulations as summarized in Fig. 2. The impulsivity trait in adult rats and mice becomes appreciable in the 5CSRTT when the ITI is increased from 5 to 7 seconds [34- 38]. To test whether a similar outcome could be obtained in adolescent mice, we tested in our modified 5CSRTT paradigm this same 5–7 ITI challenge (Fig. 2: impulsivity paradigm).

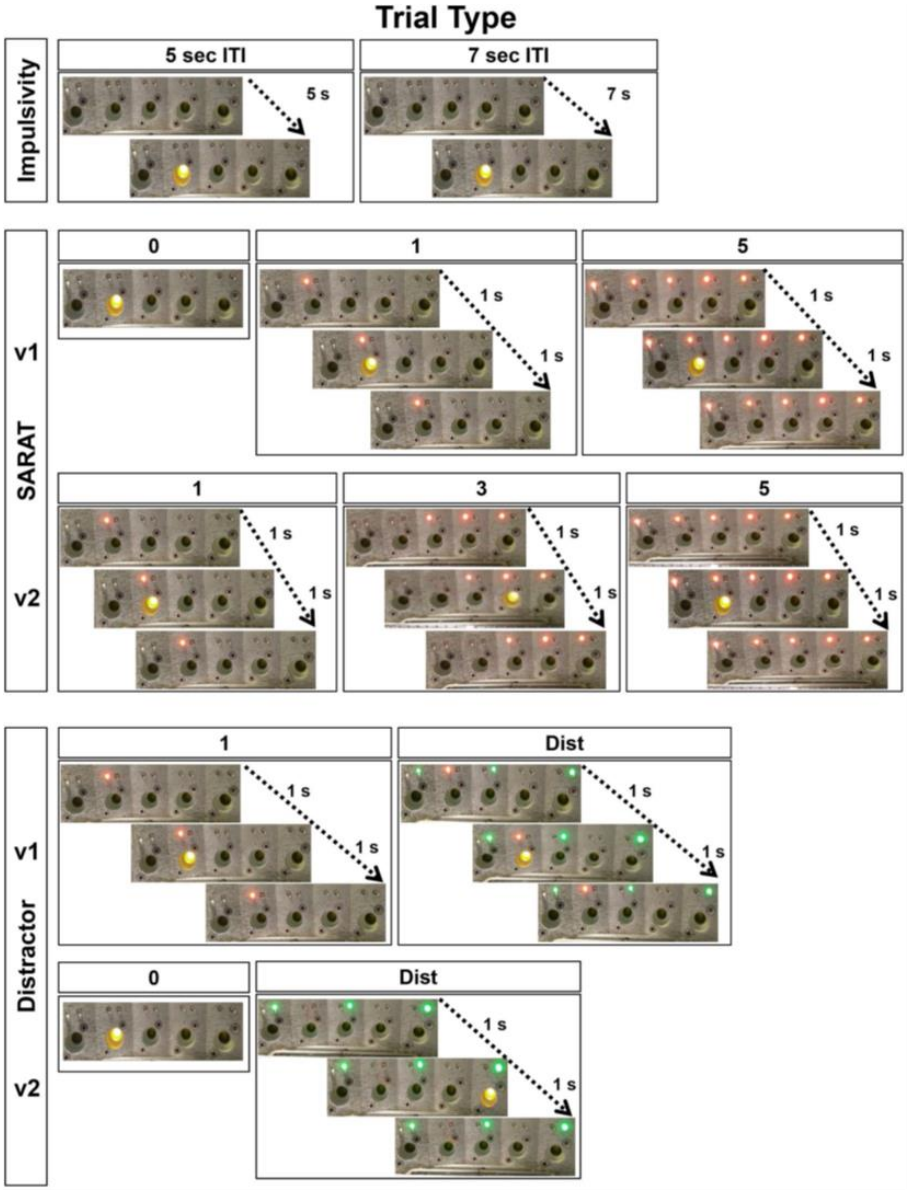

Figure 2. Schematic diagrams of the trials type that were presented to the mice during the three different test manipulation paradigms: Impulsivity; SARAT v1 and v2 and Distractor v1 and v2.

A significant ITI-by-sex interaction effect was evident for accuracy ($F_{2,54} = 4.94$, $p = 0.01$) and premature responses ($F_{2,54} = 6.78$, $p = 0.002$). Post-hoc analyses revealed no significant effects for accuracy ($p = 0.6$; Fig. 3b), but an increase in premature responding in the 7-s trials in female adolescent mice ($p = 0.008$; Fig. 3d), but not in males ($p = 0.5$; Fig. 3d). As shown in Fig. 3, the 5- to 7-s ITI shift did not influence any other parameter including correct responses ($F_{2,54} = 0.3$, $p = 0.7$), omissions ($F_{2,54} = 0.1$, $p = 0.8$), perseverative responses ($F_{2,54} = 0.2$, $p = 0.8$), time-out responses ($F_{2,54} = 1.58$, $p = 0.2$), latencies to correct responses ($F_{2,54} = 0.1$, $p = 0.8$) and reward retrieval ($F_{2,54} = 0.2$, $p = 0.8$). These results indicate that this manipulation was less effective in inducing impulsive-like behaviors in adolescent mice than in adult mice. Moreover, similarly to what was reported for adult mice [37], females showed more vulnerability to impulsivity challenges than males.

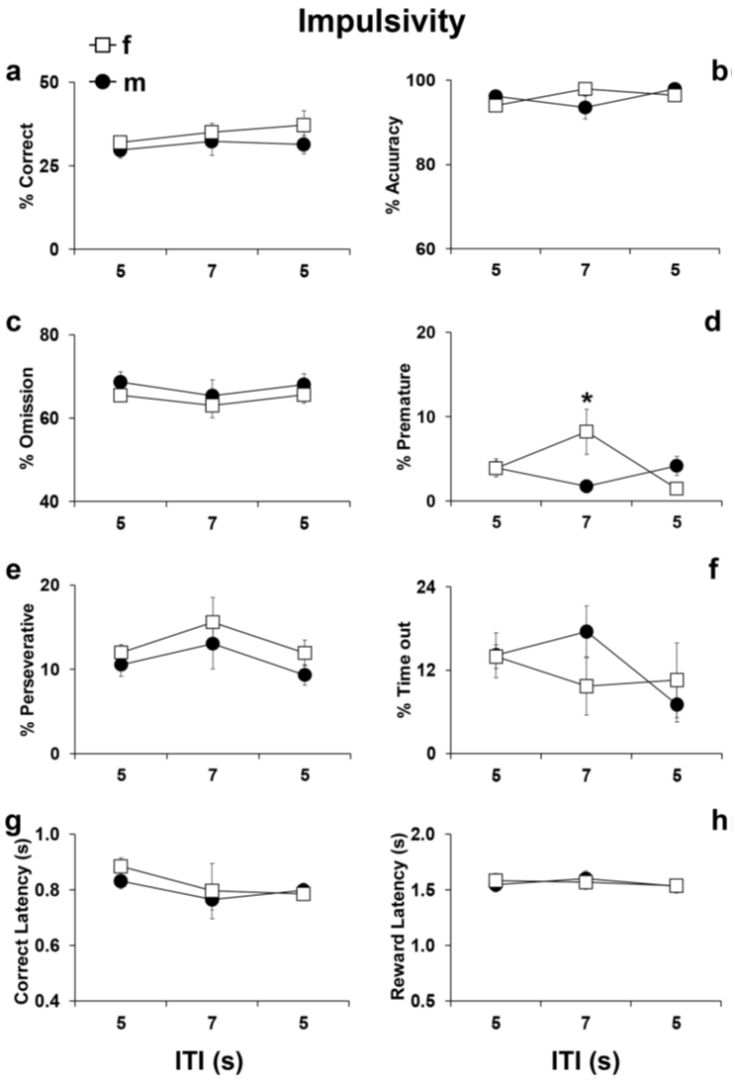

Figure 3. Performance displayed by C57Bl/6J male and female adolescent mice during the Impulsivity screening at different Inter-trial Interval delays (i.e. ITI of 5 or 7 seconds). Percentage of (a) correct responses (correct responses/total number of trials*100), (b) accuracy (correct responses/(correct + incorrect responses)*100),(c) (omitted trials/total number of trials*100), (d) premature responses (premature responses/ (correct + incorrect + premature + perseverative + time-out responses)*100), (e) perseverative responses (perseverative responses/(correct + incorrect + premature + perseverative + time-out responses)*100), (f) timeout responses (time-out responses/(correct + incorrect + premature + perseverative + time-out responses)*100), (g) correct latency (time in seconds from onset of light stimulus to the performance of a correct response/number of correct responses) and (h) reward latency (time in seconds from the performance of a correct response to the retrieval of the food reward from the food magazine/number of correct responses). Data from consecutive sessions were averaged within each trial type. For clarity, the first depicted trial type represents the performance during the previous days of only Cued 0 trials, while the other two depicted trial types were the performance during the day of impulsivity screening. Ns = 15 males and 14 females. *$p < 0.05$ versus trials with a 5-second ITI. *$p < 0.05$ versus performance at 5-ITI trials and versus males performance at the 7-ITI trials.

3.3.2. Adolescent mice showed faster reaction time with a valid pre-cue, but difficulties distributing attention broadly.

The Spatial Attentional Resource Allocation Task (SARAT) has been described as a visuospatial attention paradigm in humans able to selectively investigate broad monitoring abilities and discriminate dysfunctions in patients with psychiatric disorders such as schizophrenia [4], [6], [7], [9], [20], [38]. Notably, visuospatial functioning is impaired in children and adolescents with psychiatric disorders such as schizophrenia, ADHD, autism and 22q11.2DS [39]. Thus, here we implemented a variation of the 5CSRTT modelled after the human SARAT protocol (Fig. 2: SARAT v1 paradigm). The number of cued locations defined the predictability of the target location. Only 1 cued location (i.e. *Cued 1* trials) provided a precise information about the target, allowing a narrower and more selective attentional focus. Conversely, the *Cued 5* trials increased spatial uncertainty and the need to monitor broadly. As reported in Fig. 4, a trial effect was evident for correct responses ($F_{3,81}$ = 23.4, $p < 0.0001$), accuracy ($F_{3,81}$ = 123.5, $p < 0.0001$), omissions ($F_{3,81}$ = 13.13, $p < 0.0001$), premature responses ($F_{3,81}$= 54.7, $p < 0.0001$), perseverative responses ($F_{3,81}$= 18.31, $p < 0.0001$), time out responses ($F_{3,81}$= 13.54, $p < 0.0001$), and correct latency ($F_{3,81}$= 23.54, $p < 0.0001$). In particular, the Cued 5 trials produced a consistent decrease in correct responses ($p = 0.0001$; Fig. 4a) and accuracy ($p = 0.0001$; Fig. 4b). Both Cued 1 and Cued 5 trials decreased omissions ($p < 0.05$; Fig. 4c), increased time out responses ($p < 0.005$; Fig. 4f), increased premature responses ($p < 0.05$; Fig. 4d) and decreased perseverative responses ($p < 0.05$; Fig. 4e). The Cued 1 trials selectively triggered faster correct responses ($p = 0.0001$ Fig. 4g). No trial effect was evident for reward latencies ($F_{3, 81}$ = 2.53, $p = 0.06$; Fig. 4h). Moreover, no sex-dependent effects were evident for any parameter ($p > 0.4$). These findings provide evidence that this SARAT paradigm can be applied to adolescent C57BL/6J mice. Indeed, as well as that of adolescent mice, the performance of healthy humans displays faster reaction times in trials with more precise pre-cues while attentional control is disrupted in trials where the pre-cues provide invalid information about the target [39]–[42].

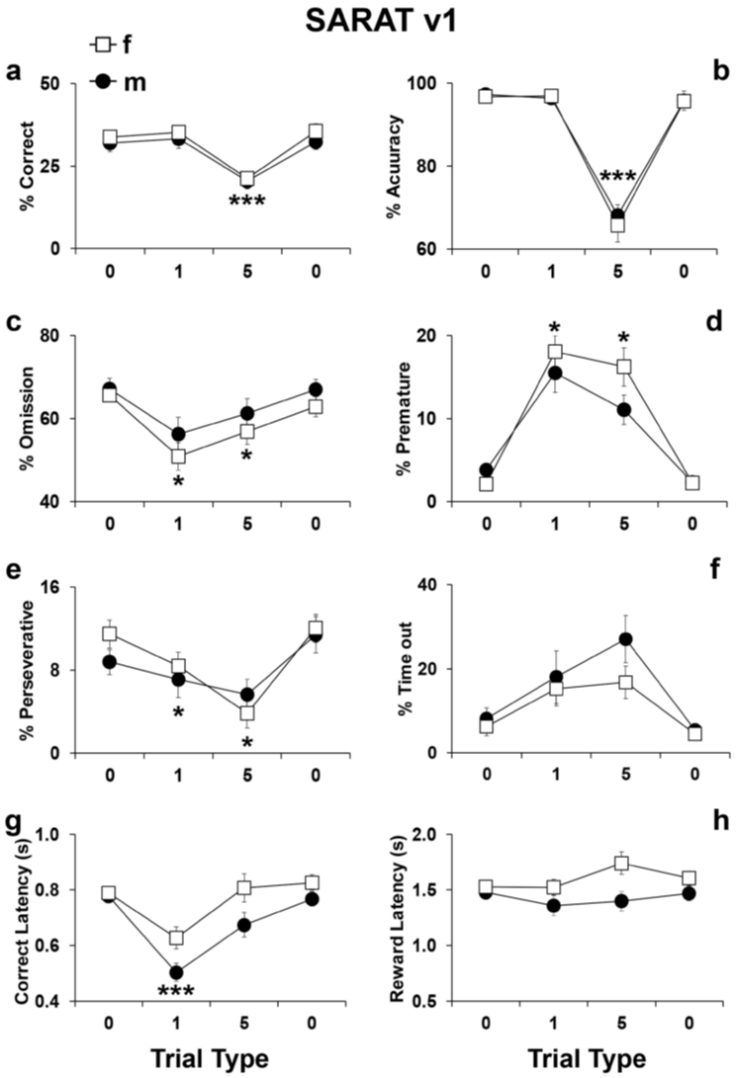

Figure 4. Performance displayed by C57Bl/6J male and female adolescent mice during the SARAT test version 1. Percentage of (**a**) correct responses (correct responses/total number of trials*100), (**b**) accuracy (correct responses/ (correct + incorrect responses)*100), (**c**) omissions (omitted trials/total number of trials*100), (**d**) premature responses (premature responses/(correct + incorrect + premature + perseverative + time-out responses)*100), (**e**) perseverative responses (perseverative responses/(correct + incorrect + premature + perseverative + time-out responses)*100), (**f**) time-out responses (time-out responses/(correct + incorrect + premature + perseverative + timeout responses)*100), (**g**) correct latency (time in seconds from onset of light stimulus to the performance of a correct response/number of correct responses) and (**h**) reward latency (time in seconds from the performance of a correct response to the retrieval of the food reward from the food magazine/number of correct responses). Data from consecutive sessions were averaged within each trial type. For clarity, the first depicted trial type represents the performance during the previous days of only *Cued 0* trials, while the other two depicted trial types were the performance during the day of SARAT screening. Ns = 15 males and 14 females. *$p < 0.05$ and ***$p < 0.0005$ versus performance at all other trials type

3.3.3. Distracting cues selectively disrupted attentional accuracy in adolescent mice.

Adolescents show less control and more distractibility during cognitive tasks that require high demand of attention[43]. Moreover, increased distractibility during adolescence has been identified as a possible risk factor for psychiatric diseases [44]–[46]. Thus, we developed a protocol able to assess the impact of distracting cues on the cognitive performance of adolescent mice (Fig. 2: Distractor v1 paradigm). As shown in Fig. 5, a trial effect was evident for correct responses ($F_{3,81}$ = 9.32, $p < 0.0001$), accuracy ($F_{3,81}$= 52.59, $p < 0.0001$), omissions ($F_{3,81}$= 14.68, $p < 0.0001$), premature responses ($F_{3,81}$ = 61.39, $p < 0.0001$), perseverative responses ($F_{3,81}$ = 14.63, $p < 0.0001$) and correct latencies ($F_{3,81}$ = 23.54, $p < 0.0001$). In particular, trials with the distracting cues produced a decrease in correct responses ($p < 0.0002$; Fig. 5a) and accuracy ($p < 0.0001$; Fig. 5b). Both the Cued 1 and distractor trials triggered more premature ($p < 0.0001$; Fig. 5d) and less perseverative responses ($p < 0.0001$; Fig. 5e). Finally, consistent with the SARAT results, in the Cued 1 trials less omissions ($p < 0.0001$; Fig. 5c) and faster correct responses were made ($p = 0.0005$; Fig. 5g).

A marked trial-by-sex interaction effect was evident in the time-out responses ($F_{3,81}$= 14.79, $p < 0.0001$) and reward latency ($F_{3,78}$ = 4.84, $p < 0.05$). Adolescent female mice made more time-out responses ($p < 0.05$; Fig. 5f) and needed more time to retrieve the food pellet in the distractor trials ($p < 0.05$; Fig. 5h). These findings highlight the ability of the distracting manipulation to disrupt attentional control in both male and female adolescent mice.

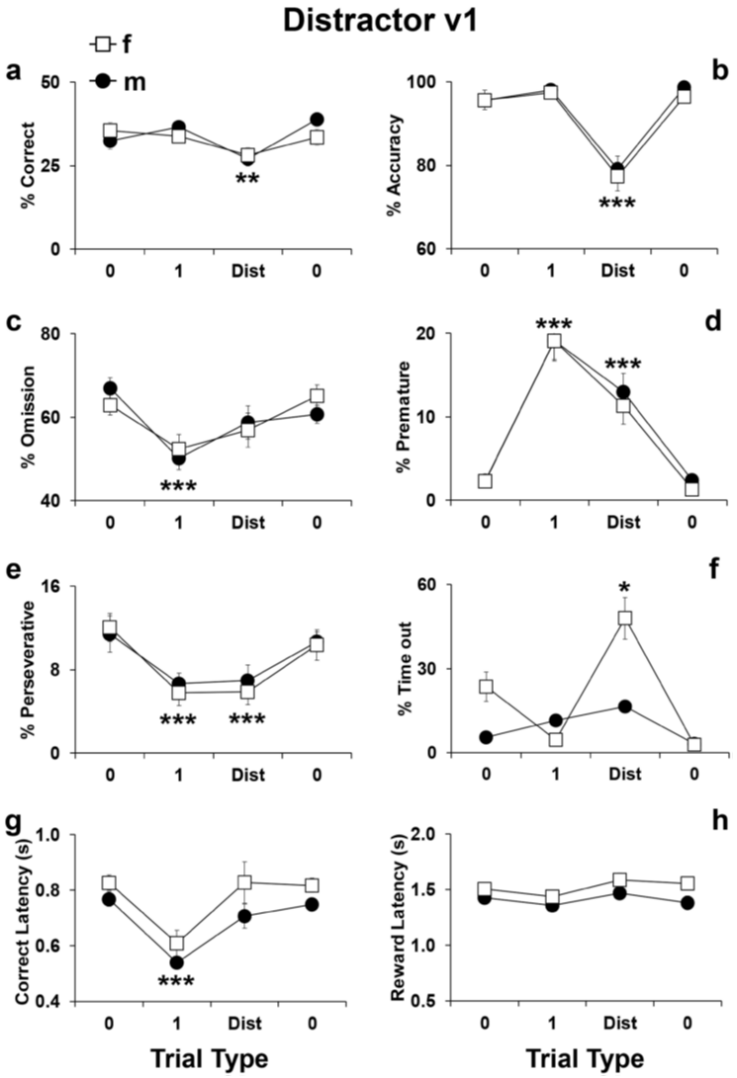

Figure 5. Performance displayed by C57Bl/6J male and female adolescent mice during the Distractor test version 1. Percentage of (**a**) correct responses (correct responses/total number of trials*100), (**b**) accuracy (correct responses/(correct + incorrect responses)*100), (**c**) omissions (omitted trials/total number of trials*100), (**d**) premature responses (premature responses/(correct + incorrect + premature + perseverative + time-out responses)*100), (**e**) perseverative responses (perseverative responses/(correct + incorrect + premature + perseverative + time-out responses)*100), (**f**) time-out responses (time-out responses/(correct + incorrect + premature + perseverative + time-out responses)*100), (**g**) correct latency (time in seconds from onset of light stimulus to the performance of a correct response/number of correct responses) and (**h**) reward latency (time in seconds from the performance of a correct response to the retrieval of the food reward from the food magazine/ number of correct responses). Data from consecutive sessions were averaged within each trial type. For clarity, the first depicted trial type represents the performance during the previous days of only *Cued 0* trials, while the other two depicted trial types were the performance during the day of Distractor screening. Ns = 15 males and 14 females. *$p < 0.05$, **$p < 0.05$, and ***$p < 0.0005$ versus performance at all other trials type.

3.3.4 The SARAT and Distractor are distinct paradigms assessing selective attentional control processes.

To test whether the SARAT and the Distractor paradigms could grasp distinct aspects of attentional control in adolescent mice, we further implemented these two paradigms (Fig. 2: SARAT v2 and Distractor v2 paradigms), as done in adult mice [47].

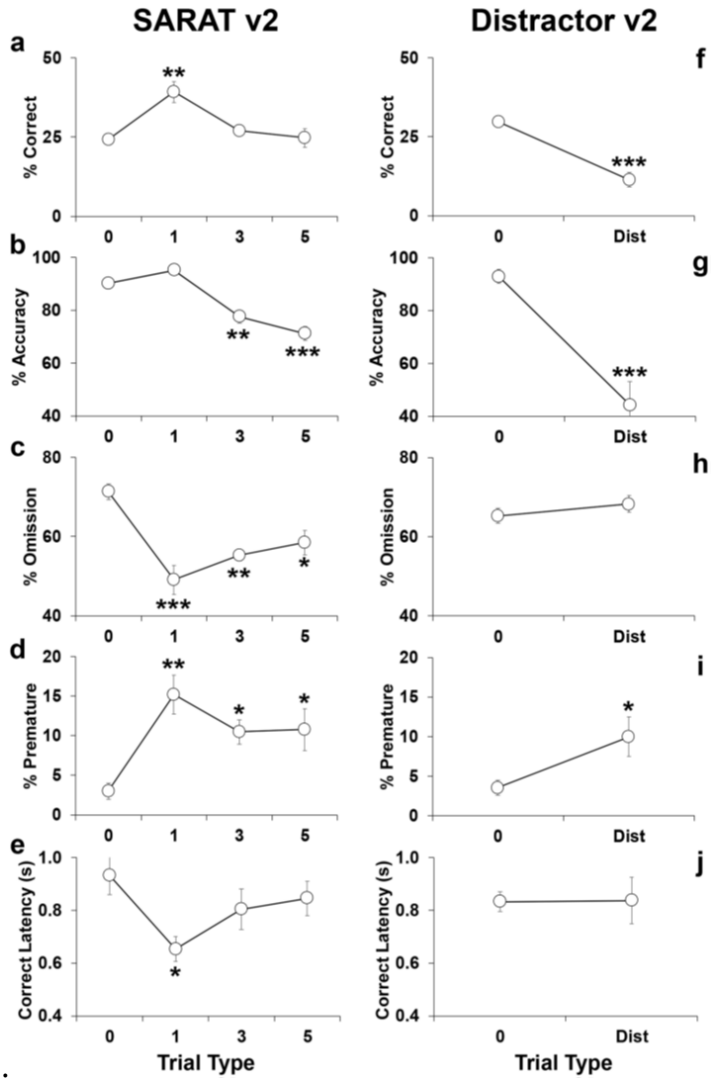

Figure 6. Comparison of the performance in key parameters displayed by C57Bl/6J male and female adolescent mice between the SARAT version 2 and the Distractor version 2 paradigms. Percentage of (**a** and **f**) correct responses (correct responses/total number of trials*100), (**b** and **g**) accuracy (correct responses/ (correct + incorrect responses)*100), (**c** and **h**) omissions (omitted trials/total number of trials*100), (**d** and **i**) premature responses (premature responses/(correct + incorrect + premature + perseverative + time-out responses)*100), (**e** and **j**) correct latency (time in seconds from onset of light stimulus to the performance of a correct response/number of correct responses). Data from consecutive sessions were averaged within each trial type. Ns = 6 mice. *p < 0.05, **p < 0.005 and ***p < 0.0005 versus performance at all other trials type.

In particular, with the SARAT version 2, we directly linked mice performance to the number of presented pre-cues (i.e. 0, 1, 3 or 5) as is reported in human studies[39]–[41]. Instead, in the second version of the Distractor, we eliminated the overlapping presence of the distracting green lights with the valid red pre-cue light that could generate conflicting information to the mice (see Fig. 2 for trials illustrations and comparisons). The SARAT v2 demonstrated that cognitive performance is tightly related to the number of valid pre-cues. Indeed, increasing spatial uncertainty to three and five pre-cues proportionately decreased the accuracy ($F_{3,15}$ = 30.32, $p < 0.0001$; Fig. 6b), and increased omissions ($F_{3,15}$ = 16.70, $p < 0.0001$; Fig. 6c). Instead, providing a more precise predicting cue (*Cued 1* trials) greatly ameliorated the performance of adolescent mice increasing the amount of correct responses ($F_{3,15}$ = 9.82, $p < 0.0008$; Fig. 6a), decreasing the omissions ($F_{3,15}$ = 16.70, $p < 0.0001$; Fig. 6c) and fastening the speed of a given correct answer ($F_{3,15}$ = 3.28, $p < 0.05$; Fig. 6e). Other parameters were not altered by this manipulation. In contrast, the distracting stimuli in the Distractor v2 decreased correct responses ($t = 7.82$; $df = 5$; $p < 0.0006$; Fig. 6f) and accuracy ($t = -6.11$; $df = 5$; $p < 0.001$; Fig. 6g) with a stronger effect compared to Distractor v1 (Fig. 5). Other parameters were not altered by this manipulation. Notably, a direct comparison between the SARAT v2 and Distractor v2 (Fig. 6 first column compared to second) highlighted the distinct pattern of performance triggered by the different stimuli. Overall, these data demonstrate that attentional control in adolescent mice can be selectively and differentially assessed by the SARAT and Distractor paradigms.

3.3.5 Attentional control performance in adolescent DAT and COMT genetically hypo-functioning mice.

To accentuate the effectiveness of this novel task for adolescent genetically modified mice, we tested two mice lines which we previously assessed in the 5CSRTT at adult age [47]. Specifically, we tested dopamine transporter (DAT) and catechol-O-methyltransferase (COMT) heterozygous (+/−) knockout mutant male mice, because they are clinically relevant mouse models with effects on cognitive functions that recapitulate the effects of similar genetic variations in humans [34], [48], [49]. The performance of DAT+/+ and COMT+/+ wild-type littermates followed an identical pattern of performance as that of the C57BL/6J mice shown in Figs. 1–5. In contrast, compared to +/+ littermates, DAT+/− adolescent mice showed reduced accuracy during the training phase of the task ($F_{2,29}$ = 4.37, $p < 0.02$; Fig. 7a), reduced levels of perseverative responses in the basic cued 0 trials ($t = 2{,}29$; $df = 17$; $p < 0.04$; Fig. 7b),

and increased premature responding following the 5–7 ITI challenge ($F_{4,48} = 3.33$, $p < 0.01$; Fig. 7c).

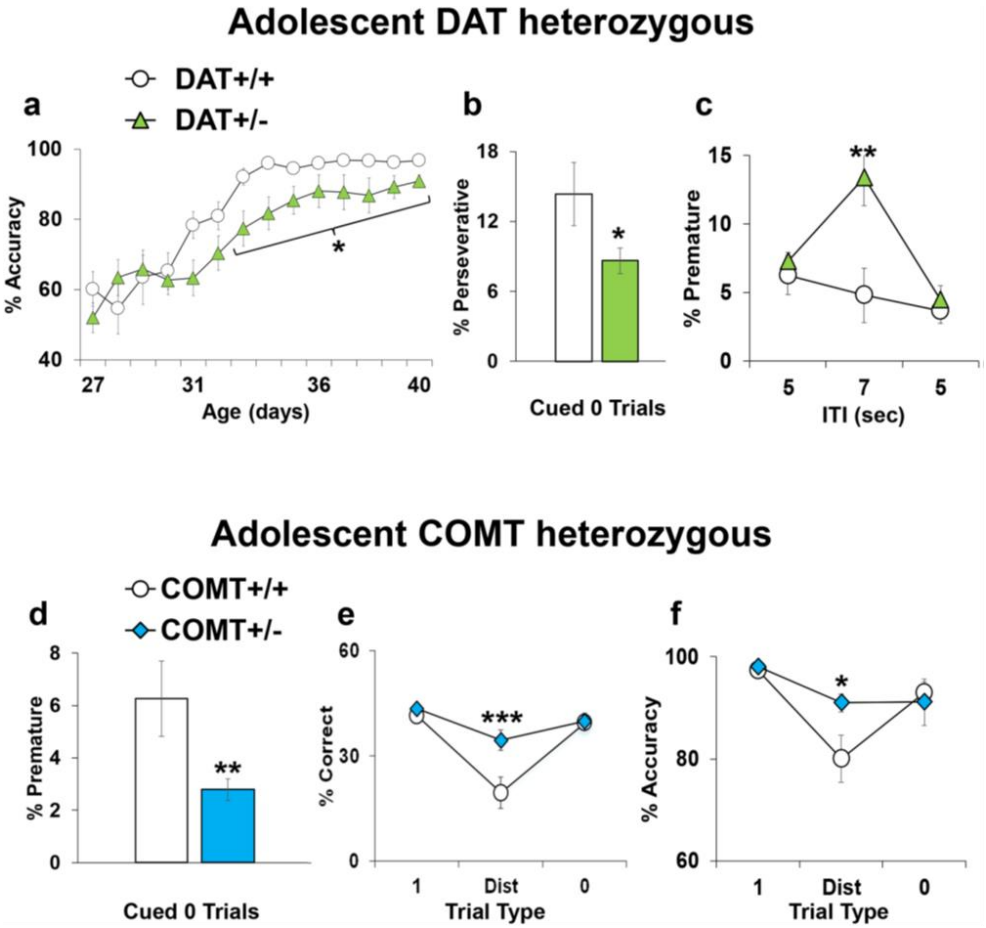

Figure 7. (a–c) Performance displayed by DAT+/+ and DAT+/− adolescent littermates in the modified 5CSRTT in key parameters which showed a genotype effect. (a) Percentage of accuracy (correct responses/ (correct + incorrect responses)*100) during the training phase of the test. (b) Percentage of perseverative responses (perseverative responses/(correct + incorrect + premature + perseverative + time-out responses)*100) during the basic phase of the test with only trial type 0 without any extra cue. (c) Percentage of premature responses (premature responses/(correct + incorrect + premature + perseverative + timeout responses)*100) during the 5–7 ITI challenge paradigm. DAT+/+ Ns = 12, DAT+/− Ns = 12. *$p < 0.05$ and **$p < 0.005$ versus performance of DAT+/+ at the same trial type. (d–f) Performance displayed by COMT+/+ and COMT+/− adolescent littermates in the modified 5CSRTT in key parameters which showed a genotype effect. (d) Percentage of premature responses (premature responses/ (correct + incorrect + premature + perseverative + time-out responses)*100) during the basic phase of the test with only trial type 0 without any extra cue. (e) percentage of correct responses (correct responses/total number of trials*100) and (f) accuracy (correct responses/(correct + incorrect responses)*100) during the Distractor paradigm. Ns: COMT+/+ = 7 and COMT+/− = 8. *$p < 0.05$, **$p < 0.005$ and ***$p < 0.0005$ versus performance of COMT+/+ at the same trial type.

No other DAT-dependent effects were evident in any of the other parameters in all other paradigm manipulations (data not shown). Consistent with data from adult mice [48], these results highlight that genetic variations reducing DAT produced attentional and impulsive control deficits since adolescence. Notably, despite the 5–7 ITI shift was confirmed to be ineffective in wild-type mice, it triggered a consistent increase in premature responding in DAT+/− mice, suggesting that this challenge is still effective in vulnerable subjects. Finally, an unexpected DAT effect in reducing compulsive-related phenotypes during adolescence was detected. Instead, compared to their +/+ littermates, COMT+/− adolescent mice showed reduced levels of premature responses in the basic Cued 0 trials ($t = 2{,}31$; $df = 14$; $p < 0.05$; Fig. 7d), and increased correct responses ($F_{2,26} = 8.54$, $p < 0.005$; Fig. 7e) and accuracy ($F_{2,26} = 3.64$, $p < 0.05$; Fig. 7f) in the distractor trials. No other COMT-dependent effects were evident in all other parameters in the other paradigm manipulations (data not shown). In contrast to what was found in adult mice [33], these results highlight that genetic variations reducing COMT are associated with reduced levels of basal impulsivity, and an attentional control that is more resilient to the detrimental effects of distracting cues in adolescence.

3.4. Discussion

The data reported here demonstrate that this modified 5CSRTT can effectively test attentional control abilities in adolescent mice. Moreover, different challenges in the test were able to detect in adolescent mice: (i) impulsive-like behaviors defined as the ability to refrain to make a preponderant response, (ii) the ability to maintain focused or broad attention when different pre-cue stimuli were presented (SARAT) and (iii) the attentional vulnerability to distractors. While developing the task, preserving the chance to train mice in less than 12 days was crucial for the effectiveness of the task itself. Training in the 5CSRTT for adult mice usually requires from thirty days up to several months [33], [36], [50]. However, paradigms longer than twenty days would exceed the rodents' short "adolescence" period which is considered to span from about 28 to 45 days of age [1], [36]. Notably, we were able to achieve this also maintaining the physiological curve of adolescent-growing body size, limiting the amount of stress and potential metabolic deficits that could derive from scarce food intake during this developmental period. Indeed, most of similar operant-based tasks in adult rodents require a food restriction protocol [33], [35], [51]. However, adolescence is a peculiar period for the vulnerability related to nutritional factors [22]. For example, an increasing body of literature illustrates a direct connection between an appropriate nutrition during adolescence and optimal cognitive and brain function [52]–[54]. Therefore, this novel paradigm can assess higher order cognitive functions such as attention, compulsivity, impulsivity, distractibility, decision making and processing speed in adolescent rodents with very few confounding factors. There is very scarce evidence regarding complex cognitive tasks designed for adolescent rodents. For example, intra-/extra-dimensional (ID/ED) set-shifting tasks or a two-choice visual discrimination task (2-CVDT) have been used in adolescent rats [55], [56]. However, in the ID/ED task, adolescent rats were also impaired in basic compound discrimination and in such studies food restriction was applied with no regard to the normal body weight growth of this developmental period. More recently, an all-day and self-pace testing in a similar 5CSRTT have been tested for adolescent mice[57]. In contrast to our setting, the latter task did not reveal any difference in performance of adolescents compared to adults, needed an additional cage attached to the 5CSRTT apparatus, required continuous single-housing, and its testing schedule differed from the one used in humans which is restricted in a consecutive and limited time period. This latter factor is critical when assessing sustained attention as the self-pace regime greatly reduce the attentional load. To note, we are not aware of other similar studies using distracting cues in adolescent mice. However, we chose extra visual cues (i.e. green flashing lights) randomly

presented within the same session to more directly compare attentional performance with that of non-distractor trials, in order to avoid potential habituation processes found with noises [57] and confusion with the use of house lights [58] as reported in adults. Finally, as also previously discussed [47], we were able to demonstrate clear differences between the SARAT and distractor paradigms. Briefly, the combination of tasks used in the current work show that the cue lights were not simply treated as target stimulus lights, as only non-predictive cues decreased the accuracy and that this was directly proportional to the degree of unpredictability (e.g. 3 vs 5 vs distracting cues). The fact that accuracy and the speed of making a correct response were both directly and proportionately modulated depending on the number of valid pre-cues presented also suggested that the mice used the cues to orient attention in anticipation of a target, and that there was a difference in this process between predictive and non-predictive cues. Moreover, our data demonstrate that faster reaction time for correct responses in cued trials were not a reflection of trials with responses initiated by the cue and executed after the target light came on. Indeed, the speed of correct responses was proportional on the number of cues presented (*Cued 1* > *Cued 3* > *Cued 0* and *5* trials), and distracting cues did not trigger faster reaction responses compared to *Cued 0* trials. Overall, all the characteristics of our modified automatic task makes it well suitable to dissect different attentional control processes in adolescent rodents also for large genetic or pharmacological screenings. This could be relevant in the context of testing early intervention/pharmacological strategies while also understanding their mechanisms. Indeed, early intervention on cognitive deficits could potentially be more effective in mitigating or reversing pathological trajectories and ameliorate the quality of life of individuals at risk for psychiatric disorders [59]. With three different variations, we were able to selectively measure in adolescent mice subtypes of attentional control such as impulsivity, focused or broad attentiveness, processing speed and distractibility. In the "impulsivity" paradigm, adolescent female mice, but not males, increased the premature responses impulsivity index when the ITIs were changed from 5 to 7 seconds. Previous literature using delay-discounting tests found that both male and female adolescent rats exhibited greater levels of impulsive-like behaviors compared to adults [60]. However, other evidence accounted for a substantial impact of hormones in producing sex-dependent differences in impulsive actions in rodents[61]. Moreover, it has been demonstrated that in delay-discounting tasks under mild food restriction, adult female mice are more impulsive than males63. Premature responses are thought to reflect a failure of inhibitory response control that occurs when preparatory response mechanisms are disrupted [62]. Thus, the 5-to-7 ITI shift in adolescent mice might be applied to assess sex-dependent vulnerability to this kind of

impulsive control. Notably, our protocol offers another option to study impulsive control in mice. In particular, in contrast with the 5–7 ITI shift, the pre-cued trials of the SARAT protocol triggered a consistent increase in premature responding in both male and female mice. This kind of motor impulsivity is qualitatively different from the one triggered by the increase in ITI. Indeed, the pre-cue visual stimuli put forth a pre-potent response, which the mice must withhold from making in order to receive a food reward and then make a correct response. Thus, this measure of motoric impulsivity is potentially analogous to "false alarm" errors made in corresponding human tasks. Both these manipulations might constitute a valuable tool to assess impulsive behaviors in adolescent mice. In the SARAT paradigm, adolescent mice showed a decreased accuracy in the trials where all the red cue light were turned on (Cued 5), while faster speed of processing for target cues were evident in trials with more precise pre-cues (Cued 1). This pattern of performance was very similar to that of human healthy subjects tested in the original SARAT, where faster reaction times are evident in trials with more precise pre-cues, while cognitive performance is disrupted in trials where the pre-cues provide invalid information about the target [39]–[41]. Thus, this SARAT paradigm might be useful to distinguish deficits in selective attention from deficits in broad monitoring in adolescent mice with good translational validity concerning human studies. In particular, this could be relevant for schizophrenia, as patients demonstrate more selective attentional deficits when broad focus of attention is required, rather than when attention must be focused narrowly [41]. To date, no study specifically assessed such kind of abilities in adolescent mice, making this an additional tool in preclinical investigations designed to specifically manipulate spatial selective attention. In the Distractor test, we observed a selective disruption of attentional accuracy and increased time out responses in the distractor trials, where non-predictive flashing lights were turned on. Adolescence is considered to be a time during which many aspects of behavior including planning, multitasking and the ability to resist distractions, are profoundly shaped [63]. For instance, teenagers have more difficulties to concentrate and are easily distracted [43]. The maturation in the resistance to distractors has been associated with a decreased activation in the superior frontal sulcus between childhood and adulthood [64], possibly linked with developmental changes in grey matter architecture and long-range connections [65]. In particular, it seems like cortical brain region fully developed in humans up to the late twenties or even the early thirties, which is much later than previously thought [43]. Thus, in adolescent mice as well as in humans, it would be important to unravel the mechanisms of cognitive vulnerability to distractors, and our modified 5CSRTT might constitute a valid tool in this respect. A comparison between adolescents' and adults' performance in an equivalent task

might highlight interesting developmental peculiarities. Overall, the performance of adolescent mice here described was similar to that of adult mice tested in an identical 5CSRTT47, with few important exceptions. In particular, the sex-dependent differences in adults showing better performances in females compared to males [47] were not evident in adolescent mice. This might reflect long-lasting effects of the sexual hormonal changes that start to appear during puberty [66]–[68], and that are thought to play a critical role in the adult maturation of the cortex and complex cognitive behaviors [67]–[69]. In line with this and again in contrast with responses in adults [33], [70], we did not find any effect in C57BL6 male mice in premature responses when the ITIs were changed from 5 to 7 seconds. Furthermore, adolescent COMT+/− males showed decreased levels of premature responses (Fig. 7), while adult COMT+/− have been reported to have increased levels of premature responses [33], even if a direct comparison with the same 5CSRTT version is still missing. However, these effects parallel recent findings unraveling a divergent dopaminergic maturation of the PFC from adolescence to adulthood between males and females [67]. Moreover, these findings raise the intriguing possibility that the COMT-dependent impact on stress vulnerability in terms of cognitive responses (e.g. impulsivity as in ref. 34) might develop in male subjects only after adolescence. This adds to previous evidence reporting that COMT-by-sex interacting effects are noticeable only between puberty and menopause [67], [71], [72] and with data reporting a different maturation of the dopaminergic system in males compared to females [67], [71], [73], [74]. Notably, the SARAT paradigm with only one predictive cue produced attentional advantages in adolescent mice that were not evident in adults. Indeed, the increase in correct responses and the decreased omissions seen in adolescents (Fig. 6) were not apparent in adults [47], indicating an higher attentiveness to extra-cues in adolescents. Other developmental differences in the 5CSRTT performance were then evident in the distractor paradigm, as distractor trials triggered larger deleterious effects in adolescents than in adults. Indeed, additional parameters (i.e. premature, perseverative and time out responses) were altered in adolescent mice other than just accuracy and correct choices as in adults [47], and accuracy level was diminished to ≈40% in adolescents in contrast with ≈70% in adults. This might be related to human findings reporting higher vulnerability to distraction in adolescent subjects compared to adults [45]. Finally, in line with human findings [67], [75][70], [78], the better performance of COMT+/− in the distractor trials suggests that this manipulation might be a more sensible tool in order to highlight the cognitive advantages of COMT genetic reduction in males that were difficult to assess with classical 5CSRTT34. Future studies might want to address trial-by-trial analyses in order to address whether adolescents might emotionally respond differently from adults following correct or

incorrect responses. In conclusion, our results demonstrate that even within the brief duration of rodent adolescence, it is possible to assess different attentional control facets by a modified 5CSRTT paradigm. Indeed, the adopted manipulations allowed to assess different subtypes of attentional control including impulsivity, focused or broad attentiveness, processing speed and distractibility. These features suggest that this task could be a useful tool with potential translational validity concerning human studies, applicable to genetic and pharmacological studies in mouse models relevant to cognitive abnormalities and psychiatric disorders.

3.5. References

[1] M. Schneider, “Adolescence as a vulnerable period to alter rodent behavior,” *Cell Tissue Res.*, vol. 354, no. 1, pp. 99–106, 2013.

[2] C. L. Sisk and D. L. Foster, “The neural basis of puberty and adolescence,” *Nat. Neurosci.*, vol. 7, no. 10, pp. 1040–1047, 2004.

[3] L. Steinberg, “Cognitive and affective development in adolescence,” *Trends Cogn. Sci.*, vol. 9, no. 2, pp. 69–74, 2005.

[4] D. Yurgelun-Todd, “Emotional and cognitive changes during adolescence,” *Curr. Opin. Neurobiol.*, vol. 17, no. 2, pp. 251–257, 2007.

[5] R. D. Q. G. Prwlrqdo, R. Ri, and D. Q. G. Grohvfhqfh, “) Urqwdo / Reh) Xqfwlrqlqj Lq & Klogkrrg.”

[6] J. M. Gold and G. K. Thaker, “Current progress in schizophrenia research cognitive phenotypes of schizophrenia: Attention,” *J. Nerv. Ment. Dis.*, vol. 190, no. 9, pp. 638–639, 2002.

[7] M. Zvyagintsev, C. Parisi, N. Chechko, A. R. Nikolaev, and K. Mathiak, “Attention and multisensory integration of emotions in schizophrenia,” *Front. Hum. Neurosci.*, vol. 7, no. October, pp. 1–7, 2013.

[8] J. M. Gold, B. Hahn, G. P. Strauss, and J. A. Waltz, “Turning it upside down: Areas of preserved cognitive function in schizophrenia,” *Neuropsychol. Rev.*, vol. 19, no. 3, pp. 294–311, 2009.

[9] S. C. L. Deoni *et al.*, “Mapping Infant Brain Myelination with Magnetic Resonance Imaging,” *J. Neurosci.*, vol. 31, no. 2, pp. 784–791, 2011.

[10] E. M. Gordon *et al.*, “Strength of default mode resting-state connectivity relates to white matter integrity in children,” *Dev. Sci.*, vol. 14, no. 4, pp. 738–751, 2011.

[11] A. Manuscript, “<2006 - 4-13 yo - Davidson et al., Neuropsychologia.pdf>,” vol. 44, no. 11, pp. 2037–2078, 2006.

[12] J. Adamek, H. Herrlich, G. S. Abstract, and C. Catego-, “References 1.,” *Form. Asp. Comput.*, vol. 6, no. 1990, pp. 62–77, 1999.

[13] M. Ernst, T. Daniele, and K. Frantz, “New perspectives on adolescent motivated behavior: Attention and conditioning,” *Dev. Cogn. Neurosci.*, vol. 1, no. 4, pp. 377–389, 2011.

[14] T. E. Moffitt *et al.*, “A gradient of childhood self-control predicts health, wealth, and public safety,” *Proc. Natl. Acad. Sci.*, vol. 108, no. 7, pp. 2693–2698, 2011.

[15] T. Dalgleish *et al.*, “[No Title],” *J. Exp. Psychol. Gen.*, vol. 136, no. 1, pp. 23–42, 2007.

[16] A. B. Clohessy, M. I. Posner, and M. K. Rothbart, “Development of the functional visual field,” *Acta Psychol. (Amst).*, vol. 106, no. 1–2, pp. 51–68, 2001.

[17] K. M. Thomas and C. A. Nelson, “Serial Reaction Time Learning in Preschool- and School-Age Children,” *J. Exp. Child Psychol.*, vol. 79, no. 4, pp. 364–387, 2001.

[18] P. O'Donnell, “Cortical disinhibition in the neonatal ventral hippocampal lesion model of schizophrenia: New vistas on possible therapeutic approaches,” *Pharmacol. Ther.*, vol. 133, no. 1, pp. 19–25, 2012.

[19] F. Papaleo *et al.*, “Dysbindin-1 modulates prefrontal cortical activity and schizophrenia-like behaviors via dopamine/D2 pathways,” *Mol. Psychiatry*, vol. 17, no. 1, pp. 85–98, 2012.

[20] L. Steinberg and A. S. Morris, “Adolescent Devolepmental,” *Annu. Rev. Psychol.*, vol. 52, pp. 83–110, 2001.

[21] S. L. Andersen, A. T. Thompson, M. Rutstein, J. C. Hostetter, and M. H. Teicher, “Dopamine receptor pruning in prefrontal cortex during the periadolescent period in rats,” *Synapse*, vol. 37, no. 2, pp. 167–169, 2000.

[22] “Spear - 2002.pdf.” .

[23] D. Wahlstrom, T. White, and M. Luciana, “Neurobehavioral evidence for changes in dopamine system activity during adolescence,” *Neurosci. Biobehav. Rev.*, vol. 34, no. 5, pp. 631–648, 2010.

[24] L. P. Spear, *<Spear LP 2000 - Review.pdf>*, vol. 24. 2000.

[25] C. S. Monk *et al.*, “Adolescent immaturity in attention-related brain engagement to emotional facial expressions,” *Neuroimage*, vol. 20, no. 1, pp. 420–428, 2003.

[26] K. Rubia *et al.*, “Functional frontalisation with age: Mapping neurodevelopmental trajectories with fMRI,” *Neurosci. Biobehav. Rev.*, vol. 24, no. 1, pp. 13–19, 2000.

[27] L. Tamm, V. Menon, and A. L. Reiss, “LIHI Certificate #58 - Union Gas | Low Impact Hydropower Institute,” vol. 20, pp. 1231–1238, 2017.

[28] M. Ernst *et al.*, “Amygdala and nucleus accumbens in responses to receipt and omission of gains in adults and adolescents,” *Neuroimage*, vol. 25, no. 4, pp. 1279–1291, 2005.

[29] A. Galvan *et al.*, “Earlier Development of the Accumbens Relative to Orbitofrontal Cortex Might Underlie Risk-Taking Behavior in Adolescents,” *J. Neurosci.*, vol. 26, no. 25, pp. 6885–6892, 2006.

[30] M. P. Purdue *et al.*, "Erratum: A prospective study of 67 serum immune and inflammation markers and risk of non-Hodgkin lymphoma (Blood (2013) 122:6 (951-957))," *Blood*, vol. 123, no. 18, p. 2901, 2014.

[31] J. McGaughy, J. W. Dalley, C. H. Morrison, B. J. Everitt, and T. W. Robbins, "Selective behavioral and neurochemical effects of cholinergic lesions produced by intrabasalis infusions of 192 IgG-saporin on attentional performance in a five-choice serial reaction time task.," *J. Neurosci.*, vol. 22, no. 5, pp. 1905–13, 2002.

[32] J. McGaughy, J. Turchi, and M. Sarter, "Crossmodal divided attention in rats: effects of chlordiazepoxide and scopolamine," *Psychopharmacology (Berl).*, vol. 115, no. 1–2, pp. 213–220, 1994.

[33] F. Papaleo, L. Erickson, G. Liu, J. Chen, and D. R. Weinberger, "Effects of sex and COMT genotype on environmentally modulated cognitive control in mice," *Proc. Natl. Acad. Sci.*, vol. 109, no. 49, pp. 20160–20165, 2012.

[34] D. Scheggia, A. Bebensee, D. R. Weinberger, and F. Papaleo, "The ultimate intra-/extra-dimensional attentional set-shifting task for mice," *Biol. Psychiatry*, vol. 75, no. 8, pp. 660–670, 2014.

[35] A. Bari, J. W. Dalley, and T. W. Robbins, "The application of the 5-choice serial reaction time task for the assessment of visual attentional processes and impulse control in rats.," *Nat. Protoc.*, vol. 3, no. 5, pp. 759–767, 2008.

[36] W. Adriani and G. Laviola, "Elevated levels of impulsivity and reduced place conditioning with d-amphetamine: Two behavioral features of adolescence in mice," *Behav. Neurosci.*, vol. 117, no. 4, pp. 695–703, 2003.

[37] F. Papaleo, L. Erickson, G. Liu, J. Chen, and D. R. Weinberger, "Effects of sex and COMT genotype on environmentally modulated cognitive control in mice," pp. 2–7, 2012.

[38] V. Anderson, "Assessing executive functions in children: Biological, psychological, and developmental considerations," *Pediatr. Rehabil.*, vol. 4, no. 3, pp. 119–136, 2001.

[39] T. J. Simon, Z. Wu, B. Avants, H. Zhang, J. C. Gee, and G. T. Stebbins, "Atypical cortical connectivity and visuospatial cognitive impairments are related in children with chromosome 22q11.2 deletion syndrome," *Behav. Brain Funct.*, vol. 4, pp. 1–11, 2008.

[40] B. Hahn, T. J. Ross, and E. A. Stein, "Neuroanatomical dissociation between bottom-up and top-down processes of visuospatial selective attention," *Neuroimage*, vol. 32, no. 2, pp. 842–853, 2006.

[41] B. Hahn *et al.*, “Visuospatial attention in schizophrenia: Deficits in broad monitoring.,” *J. Abnorm. Psychol.*, vol. 121, no. 1, pp. 119–128, 2012.

[42] E. Macaluso and F. Doricchi, “Attention and predictions: control of spatial attention beyond the endogenous-exogenous dichotomy,” *Front. Hum. Neurosci.*, vol. 7, no. October, pp. 75–80, 2013.

[43] I. Dumontheil, B. Hassan, S. J. Gilbert, and S.-J. Blakemore, “Development of the Selection and Manipulation of Self-Generated Thoughts in Adolescence,” *J. Neurosci.*, vol. 30, no. 22, pp. 7664–7671, 2010.

[44] C. L. Frame and T. F. Oltmanns, “Serial recall by schizophrenic and affective patients during and after psychotic episodes,” *J. Abnorm. Psychol.*, vol. 91, no. 5, pp. 311–318, 1982.

[45] P. Harvey, K. C. Winters, S. Weintraub, and J. M. Neale, “Distractibility in children vulnerable to psychopathology,” *J. Abnorm. Psychol.*, vol. 90, no. 4, pp. 298–304, 1981.

[46] O. Slobodin, H. Cassuto, and I. Berger, “Age-Related Changes in Distractibility: Developmental Trajectory of Sustained Attention in ADHD,” *J. Atten. Disord.*, 2015.

[47] H. Huang *et al.*, “A schizophrenia relevant 5-Choice Serial Reaction Time Task for mice assessing broad monitoring, distractibility and impulsivity,” *Psychopharmacology (Berl).*, vol. 234, no. 13, pp. 2047–2062, 2017.

[48] M. Mereu *et al.*, “Dopamine transporter (DAT) genetic hypofunction in mice produces alterations consistent with ADHD but not schizophrenia or bipolar disorder,” *Neuropharmacology*, vol. 121, pp. 179–194, 2017.

[49] D. Scheggia, S. Sannino, M. Luisa Scattoni, and F. Papaleo, “COMT as a Drug Target for Cognitive Functions and Dysfunctions,” *CNS Neurol. Disord. - Drug Targets*, vol. 11, no. 3, pp. 209–221, 2012.

[50] N. M. W. J. de Bruin, F. Fransen, H. Duytschaever, C. Grantham, and A. A. H. P. Megens, “Attentional performance of (C57BL/6J × 129Sv)F2 mice in the five-choice serial reaction time task,” *Physiol. Behav.*, vol. 89, no. 5, pp. 692–703, 2006.

[51] T. W. Robbins, “The 5-choice serial reaction time task: Behavioural pharmacology and functional neurochemistry,” *Psychopharmacology*, vol. 163, no. 3–4. pp. 362–380, 2002.

[52] J. Davis *et al.*, “A review of vulnerability and risks for schizophrenia: Beyond the two hit hypothesis,” *Neurosci. Biobehav. Rev.*, vol. 65, pp. 185–194, 2016.

[53] L. Desbonnet *et al.*, “Gut microbiota depletion from early adolescence in mice:

Implications for brain and behaviour," *Brain. Behav. Immun.*, vol. 48, pp. 165–173, 2015.

[54] M. B. Zimmermann, "The role of iodine in human growth and development," *Semin. Cell Dev. Biol.*, vol. 22, no. 6, pp. 645–652, 2011.

[55] P. E. Orndorff, T. S. Hamrick, I. W. Smoak, and E. A. Havell, "Host and bacterial factors in listeriosis pathogenesis," *Vet. Microbiol.*, vol. 114, no. 1–2, pp. 1–15, 2006.

[56] L. A. Newman and J. Mcgaughy, "Adolescent rats show cognitive rigidity in a test of attentional set shifting," *Dev. Psychobiol.*, vol. 53, no. 4, pp. 391–401, 2011.

[57] E. Remmelink, U. Chau, A. B. Smit, M. Verhage, and M. Loos, "A one-week 5-choice serial reaction time task to measure impulsivity and attention in adult and adolescent mice," *Sci. Rep.*, vol. 7, no. January, pp. 1–13, 2017.

[58] N. Amitai and A. Markou, "Comparative effects of different test day challenges on performance in the 5-choice serial reaction time task," *Behav. Neurosci.*, vol. 125, no. 5, pp. 764–774, 2011.

[59] M. J. Millan *et al.*, "Altering the course of schizophrenia: Progress and perspectives," *Nat. Rev. Drug Discov.*, vol. 15, no. 7, pp. 485–515, 2016.

[60] J.-Q. Engle, K. M.; Mei, T-S.; Wasa, M.; Yu, "NIH Public Access," *Acc. Chem. Res.*, vol. 45, no. 6, pp. 788–802, 2008.

[61] D. W. Bayless, J. S. Darling, W. J. Stout, and J. M. Daniel, "Sex differences in attentional processes in adult rats as measured by performance on the 5-choice serial reaction time task," *Behav. Brain Res.*, vol. 235, no. 1, pp. 48–54, 2012.

[62] Y. Chudasama and T. W. Robbins, "Dissociable contributions of the orbitofrontal and infralimbic cortex to pavlovian autoshaping and discrimination reversal learning: further evidence for the functional heterogeneity of the rodent frontal cortex.," *J. Neurosci.*, vol. 23, no. 25, pp. 8771–8780, 2003.

[63] S. J. Blakemore and S. Choudhury, "Development of the adolescent brain: Implications for executive function and social cognition," *J. Child Psychol. Psychiatry Allied Discip.*, vol. 47, no. 3–4, pp. 296–312, 2006.

[64] P. J. Olesen, J. Macoveanu, J. Tegnér, and T. Klingberg, "Brain activity related to working memory and distraction in children and adults," *Cereb. Cortex*, vol. 17, no. 5, pp. 1047–1054, 2007.

[65] B. Luna, A. Padmanabhan, and K. O. Hearn, "Sturio_Publi_Symposium_Albufeira_2000.Pdf," vol. 72, no. 1, pp. 1–28, 2011.

[66] G. C. Harris and J. E. Levine, "Pubertal acceleration of pulsatile gonadotropin-

releasing hormone release in male rats as revealed by microdialysis," *Endocrinology*, vol. 144, no. 1, pp. 163–171, 2003.

[67] S. Sannino *et al.*, "Adolescence is the starting point of sex-dichotomous COMT genetic effects," *Transl. Psychiatry*, vol. 7, no. 5, 2017.

[68] C. L. Sisk, H. N. Richardson, P. E. Chappell, and J. E. Levine, "In vivo gonadotropin-releasing hormone secretion in female rats during peripubertal development and on proestrus," *Endocrinology*, vol. 142, no. 7, pp. 2929–2936, 2001.

[69] M. Arain *et al.*, "NDT-39776-maturation-of-the-adolescent-brain," *Neuropsychiatr. Dis. Treat.*, vol. 9, pp. 449–461, 2013.

[70] J. W. Dalley *et al.*, "Nucleus Accumbens D2/3 Receptors Predict Trait Impulsivity and Cocaine Reinforcement," *Science (80-.).*, vol. 315, no. 5816, pp. 1267–1270, 2007.

[71] F. Papaleo, S. Sannino, F. Piras, and G. Spalletta, "Sex-dichotomous effects of functional COMT genetic variations on cognitive functions disappear after menopause in both health and schizophrenia," *Eur. Neuropsychopharmacol.*, vol. 25, no. 12, pp. 2349–2363, 2015.

[72] E. Jacobs and M. D'Esposito, "Estrogen Shapes Dopamine-Dependent Cognitive Processes: Implications for Women's Health," *J. Neurosci.*, vol. 31, no. 14, pp. 5286–5293, 2011.

[73] K. D. Foust and B. K. Kaspar, "NIH Public Access," vol. 8, no. 24, pp. 4017–4018, 2010.

[74] J. B. Becker, "Gender differences in dopaminergic function in striatum and nucleus accumbens," *Pharmacol. Biochem. Behav.*, vol. 64, no. 4, pp. 803–812, 1999.

[75] B. Hooks, "Feminist Theory: From Margin to Center," vol. 8, no. January, p. 179, 2000.

Chapter 4

Remote memories are enhanced by COMT activity through dysregulation of the endocannabinoid system in the prefrontal cortex

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Abstract

The prefrontal cortex (PFC) is a crucial hub for the flexible modulation of recent memories (executive functions) as well as for the stable organization of remote memories. Dopamine in the PFC is implicated in both these processes and genetic variants affecting its neurotransmission might control the unique balance between cognitive stability and flexibility present in each individual.

Functional genetic variants in the catechol-O-methyltransferase (COMT) gene result in a different catabolism of dopamine in the PFC. However, despite the established role played by COMT genetic variation in executive functions, its impact on remote memory formation and recall is still poorly explored. Here we report that transgenic mice overexpressing the human COMT-Val gene (COMT-Val-tg) present exaggerated remote memories (450 days) while having unaltered recent memories (24 h).

COMT selectively and reversibly modulated the recall of remote memories as silencing COMT Val overexpression starting from 30 days after the initial aversive conditioning normalized remote memories. COMT genetic over activity produced a selective overdrive of the endocannabinoid system within the PFC, but not in the striatum and hippocampus, which was associated with enhanced remote memories. Indeed, acute pharmacological blockade of CB1 receptors was sufficient to rescue the altered remote memory recall in COMT-Val-tg mice and increased PFC dopamine levels. These results demonstrate that COMT genetic variations modulate the retrieval of remote memories through the dysregulation of the endocannabinoid system in the PFC.

4.1. Introduction

The everyday activities of each individual are characterized by the dichotomy between the formation/manipulation of new memories (recent memories and executive functions) and the recall of remote memories. Previous studies have demonstrated that freshly formed memories are mostly dependent on hippocampal formation, whereas remote memories are mainly integrated by cortical networks converging into the prefrontal cortex (PFC) [1]–[3]. The genetic footprint of each individual can greatly affect these cognitive functions [4]–[6]. However, how selective genetic variations might impact the dichotomy between recent versus remote memories is not yet clear.

Catecholamines within the PFC have an integrative role in the flexible updating of novel information (executive functions) as well as in the formation of stable remote memories [7], [8]. Genetic variations leading to individual differences in the enzymatic activity of the catechol-O-methyltransferase (COMT) produce predominant changes of cortical dopamine, but not norepinephrine metabolism [8]–[12]. In particular, because more than half of the dopamine flux in the PFC is accounted by COMT activity, this enzyme has a privileged position in relationship to PFC-dependent cognitive functions [9], [10], [13].

In agreement, functional COMT genetic variations in both humans and rodents [4], [11], [14] have been consistently reported to modulate PFC-dependent cognition such as working memory and executive functions [11], [15], [16].

Furthermore, COMT genetic variations have been associated with mental disorders characterized by specific alterations in cognitive performance and physiology such as schizophrenia [17], panic disorder [18] and posttraumatic stress disorder [19]. Despite this, the impact of COMT genetic variations on remote memory formation and recall is still poorly understood. Based on the established role played by COMT genetics in the modulation of PFC-dependent cognitive processes, here we hypothesized that functional COMT genetic variations might also influence remote memories sparing short-term, hippocampus dependent memories. In particular, we used a humanized conditional mouse expressing the human COMT-Val gene (COMT-Val-tg) that simulates human genetic conditions resulting in relative increased COMT enzymatic activity during the life span. COMT-Val-tg mice recapitulate salient aspects of human behaviors associated with COMT polymorphisms and establish the biological validity of these associations. That is, a modest increase of COMT activity disrupts executive functions while reducing the reactions from anxiety-like states and stressful situations [11]. The endocannabinoid system has a central role in formation and extinction of long-lasting

memories [20]–[22] and clinical studies have suggested an interaction of this system with the human COMT Val genotype [23]. However, the impact of the COMT-Val genotype on this signaling complex and its potential link with memory functions is still unknown. Thus, transgenic (tg) mice overexpressing the human COMT-Val-allele provide an unequivocal model to evaluate the role of genetically determined increases in COMT and the underlying biology of the observed behavioral abnormalities.

4.2. Materials and Methods

4.2.1. Mice

All procedures were approved by the National Institute of Mental Health Animal Care and by the Italian Ministry of Health (permit no. 230/2009-B) and Animal Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the European Community Council Directives. COMT-Val-tg mice were mated with control littermates. Construction and development of human COMTVal-tg mice has been previously described [11]. Mice were identified by PCR analysis of tail DNA. Mice were group housed (two to four per cage) in a climate-controlled animal facility (22 ± 2 °C) and maintained on a 12 h light/dark cycle with ad libitum access to food and water. Testing was conducted in male mice, 3 to 6 months old, during the light phase. Distinct cohorts of naive mice were used for each experiment. Experiments were blind to the genotype during behavioral testing. Mice were handled by the experimenter on alternative days during the week preceding the tests. At least 1 h before any test manipulation, mice were habituated in a room adjacent to the testing room.

4.2.2 Behavior

4.2.2.1 Fear conditioning.

Fear conditioning took place in a standard conditioning box (TSE Systems, Bad Homburg, Germany). The conditioned stimulus (CS) was a tone (4 kHz, 80 dB sound pressure level, 30 s) and the unconditioned stimulus (US) was a scrambled shock (0.7 mA) delivered through the grid floor that terminated simultaneously with the tone (2 s). On experimental day 1, mice were placed in the training chamber and after a 2 min habituation period (baseline), three conditioning trials were presented (tones paired with shock) with an intertrial interval of 90 s. Then, the animals were returned to their home cages ~ 2 min after the last CS–US pairing. After 24 h, mice were retested in the same chamber for 5 min without tone or foot shock (recent contextual memory). At 50 days following training, the mice were returned to the same conditioning chamber for contextual fear memory test (remote contextual memory). At 1 h after the recent and remote contextual memory recall, mice were placed in a new chamber and after 2 min of habituation (baseline) exposed to the conditioning tone for 2 min (cue) to test for recent and remote cued memory. Then, the animals were returned to their home cages after 2 min (post cue).

4.2.2.2. Fear extinction.

Mice were tested for fear extinction in the apparatus previously described. On day 1, animals were placed in the conditioning chamber and after 90 s without tone or shock, received 5 habituation trials (tone alone, 4 kHz, 80 dB sound pressure level, 30 s), immediately followed by fear conditioning consisting of 5 presentations of the tone that coterminated with foot shocks (2 s, 0.7 mA). Approximately 90 s after the last CS–US pairing, mice were replaced in their home cages. After 2 h, mice were returned to the same conditioning chamber and were given extinction training consisting of 20 tone-alone trials. The extinction training lasted ~ 60 min. The chamber was cleaned with 70% ethanol and wiped dry before running the first mouse and after each animal completed the test. Behavior during fear conditioning, fear extinction, recent and remote recall of contextual and cued fear memories was recorded with digital video cameras for offline scoring of freezing with stopwatches. The percent time spent freezing during each trial (the absence of all movements except for respiration) was measured by an observer blind with respect to group assignment.

4.2.2.3 Conditioned place aversion.

The conditioned place aversion (CPA) was performed in a rectangular Plexiglas box (length, 42 cm; width, 21 cm; height, 21 cm) divided by a central partition into two chambers of equal size (21 × 21 × 21 cm). One compartment had black walls and a smooth Plexiglas floor, whereas the other one had vertical black and white striped (2 cm) walls and a slightly rough floor. During the test sessions, an aperture (4 × 4 cm) in the central partition allowed the mice to enter both sides of the apparatus, whereas during the conditioning sessions the individual compartments were closed off from each other. To measure time spent in each compartment a video tracking system (ANYMAZE) was used. The CPA experiment lasted 52 days and consisted of three phases: preconditioning test, conditioning phase and post conditioning test. On day 0, each mouse was allowed to freely explore the entire CPA apparatus for 20 min, and time spent in each of the two compartments was measured (preconditioning test). Within each genotype, mice were divided in two groups with similar preconditioning time values in the preferred and non-preferred compartment of the CPA apparatus. Conditioning sessions took place on days [1], [3], [24]. During the morning session mice were injected with vehicle (Veh) and then confined in the non- preferred compartment of CPA apparatus for 30 min a day. After 4 h, mice were treated with naloxone (5 mg kg− 1) and confined for 30 min a day into their preferred compartment, as determined on preconditioning test. To test recent memory, post conditioning test was performed on day 4 in the same condition of preconditioning test. On day

52, mice were treated with Veh or AM251 (1 mg kg− 1) and tested again on postconditioning test. For each mouse, a place aversion score was calculated as the post conditioning time minus the preconditioning time (seconds) spent in the conditioning compartment of the CPA apparatus.

4.2.2.4. Drugs

Doxycycline hyclate (Sigma Aldrich, Dorset, UK) was freshly prepared every day and administered in drinking water (100 μg ml− 1) for 20 days before testing remote contextual fear memory. Cannabinoid receptor-1 (CB1R) antagonist AM251 (1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1- piperidinyl-1H-pyrazole-3-carboxamide, 1 or 2 mg kg^{-1} intraperitoneal [25]; Sigma Aldrich, UK) was first dissolved in polyethylene glycol 400 (2:8), then made up to volume with physiological saline (0.9% NaCl) and injected in a volume of 10 ml kg^{-1} of body weight. Control mice were injected with the same volume of saline.

4.2.3. *In vivo* microdialysis

Concentric dialysis probe, with a dialysis portion of 2.0 mm, were prepared as previously described [26]. Mice were anesthetized with isoflurane and then placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) for the probe implantation. The probe was implanted into the medial PFC (mPFC), according to the Paxinos and Franklin mouse brain atlas [27] (AP: ± 1.9; ML: ± 0.1; DV:-3.0 from Bregma). Microdialysis sessions started 24 h after the surgical procedures. Probes were perfused with Ringer's solution (147.0 mM NaCl, 2.2 mM CaCl2 and 4.0 mM KCl) at a constant flow rate of 1 μl min− 1. Collection of basal dialysate samples (20 μl) started 30 min after. To measure dopamine release, after 60 min of basal sampling, we injected amphetamine (1.5 mg kg− 1, intraperitoneal) followed by another 2 h of sampling collection. Dialysate samples (20 μl) were injected into high-performance liquid chromatography equipped with a reverse phase column (C8 3.5 μm, Waters, Milford, MA, USA) and dopamine was quantified by a coulometric detector (ESA, Coulochem II, Bedford, MA, USA). At the end of the experiment, mice were anesthetized with isoflurane and killed. Brains were removed and mPFC serial coronal sections were prepared with a vibratome to identify the location of the probes. All measurements were performed blind to the treatment and the genotype of the animals.

4.2.4. Lipid extraction

Tissue fatty acyl ester levels were measured as previously described [28]. Briefly, prefrontal cortex was weighed (5–7 mg) and homogenized in methanol (1 ml) containing [2H4]-AEA, [2H4]-OEA, [2H4]-PEA and [2H8]-2-AG as internal standards (Cayman Chemical, Ann Arbor, MI, USA). Lipids were extracted with chloroform (2 ml) and washed with water (1 ml). After centrifugation (2500 × g, 15 min, 4 °C), organic phases were collected and dried under nitrogen. The organic extracts were fractionated by silica gel column chromatography. Anandamide (AEA), oleoylethanolamide (OEA), palmitylethanolamide (PEA) and 2-arachidonoylglycerol (2-AG) were eluted with chloroform/methanol (9:1, v/v). Organic phases were evaporated under nitrogen and reconstituted in methanol/chloroform (9:1, v/v). Liquid chromatography/mass spectrometry (LC/MS) analyses were conducted on a Xevo TQ UPLC-MS/MS system (Waters) equipped with a reversed-phase BEH C18 column (Waters), using a linear gradient of acetonitrile in water. Quantifications were performed monitoring the following transitions (parent m/z-.daughter m/z, collision energy eV): AEA 348-.62, 20; [2H4]- AEA 352-.66, 20; OEA 326-.62, 20; [2H4]-OEA 330-.66, 20; PEA 300-.62, 20; [2H4]-PEA 304-.66, 20; 2-AG 379-.287, 15; [2H8]-2-AG 387-.295, 15. Analyte peak areas were compared with a standard calibration curve (0.1 nM to 1 mM).

4.2.5. Western blot

For western blot analyses, mice were killed by cervical dislocation and brains were quickly removed. The cerebral areas of interest (PFC, hippocampus and striatum) were obtained by regional dissection on ice, immediately frozen in liquid nitrogen and stored at − 80 °C until processing. The experiments were carried out as previously reported [29]. Briefly, equal amounts of protein lysates (30 μg) were run on a 10% SDS–polyacrylamide gel. The proteins were then transferred to polyvinylidene difluoride membranes and blocked for 2 h at room temperature before incubation overnight at 4 °C with the primary antibody. The following primary antibodies were used: rabbit polyclonal anti-CB1 (1:1000; Cayman Chemical), rabbit polyclonal anti-NAPE-PLD (1:3000; Cayman Chemical), rabbit polyclonal anti-FAAH (1:2000; Cayman Chemical), goat polyclonal anti-DAGL-α (1:1000; Abcam, Cambridge, UK), rabbit polyclonal anti-MAGL (1:1000; Cayman Chemical) and rabbit polyclonal anti-human COMT (1:5000; Chemicon International, Temecula, CA, USA). Bound antibodies were detected with horseradish peroxidase-conjugated secondary anti-rabbit or anti-goat antibody (1:2000–1:5000; Chemicon International). For normalization, the blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, USA) and reblotted

with mouse anti-β-actin monoclonal antibody (1:10 000; Sigma Aldrich, Milan, Italy) overnight at 4 °C. Bound antibodies were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) and bands were detected with a GBOX XT camera (Syngene, Cambridge, UK). Optical density of the bands was quantified using Image Pro Plus 7.0 software (MediaCybernetics, Bethesda, MD, USA). The density of the bands was normalized to β-actin and expressed as percentage of controls.

4.2.6. Immunofluorescence

Mice were deeply anesthetized with a 400 mg kg− 1 dose of chloral hydrate and then perfused with 4% paraformaldehyde. Following extraction, brains were stored at 4 °C in 4% paraformaldehyde for 24 h and cryoprotected in 30% sucrose for a minimum of 24 h. Brains were included in O.C.T. Compound (Sakura, Alpheen aan den Rijn, The Netherlands), cut in 40 μm-thick sections using a cryostat and stored at − 20 °C in anti-freezing solution (30% glycerol, 30% ethylene glycol and 0.02% sodium azide in phosphate-buffered saline (PBS)). For CB1 receptor, fatty acid amide hydrolase (FAAH) and N-acylphosphatidyl-lethanolamine phospholipase D (NAPE-PLD) immunofluorescence, free-floating sections were incubated in sodium citrate 50 mM, pH 9, for 10 min at 90 °C for antigen retrieval. After blocking peroxidase activity with 3% H2O2 in PBS for 20 min, sections were incubated in blocking buffer (10% normal goat serum, 0.3% Triton X-100 in PBS) for 3 h at room temperature and then with rabbit polyclonal anti-CB1 antibody (1:200, Cayman Chemical), rabbit polyclonal anti-FAAH antibody (1:500, Cayman Chemical) or rabbit polyclonal anti-NAPE-PLD antibody (1:300, Cayman Chemical) in blocking solution overnight at 4 °C. On the second day, the sections were washed with PBS-Tryton 0.5% and signal was revealed by incubating sections with Alexa Fluor 594 goat anti-rabbit antibody (1:2000; Invitrogen, Eugene, OR, USA) for 2 h at 4 °C. After several washes in PBS, sections were mounted onto Superfrost slides, dehydrated and coverslipped with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen). For double immunofluorescence, free-floating sections were processed for antigen retrieval, preincubated in blocking solution for 2 h at room temperature and then incubated with goat polyclonal anti-CB1 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking solution overnight at 4 °C. On the second day, the sections were washed with PBSTryton 0.5% and incubated with Alexa Fluor 488 donkey anti-goat antibody (1:2000; Invitrogen) for 2 h at 4 °C and then with rabbit polyclonal anti-VGLUT (1:1000; Abcam), vesicular GABA transporter (1:1000; Novus Biologicals, Littleton, CO, USA) or dopamine transporter (1:500; Sigma Aldrich, Italy) in blocking solution overnight at

4 °C. After washing, signal was revealed by incubating sections with Alexa Fluor 594 goat anti-rabbit antibody (1:2000; Invitrogen) for 2 h at 4 °C. After several washes in PBS, sections were mounted onto Superfrost slides, dehydrated and coverslipped with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen). Digital Images were captured using Retiga R1 CCD camera (QImaging, Surrey, BC, Canada) attached to an Olympus BX51 (Tokyo, Japan). polarizing/light microscope. Ocular imaging software (QImaging) was used to import images from the camera. Images were acquired by first delineating the brain sections and the regions of interest at low magnification (×4 objective) and the region of interest outlines were further refined under a × 40 objective. Three sections per mouse were analyzed. Digital images were adjusted only for contrast and brightness and digitally merged to visualize the colocalization of signals using Adobe Photoshop (5.0, San Jose, CA, USA). JACoP plugin embedded in the ImageJ software (NIH, Bethesda, MD, USA) was used to calculate colocalization statistics [30]. Merged color images were separated into individual red, green and blue components using the RGB split feature, and Pearson's coefficient, overlap coefficient and Mander's coefficients were calculated as quantitative measures of colocalization.

4.2.7. Statistical analysis

Results are expressed as mean ± s.e.m. throughout. No statistical methods were used to predetermine sample size. No exclusion criteria were used and all animals and samples tested were included in the analysis. All behavioral manipulations performed on each animal were determined randomly. All randomization was performed by an experimenter, and no explicit randomization algorithm was used. Two-way analysis of variance with genotype (controls and COMT-Val-tg) as the between-subject factor and time as the within-subject repeated measure factor was used to examine the percentage of freezing during the repeated exposure to the context environment, and during the habituation, conditioning, extinction and recall phases of the fear extinction paradigm. As normality tests have little power to detect non-Gaussian distributions with small data sets, we did not explicitly test for the normality of our data sets. For the analysis of lipids (AEA, OEA, PEA and 2-AG) and of enzymes responsible for synthesis and degradation/reuptake of the endocannabinoids (NAPE-PLD, diacylglycerol lipase-α (DAGL-α), FAAH, monoacylglycerol lipase (MAGL)) we found similar results in naive animals and in mice after remote memory recall, and thus data were pooled together. Data were expressed as fold changes compared with control Veh. The post hoc analyses for individual group comparisons employed Newman–Keuls analyses. The accepted value for significance was $P \leqslant 0.05$. Result sheets of statistical tests detailing (wherever applicable)

estimates of variance within each group, confidence intervals and comparison of variances across groups are available on request.

4.3.Results

4.3.1 Increased remote fear memory in COMT Val-tg mice

To study the effect of genetically dependent increase in COMT enzymatic activity on remote memories, we tested COMT-Val-tg mice in a Pavlovian fear conditioning. This paradigm combines the advantages of the formation of long-lasting memories following a single training experience with a well-known knowledge of timedependent brain circuits involved [24]. Re-exposure to the same context.Contextual fear memories have been shown to strongly depend on hippocampus functioning up to 28 days after training [31]. Thus, to tackle remote memories that are critically dependent on the functioning of the PFC [32], [33], we re-exposed the mice to the conditioning context again after 50 days. In contrast to normal recent memory, COMT-Val-tg mice showed higher expression of fear during the remote memory recall compared with their control littermates (Figure 1a). Indeed, while control mice showed a normal weakening of freezing behavior, COMT-Val-tg mice showed the same levels of freezing at 24 h and 50 days after conditioning. Fear reaction in both genotypes groups was context specific. Indeed, when we exposed the mice to a modified context, very little COMT-independent freezing behavior was observed 24 h and 50 days post conditioning (Figure 1b). Similarly, COMT independent reactions to cued fear memory were evident in a novel modified context (Supplementary Figures 1a and b). These results indicate that genetic variations resulting in increased COMT enzymatic activity were associated with a more stable recall of contextual remote memory. To rule out the possibility that COMT over activity might alter the ability to extinguish conditioned fear memories, we next tested fear extinction in COMT-Val-tg and control mice. During training, conditioned responses increased gradually in control and COMT-Val-tg mice (Figure 1c), demonstrating a rapid acquisition of conditioned freezing to the tone independent on the COMT genotype. Similarly, a classical extinction procedure [34] demonstrated that both control and COMT- Val-tg ce were able to rapidly extinguish the conditioned freezing in a COMT-independent way (Figure 1d). Thus, genetic variations increasing COMT do not affect the ability to extinguish fear memories. Silencing COMT overexpression during late consolidation/recall re-established a normal expression of remote memories. To determine whether the enhanced expression of remote memories in COMT-Val-tg mice was determined by concurrent increase of COMT activity or to chronic and/or developmental effects, we silenced the expression of the COMT transgene. Administration of doxycycline for 20 days, in drinking water, turned off the tetracycline-regulated COMT transgene expression (Figure 1e). Moreover, silencing of the COMT transgene also restored

normal levels of extracellular dopamine in the mPFC that were reduced in COMT-Val-tg mice (Figure 1f). In agreement with previous literature [9], [12], [11], [35] norepinephrine tissue content was unaffected in COMT-Val-tg mice compared with controls (Supplementary Figures 2b and c).

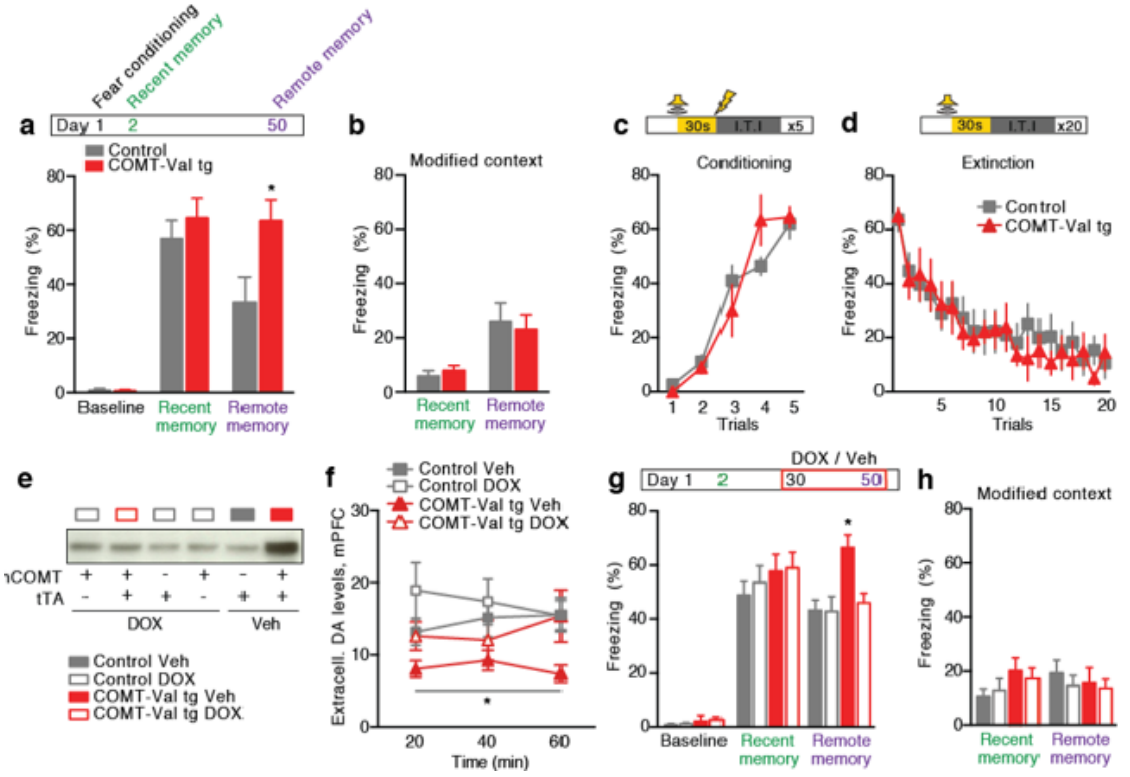

Figure 1. Increased remote memories in catechol-O-methyltransferase (COMT) Val-transgenic mice. (a) Behavioral paradigm: after 2 min of habituation in the conditioning chamber (Baseline), animals were presented with three tone–shock pairings. Mice were then tested for recent and remote memory in the same conditioning context respectively after 24 h and 50 days. Increased freezing behavior of COMT-Val tg mice (n=8) on the remote memory recall compared with control mice (n=11; two-way analysis of variance (ANOVA), $F_{(2, 34)}$= 3.54, p< 0.05), but not on recent memory (p= 0.64). No difference between controls and COMT-Val-tg mice on baseline freezing behavior (p =0.99). *p < 0.05 vs control at same stage. (b) Freezing reaction of COMT-Val-tg (n=8) and wild-type control mice (n=11) during exposure to a modified context 24 h and 50 days after conditioning. Fear reaction to novel context after 24 h was negligible in both groups. At 50 days after the conditioning training, COMT-Val-tg and control mice showed relatively little level of freezing. No differences between groups (two-way ANOVA: $F_{(1, 17)}$= 0.00, p= 0.92). (c) Freezing behavior observed during fear conditioning and (d) extinction in COMT-Val-tg (n=6) and their control littermates (n=10). No COMT-dependent difference was present during the habituation phase in the experimental chamber (two-way ANOVA: $F_{(1, 14)}$= 0.87, p= 0.37). We found no COMT genotype difference in both conditioning training (two-way ANOVA: $F_{(1, 14)}$= 0.03, p= 0.86) and extinction (two-way ANOVA: $F_{(1, 14)}$= 0.06, p= 0.81). (e) Representative western blot of the effect of vehicle (Veh) and doxycycline (DOX) on COMT levels in the prefrontal cortex (PFC) of control and COMT-Val tg mice. (f) Basal extracellular dopamine levels in the medial PFC (mPFC) of control (Veh n=7, DOX n=6) and COMT-Val-tg mice

(Veh n =7, DOX n=5) over a period of 60 min. Dopamine levels were lower in COMT-Val tg compared with control vehicle-treated mice (two-way RM ANOVA: $F_{(1, 11)}= 7.02$, $p< 0.05$). Silencing of the COMT Val transgene restored dopamine up to control-like levels (two-way RM ANOVA: $F_{(1, 11)}=1.16$, $P =0.30$). *$p< 0.05$ COMT-Val-tg vs control after same treatment (Veh- DOX). (g) Freezing behavior in controls (Veh n=12, DOX n =11) and COMT-Val-tg (Veh n=8, DOX n =13) on recent and remote memory recall. Silencing of COMT transgene by DOX administration reduced freezing behavior in COMT-Val-tg mice compared with Veh treatment (two-way ANOVA: $F_{(6, 80)}= 2.39$, $p< 0.05$). No effect of DOX treatment in control mice ($p =0.86$). We observed no COMT genotype ($p =0.31$) and DOX treatment ($p =0.43$) effects during recent context memory test. *$p< 0.05$ vs all other conditions at same stage. (h) No differences of freezing behavior during exposure to a novel context 24 h and 50 days after conditioning between COMT-Val-tg (Veh n=8, DOX n =13) and wild-type control mice (Veh n=12, DOX n=11) (two-way ANOVA: $F_{(3, 40)}= 0.25$, $p= 0.85$). Values represent mean ±s.e.m. throughout Figures 1, 2, 3, 4.24 h after conditioning showed no differences in the expression of recent fear memory between COMT-Val-tg and control mice (Figure 1a). These data indicate an intact learning and expression of recent fear-conditioned memories in COMT-Val-tg mice.

Then, to explore whether the reduced dopamine level in COMT-Val-tg mice was established by an increased catabolism or reduced release, we measured amphetamine-induced synaptic release of dopamine in the mPFC. We found no difference in the rate of dopamine-induced release between control and COMT-Val-tg mice (Supplementary Figure 2), further confirming that increased COMT activity elevated dopamine catabolism, but not release, in the mPFC. We next turned off the COMT transgene after 30 days from the initial conditioning until the remote memory recall (day 50). This manipulation selectively tackled the hippocampal-independent late consolidation and/or retrieval phases, but not the encoding of fear memory [2]. Silencing of the COMT transgene exclusively during late consolidation and retrieval phases was sufficient to rescue the enhanced expression of remote fear memory in COMT-Val-tg mice back to the level of their control littermates (Figure 1g). Fear reactions on recent and remote memory recall were context specific, as when we exposed the mice to a modified context we observed negligible and COMT-independent fear reactions (Figure 1h). Moreover, the altered freezing responses in COMTVal- tg mice during remote memory recall were not associated with any alteration in active coping behaviors (Supplementary Figure 1c), which have also been used to assess conditioning induced fear memory [37]. Overall, these findings demonstrate that increased COMT activity is the cause of the increased expression of remote memories. Moreover, this effect is reversible and not developmental, and it is restricted to COMT modulation on late consolidation/recall of remote memories.

4.3.2. Increased COMT activity produced an overdrive of the endocannabinoid system in the PFC

In order to explore possible molecular bases of the increased expression of remote memory in mice with increased COMT activity we first analyzed overall gene expression in the PFC. Indeed, COMT has a crucial role in the regulation of dopamine levels in the PFC [10], [13] and remote memories are thought to be stored in this cortical region [24], [38]. One of the genes for which the expression was tightly regulated by COMT was the CB1R. Indeed, we found a decreased CB1R expression in COMT-Val-tg mice (Figures 2a and b) and increased expression in COMT knockout mice (Supplementary Figure 3). The endocannabinoid system has a central function in formation and extinction of long-lasting aversive memories and CB1R deficiency is associated with a protracted expression of contextual fear memories [22]. Moreover, human COMT Val carriers are most likely to exhibit psychotic symptoms and to develop schizophreniform disorders if exposed to cannabis [23]. Thus, because the impact of the COMT Val genotype on the endocannabinoid system and its potential link with memory functions is unknown, we investigated more in detail the possible modulatory function of COMT genetics towards the endocannabinoid system. At the protein level, we confirmed a decrease of CB1R in the PFC of COMT-Val-tg mice compared with controls (Figures 2b and c) in both naive and fear-conditioned mice. A more detailed immunohistochemical analysis (Figures 2d and e and Supplementary Figure 4) revealed that the reduction of CB1R in COMT-Val-tg mice was specifically localized in the glutamatergic terminals in the PFC, the primary site of expression of COMT [11], [13]. Concurrent silencing of the COMT Val transgene was sufficient to re-establish normal levels of CB1R (Figures 2b, c and e). These data demonstrate that CB1R expression in the PFC is tightly regulated by COMT enzymatic activity. To investigate whether the decrease of CB1R levels in the PFC of COMT-Val-tg mice was associated with changes of its signaling, we measured the levels of endocannabinoids. We observed an increase of AEA in COMT-Val-tg mice that was reversed after silencing of the COMT Val transgene (Figure 2f). In contrast, we found no effects of the COMT Val genotype for the levels of the 2-AG (Figure 2g), OEA (Figure 2h) and PEA (Figure 2i). We next analyzed NAPE-PLD and FAAH, the main enzymes respectively responsible for synthesis and degradation of anandamide in the brain, and DAGL-α and MAGL that respectively modulate synthesis and degradation of 2-AG [38].

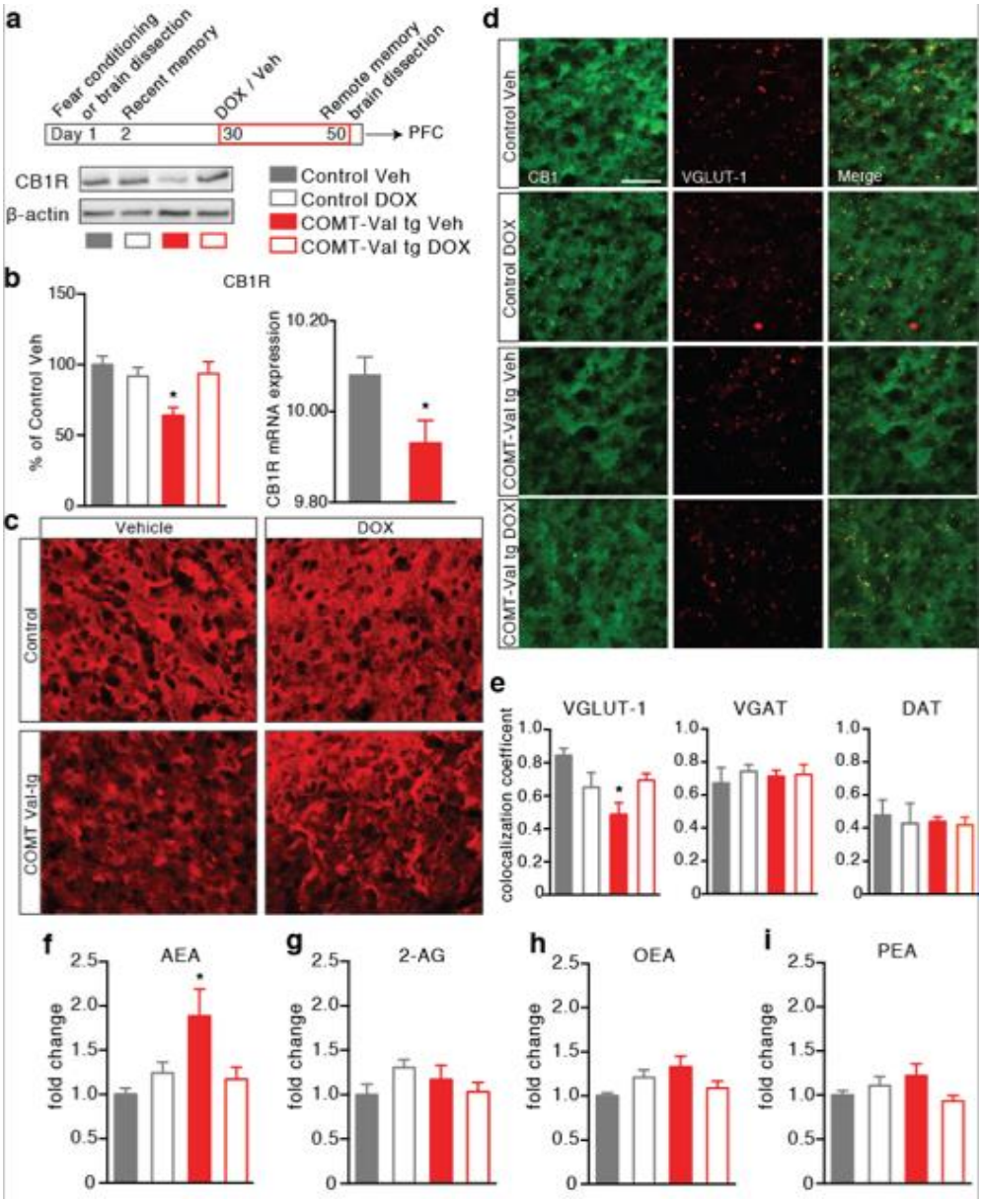

Figure 2. Increased catechol-O-methyltransferase (COMT) activity produced an overdrive of the endocannabinoid system in the prefrontal cortex (PFC). (a) Overview of the experiment. COMT-Val-tg and control mice were conditioned on day 1, and then tested for recent memory recall on day 2. On day 30, mice were administered with vehicle (Veh) or doxycycline (DOX) in drinking water for 20 days. On day 50, mice were tested for remote memory recall. After testing mice were killed and PFC was removed for analysis of the endocannabinoid system. Because we found similar results in naive (before conditioning) and in COMT-Val-tg and control mice after remote memory recall, data were pooled together. (b, left). Decrease of CB1R protein levels in the PFC of COMT-Val-tg mice (Veh n =7, DOX n=7) compared with controls (Veh n=9, DOX n=7; two-way analysis of variance (ANOVA): genotype × treatment, $F_{(1, 26)}= 8.23$, $p< 0.05$) and representative western blot images. DOX treatment in COMT-Val-tg mice restored normal levels of CB1R in the PFC ($p=0.50$ vs control Veh), whereas it has no effect on control mice ($p=0.66$). *$p<0.005$ vs all other groups. (b, right) Reduction of CB1R mRNA expression in the PFC of COMT-Val-tg (n =6) compared with control mice (n =7; unpaired t-test: $t=2.16$, d.f.=11, $p< 0.05$). (c) Immunoreactivity (n=3 mice each group, 3 slices per mice) of CB1R in the PFC of

COMT-Val-tg and control mice after Veh or DOX administration. Images are shown at × 400 magnification. (d) Immunohistochemistry on sections of PFC for CB1 receptors (green) and vesicular glutamate transporter (VGLUT, red) from controls and COMT-Val-tg after Veh or DOX treatment. Scale bar 100 μM. (e) CB1R reduction colocalized with VGLUT (two-way ANOVA: $F(1, 8)=9.48$, $P< 0.05$), but not with vesicular GABA transporter (VGAT) and dopamine transporter (DAT) in the PFC of control and COMT-Val-tg mice following Veh or DOX administration. *p< 0.005 vs control Veh. (f) Increase of anandamide (AEA) in the PFC (two-way ANOVA: genotype × treatment, $F_{(1, 25)}= 4.69$, $p< 0.05$) in COMT-Val-tg (Veh n =9, DOX n=5) compared with control mice (Veh n=10, DOX n= 5) was reversed by silencing of COMT transgene by DOX (p= 0.84 vs control Veh). *p< 0.005 vs control Veh. (g) No significant change of 2AG in the PFC (two-way ANOVA: genotype × treatment, $F_{(1, 24)}= 2.23$, P =0.14) of COMT-Val-tg (Veh n=9, DOX n=5) and control mice (Veh n=9, DOX n=5). (h) A significant interaction between COMT genotype and DOX treatment was evident for oleoylethanolamide (OEA) levels in the PFC in COMT-Val-tg (Veh n=9, DOX n=5) compared with control mice (Veh n=10, DOX n=5; two-way ANOVA: genotype × treatment, $F_{(1, 25)}=5.64$, Po0.05). However, post hoc comparisons did not show any significant difference between groups (p= 40.10). (i) No significant effect for the endocannabinoid PEA in the PFC of COMT-Val-tg (Veh n =10, DOX n=5) and control mice (Veh n =10, DOX n=5; two-way ANOVA: genotype × treatment, $F_{(1, 26)=}$ 3.07, p= 0.09). Data in (f–i) are expressed as fold changes compared with control Veh.

Both NAPE-PLD and FAAH levels (Figures 3b, c and g and Figures 3b, e and h, respectively) were elevated in the PFC of COMT-Val-tg mice. Similarly, in COMT-Val-tg mice we found a 25% increase in FAAH enzymatic activity compared with control mice (Figure 3e). Restoring the endogenous COMT levels normalized both enzymes to control-like levels (Figures 3b, c and g and Figures 3b,e and h, respectively). On the contrary, we found no COMT-dependent changes for the DAGL-α and MAGL (Figures 3d and f). Collectively, these findings indicate that genetic variations increasing COMT produce a hyperactive NAPE-PLD, anandamide and FAAH endocannabinoid pathway in the PFC. In contrast, the DAGL-α, 2-AG and MAGL endocannabinoid pathway was unaffected. These alterations were not permanent as turning off the COMT Val transgene normalized endocannabinoid signaling.

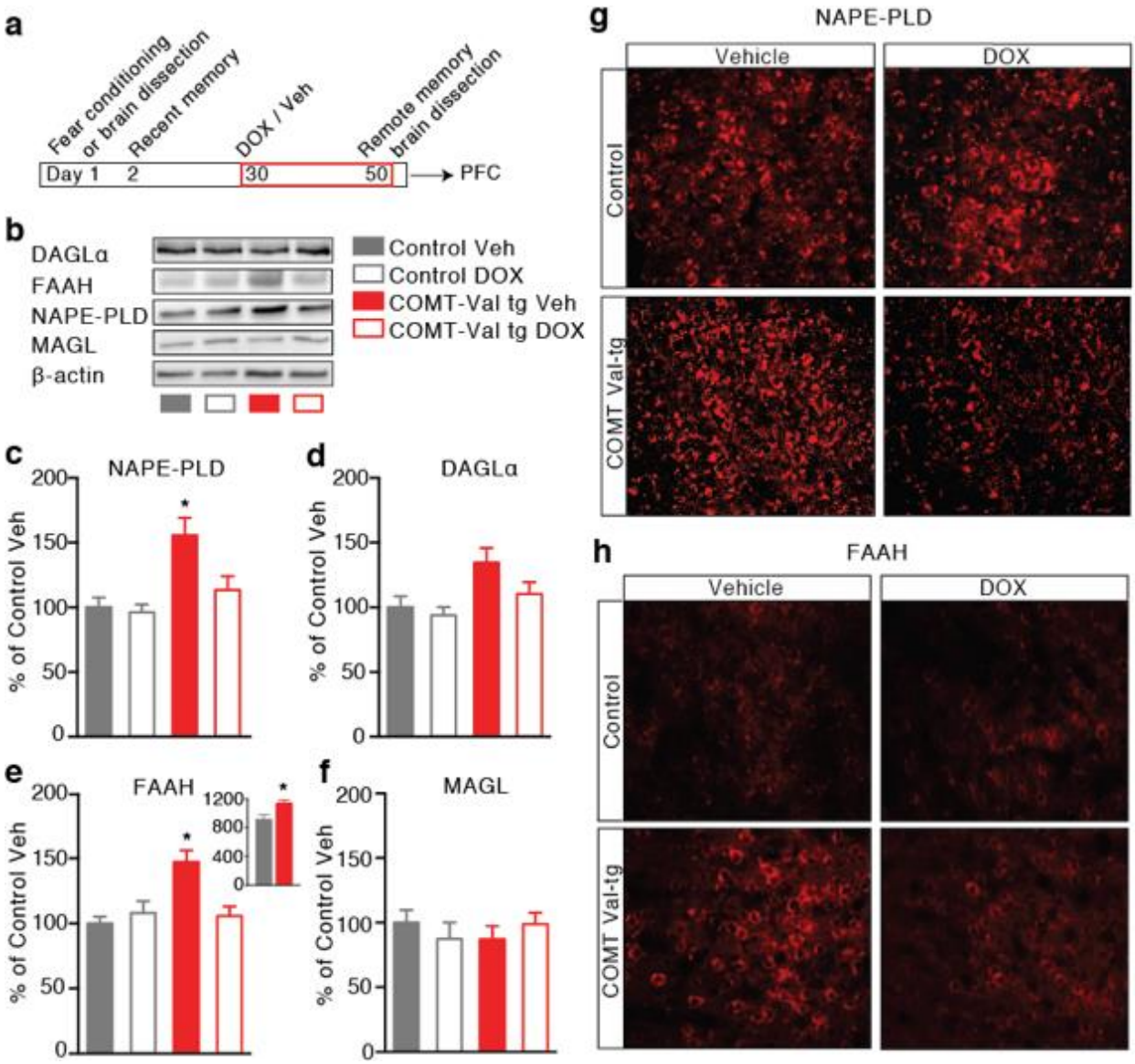

Figure 3. Increased catechol-O-methyltransferase (COMT) activity produced a hyperactive N-acyl phosphatidyl-lethanolamine phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH) endocannabinoid pathway in the prefrontal cortex (PFC). (a) Overview of the experiment. On day 50, mice were tested for remote memory recall. After testing mice were killed and PFC was removed for analysis of the NAPE-PLD, diacylglycerol lipase-α (DAGL-α), FAAH and monoacylglycerol lipase (MAGL). Because we found similar results in naive (before conditioning) and in COMT-Val-tg and control mice after remote memory recall, data were pooled together. (b) Representative western blot images of DAGL- α, FAAH, NAPE-PLD and MAGL in the PFC of control and COMT-Val-tg mice after vehicle (Veh) or doxycycline (DOX) treatment. (c) Increase of NAPE-PLD (two-way analysis of variance (ANOVA): genotype × treatment, $F_{(1, 28)}$= 4.45, p< 0.05) in the PFC of COMT-Val-tg (Veh n= 6, DOX n=8) compared with control mice (Veh n=9, DOX n=9). DOX in COMT-Val-tg mice restored NAPE-PLD to control-like levels (p =0.70 vs control Veh). *p< 0.005 vs all other groups. (d) No differences of DAGL-α (two-way ANOVA: genotype × treatment, $F_{(1, 29)}$= 1.04, p= 0.31) levels in the PFC in COMT-Val-tg (Veh n=7, DOX n=8) and controls (Veh n=9, DOX n=9). (e) Increase of FAAH levels (two-way ANOVA: genotype × treatment, F(1, 28)=9.93, Po0.005) in COMT-Val-tg (Veh n=6, DOX n=8) in the PFC compared with control

mice (Veh n=9, DOX n=9). DOX in COMT-Val-tg reversed FAAH increase back to control-like levels (p= 0.99 vs control Veh). *p < 0.005 vs all other groups. Inset shows increased FAAH enzymatic activity (expressed as pmol min− 1 mg− 1) in the PFC of COMT-Val-tg (unpaired t-test: t=2.87, d.f.=9, *p< 0.05). (f) No differences of MAGL (two-way ANOVA: genotype × treatment, $F_{(1, 30)}$= 1.31, p= 0.26) levels in the PFC in COMT-Val-tg (Veh n=7, DOX n=9) and controls (Veh n=9, DOX n=9). (g) Representative immunoreactivity (n=3 mice each group, 3 slices per mice) of NAPEPLD in the medial PFC (mPFC) in control and COMT-Val-tg after Veh or DOX administration. Images are shown at × 400 magnification. (h) Representative immunoreactivity (n=3 mice each group, 3 slices per mice) of FAAH in the mPFC in control and COMT-Val-tg after Veh or DOX administration. Images are shown at × 400 magnification.

4.3.3 Increased COMT activity did not alter the endocannabinoid system in hippocampus and striatum.

Given the role played by the hippocampus in memory consolidation [1], [39] and that a relative increase of COMT activity in mice might also alter dopamine release in the striatum [40] we also analyzed possible COMT-dependent effects on the cannabinoid system in these two brain regions. In contrast to the PFC, in the hippocampus we found no differences in CB1R levels (Supplementary Figures 5a and b), or in the levels of the endocannabinoids AEA, 2-AG, OEA and PEA (Supplementary Figures 5c–f) between COMT-Val-tg and control mice. Moreover, in the hippocampus there were no COMTdependent changes for the enzymes responsible for synthesis (NAPE-PLD and DAGL-α, Supplementary Figures 5g and j) and degradation (FAAH and MAGL, Supplementary Figures 4h and j) of the endocannabinoids. Similarly, all these measures of the endocannabinoid system did not differ between COMT-Val-tg and control mice in the striatum (Supplementary Figure 6). Thus, genetic variations increasing COMT enzymatic activity specifically modulated the endocannabinoid system in the PFC, and not in the hippocampus or in the striatum.

4.3.3. CB1R blockade restored remote memories alterations dependent on COMT genotype

A reduction of CB1R, in the context of increased NAPE-PLD, AEA and FAAH pathways found in the PFC of COMT-Val-tg mice, might indicate a compensatory mechanism to cope with an overdrive of the endocannabinoid system. To test directly the causal role of the altered endocannabinoid system on the stronger remote memory found in COMT-Val-tg mice, we tested the prediction that blocking the endocannabinoid signaling on CB1R during the remote recall could restore a normal expression of fear memory in COMT-Val-tg mice. To this end, 50 days after fear conditioning, we injected Veh or the CB1R antagonist AM251 30 min before

the remote memory recall to reach the maximum effect of the drug [25]. Blockade of CB1R increased remote memory expression in controls, such that freezing behavior did not differ from Veh-treated COMT-Val-tg mice (Figure 4a). Conversely, in COMT-Val-tg mice the CB1R antagonist AM251 reversed the enhanced remote memory back to control levels (Figure 4a). Active fear-coping behaviors during the remote memory recall test did not change depending on either the COMT genotype or AM251 treatment (Supplementary Figure 7a). Thus, blockade of CB1R signaling had opposite effects on remote memory in animals with normal or increased COMT activity. Finally, we addressed whether the COMT-by-endocannabinoid interaction found in fear conditioning paradigms could be generalized to other form of remote aversive memories. In particular, we tested control and COMT-Val-tg mice in a naloxone-induced CPA, a well-established task to assess negative affective states in mice [41]. COMT-Val-tg mice showed higher aversion for environmental cues paired with naloxone injections received 50 days earlier during conditioning (Figure 4b), whereas we did not observe genotype-dependent differences on recent memory recall (Supplementary Figure 7b). Conversely, AM251 had no effect on CPA in control mice (Figure 4b). This same treatment with AM251 increased extracellular dopamine in the mPFC, in particular we observed a peak in COMT-Val-tg 30 min after injection. Overall, these findings demonstrated that increased COMT activity modulated remote memories through an overactivation of the endocannabinoid system.

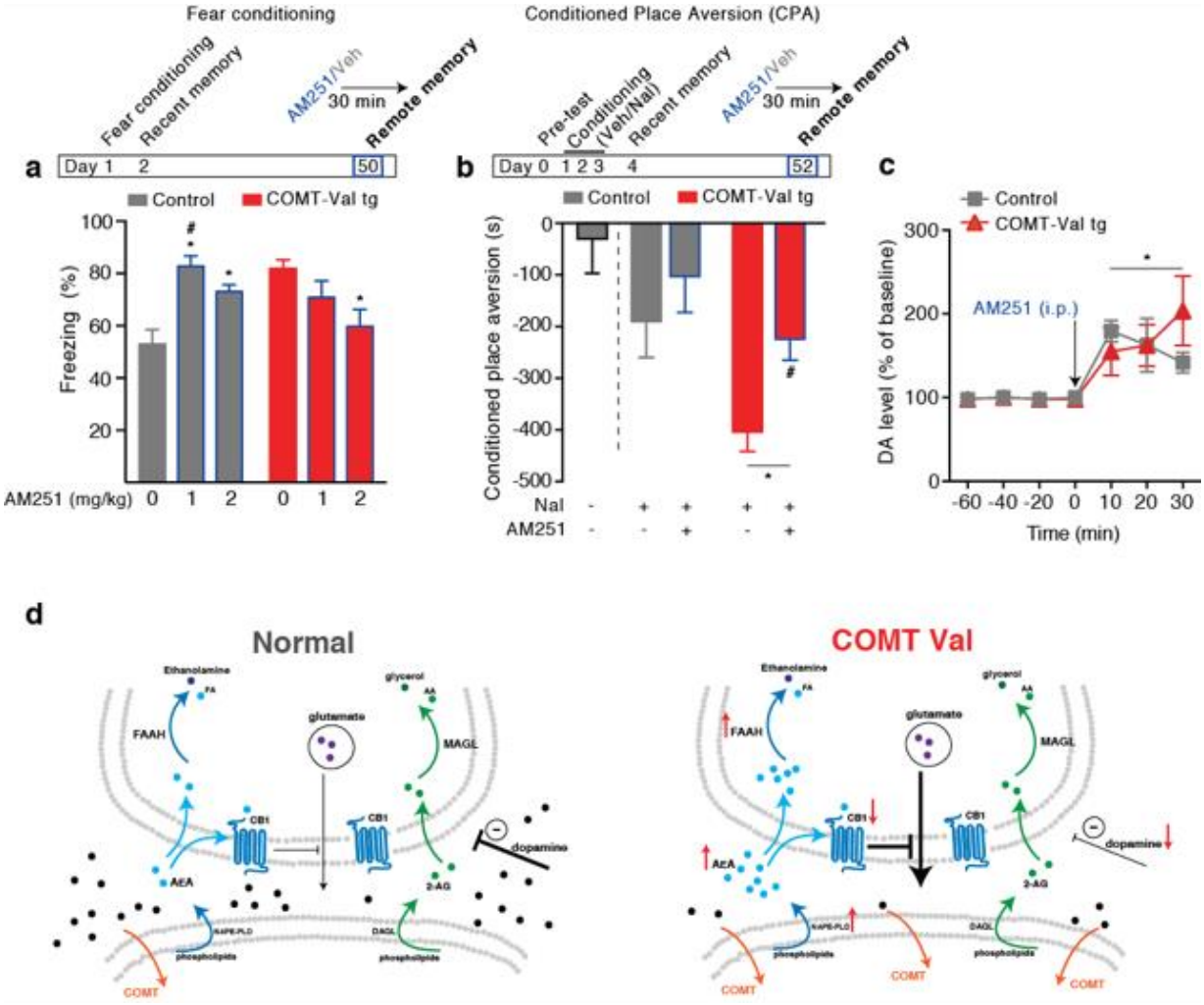

Figure 4. CB1 receptor blockade restored remote memory alterations dependent on catechol-O-methyltransferase (COMT) genotype. (a) Freezing behavior in controls and COMT-Val tg mice on remote memory recall after vehicle (Veh) or AM251 treatment (n=8–11 each group). AM251 reduced freezing behavior in COMT-Val tg mice (two-way analysis of variance (ANOVA): $F_{(2, 44)}$= 12.03, p< 0.0005) compared with COMT-Val tg Veh (p< 0.05) and Veh-treated control animals (P =0.21). Increased freezing reaction in controls after CB1-R antagonist AM251 compared with Veh (Po0.05). Control: *Po0.05 vs Veh., #p< 0.05 vs COMT-Val tg AM251 (2 mg kg− 1). COMT-Val tg: *p< 0.05 vs Veh. (b) Remote memory, assessed using conditioned place aversion (CPA), was increased in COMT-Val tg mice (two-way ANOVA: genotype, $F_{(1, 33)}$= 7.22, p< 0.05). Administration of AM251 reduced CPA in COMT-Val-tg (unpaired t-test: t =2.97, d.f.=14, p< 0.05), whereas it had no effects in controls (unpaired t-test: t =0.87, d.f.=19, P =0.39). Conditioning with Veh did not produce place aversion (one-way ANOVA: $F_{(4, 39)}$= 4.51, p< 0.05 vs control naloxone (Nal) and COMT-Val-tg Nal). (c) Extracellular dopamine levels in the medial prefrontal cortex (mPFC) of controls (n=8) and COMT-Val-tg (n=8) mice following AM251 (1 mg kg− 1, intraperitoneal). Systemic injection of CB1R blocker AM251 elevated dopamine levels in the mPFC (two-way ANOVA: time, $F_{(6, 60)}$= 10.75, p <0.0005). In particular, COMT-Val-tg showed a peak of dopamine 30 min after AM251 injection, compared with baseline (p< 0.005). (d) Proposed model of the dysregulation of endocannabinoid system by genetically increased levels of COMT. In 'Normal' conditions in the PFC dopamine exerts an inhibitory control over the activity of glutamatergic pyramidal neurons. Dopaminergic

terminals may modulate pyramidal neurons activity directly through synaptic contacts to the pyramidal neuron spines or, alternatively, inhibition may occur indirectly via activation of GABAergic (γ-aminobutyric acid) inhibitory interneurons. In subjects with increased COMT activity ('COMT-Val') extracellular dopamine in PFC is decreased, resulting in lower inhibition of cortical pyramidal neurons. To counterbalance this reduced inhibition, the system will then produce an overactive anandamide tone by acting at CB1R. However, prolonged stimulation of CB1R by anandamide in the PFC enhanced the stability of remote memories. These alterations can be rescued increasing cortical dopamine levels by either blocking COMT or CB1R over activity.

4.4. Discussion

Using a conditional humanized mouse overexpressing the COMT Val gene we demonstrated that remote memory recall is modulated by COMT genetic variations. Moreover, we established that the COMT Val genotype has a selective and reversible impact on the endocannabinoid system at the level of the PFC that was the cause of increased remote memories in COMT Val mutants. Mice with a relative increased COMT activity display an exaggerated recall of remote memories. This finding illuminates human neuroimaging studies showing a COMT-dependent effect on prefrontal activation during long-term memory encoding and retrieval [17], [42], [43]. In particular, in the present work we demonstrated that this effect on remote memories was reversible and selectively dependent on COMT concurrent activity. Furthermore, the more stable expression of remote memories in COMT-Val-tg mice was not influenced by factors such as deficits in conditioning/ extinction learning (present study) or increased levels of anxiety [11]. Notably, a wealth of evidence from humans and genetically modified mice established COMT as an exquisite and selective modulator of executive functions [11], [44]–[50]. In particular, COMT-Val-tg mice as well as humans carrying the COMT Val-allele show poorer executive functions, including deficits in cognitive flexibility and working memory [35], [51], [52]. In contrast, here we found that COMT-Val-tg mice show exaggerated remote memories recall, unraveling a more stable organization of these types of memories. This tradeoff between a genetic effect that disrupts cognitive flexibility (executive functions) but exaggerates remote memories provides a biological substrate on the dichotomy between stable maintenance of information and cognitive flexibility. COMT activity finely regulates dopamine levels in the PFC that, together with the balance between D1 and D2 receptor activation, have been proposed as the neurobiological basis regulating cognitive flexibility/stability balance [53]. In contrast, despite also being a COMT substrate, norepinephrine levels in the PFC are negligibly affected by COMT genetic variations [9]–[11], possibly because of the abundance of its norepinephrine transporter in this brain region [9]. However, even if our own data did not show any major COMT dependent effect on norepinephrine levels in the PFC, we cannot exclude a possible involvement of this neurotransmitter given its relevance in the strengthening of aversive memories [54], [55]. Thus, our results suggest that the enhanced remote memories in subjects with increased COMT activity (and in turn reduced extracellular dopamine) may reflect a sort of ‘inflexibility’ of the PFC. This hypothesis is also consistent with the current model of remote memories storage that points to a more prominent role of the PFC over the hippocampus [2]. In relationship to the

clinical setting, our findings might indicate a possible role of COMT in psychiatric conditions associated with inappropriate retention of past aversive memories, such as posttraumatic stress disorder (PTSD). COMT genetics have been associated with both PTSD vulnerability and severity of symptomatology, even if the directionality of the effects are controversial [21], [56]–[58]. The discrepant results might be related to the ethnicity of the subjects, as in Caucasians (both children and adults) the COMT Val allele was associated with higher vulnerability and more severe PTSD symptoms, whereas the opposite effect was found in African-American children [57], [58]. Another factor to consider is the interaction between sex and the developmental stage of the individuals. Indeed, COMT genetic variants affect cognitive functions in opposite way in males and females but only during adulthood (between puberty and menopause) [44], [45], [59]. However, because relatively lower COMT increases anxiety states and vulnerability to stressful events [35], [60], [61], whereas increased COMT enhances aversive remote memories (present work), both too little and too much COMT activity could contribute to PTSD development and manifestations. In agreement, in a study with 236 Iraq War veterans, both Met/Met and Val/Val subjects were at increased risk for PTSD compared with Val/Met carriers [56]. Finally, our findings uncover an interaction between the COMT Val genotype and the cannabinoid system that has been previously suggested in schizophrenia development. Specifically, individuals carrying the COMT Val allele present higher risk to develop schizophrenia if exposed to cannabis [23]. Despite this coincidental evidence, the biological bases through which COMT genetics might influence cannabis-induced psychosis vulnerability are still unknown. The endocannabinoid overdrive we found in the PFC of subjects with genetic variations increasing COMT activity (COMT Val) could be one of the biological causes of these clinical associations. Damage of the ventromedial PFC reduce the occurrence of PTSD [62] and the PFC have been consistently implicated in the manifestation of schizophrenia-relevant cognitive phenotypes [63], [64]. Moreover, endocannabinoids are important in the regulation of memory for emotional events [65]. For example, aversive experiences stimulate the release of anandamide into the medial PFC to induce the formation of a strong memory trace [66]. This is in line with our finding of increased anandamide in the PFC of animals with higher COMT activity, leading to exaggerated expression of aversive memories. The COMT-dependent dysregulation of the endocannabinoid system also included a reduction of CB1R that seemed to be restricted to the glutamatergic terminals. This is in agreement with previous evidence that a primary localization of COMT is within glutamatergic pyramidal neurons in the PFC [9], [11], and suggesting this as the main site of COMT–endocannabinoid interaction. However, because of the presence of COMT in astrocytes [13]

and the role of endocannabinoids in astrocytes [67] we cannot exclude astrocytes as a potential contributing site of action. At excitatory synapses, endocannabinoids function as retrograde messengers, binding to presynaptic CB1R that in turn mediates the suppression of glutamate release [65]. Moreover, dopamine in the mPFC inhibits glutamate outputs by acting at D2 receptors on pyramidal neurons reducing neurotransmitter release probability at glutamatergic synapse [68], [69]. Thus, in the context of reduced extracellular dopamine in the mPFC (as found in COMT Val subjects), we might interpret the increase in tonic anandamide activity at CB1R in glutamatergic neurons as an adaptive mechanism to control an excessive glutamatergic excitatory drive. In line with this hypothesis, the reduction of CB1R would be the result of a compensatory phenomenon to cope with increased endocannabinoid tone. Accordingly, acutely blocking CB1R signaling by AM251 was sufficient to normalize remote memories recall in COMT-Val-tg mice and increased extracellular dopamine levels within the mPFC (general model depicted in Figure 4b). In this study, we unraveled an outstanding role of the PFC, while hippocampus and striatal regions were seemingly not involved. The selective effects of the COMT Val genotype in the regulation of the endocannabinoid system within the PFC are in line with a more prominent role of COMT enzymatic activity in the cortex [9] and with the theory that the neocortex represent a key site for permanent storage of long-term memories [2], [32]. Afferents from the PFC coordinate the integration of CS and US pairing within the lateral amygdala [70]. Moreover, the central amygdala is widely considered the major output center that controls brainstem and hypothalamic systems necessary for the expression of emotionally related conditioned responses such as freezing [71] and place aversion [72] In our paradigm we did not find differences in remote auditory cued fear memory that is known to be stored in the amygdala [70]. However, COMT genotype has been suggested to affect the processing of emotional stimuli in the amygdala and in the cortex as well as the functional connectivity between these two brain regions [60], [73]. Furthermore, cannabinoid transmission within the basolateral amygdala strongly modulates the formation of associative fear memory via functional interactions with the PFC [74]. Thus, the significance and possible involvement of COMT dependent changes in PFC–amygdala circuits might represent an important subject for future studies. In conclusion, we showed that a genetic condition of increased COMT activity enhanced aversive remote memories. We propose that COMT activity can modulate the stability of remote memories in the PFC at the expense of cognitive flexibility. The effect of COMT on dopamine metabolism in this cognitive balance might help us to better understand in mechanistic terms the derailment of cognitive and emotional functions in nervous system diseases like PTSD and schizophrenia. In this context, we found a

selective biological interaction between COMT genotype and the endocannabinoid system in the PFC. Thus, COMT-dependent alterations in the endocannabinoid system might explain some of the individual differences in the effects of cannabis use, and more in general COMT polymorphisms could influence the response to medication targeting the cannabinoid system.

4.5. Conflict of interest

The authors declare no conflict of interest.

4.6. Acknoledgement

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4.7. References

[1] T. Kitamura *et al.*, "Adult Neurogenesis Modulates the Hippocampus-Dependent Period of Associative Fear Memory," *Cell*, vol. 139, no. 4, pp. 814–827, 2009.

[2] P. W. Frankland and B. Bontempi, "The organization of recent and remote memories," *Nature Reviews Neuroscience*. 2005.

[3] C. L. Beeman, P. S. Bauer, J. L. Pierson, and J. J. Quinn, "Hippocampus and medial prefrontal cortex contributions to trace and contextual fear memory expression over time," *Learn. Mem.*, vol. 20, no. 6, pp. 336–343, 2013.

[4] F. Papaleo, B. K. Lipska, and D. R. Weinberger, "Mouse models of genetic effects on cognition: Relevance to schizophrenia," *Neuropharmacology*, vol. 62, no. 3, pp. 1204–1220, Mar. 2012.

[5] T. W. Robbins and S. Kousta, "Uncovering the genetic underpinnings of cognition," *Trends Cogn. Sci.*, vol. 15, no. 9, pp. 375–377, 2011.

[6] W. Davies, T. Humby, S. Trent, J. B. Eddy, O. a Ojarikre, and L. S. Wilkinson, "Genetic and pharmacological modulation of the steroid sulfatase axis improves response control; comparison with drugs used in ADHD.," *Neuropsychopharmacology*, vol. 39, no. 11, pp. 2622–32, 2014.

[7] R. Cools and M. D'Esposito, "Inverted-U-shaped dopamine actions on human working memory and cognitive control," *Biol. Psychiatry*, vol. 69, no. 12, pp. e113–e125, 2011.

[8] D. Shohamy and R. A. Adcock, "Dopamine and adaptive memory," *Trends Cogn. Sci.*, vol. 14, no. 10, pp. 464–472, 2010.

[9] E. M. Tunbridge, P. J. Harrison, and D. R. Weinberger, "Catechol-o-Methyltransferase, Cognition, and Psychosis: Val158Met and Beyond," *Biol. Psychiatry*, vol. 60, no. 2, pp. 141–151, 2006.

[10] L. Yavich, M. M. Forsberg, M. Karayiorgou, J. A. Gogos, and P. T. Mannisto, "Site-Specific Role of Catechol-O-Methyltransferase in Dopamine Overflow within Prefrontal Cortex and Dorsal Striatum," *J. Neurosci.*, vol. 27, no. 38, pp. 10196–10209, 2007.

[11] F. Papaleo *et al.*, "Genetic dissection of the role of catechol-O-methyltransferase in cognition and stress reactivity in mice.," *J. Neurosci.*, vol. 28, no. 35, pp. 8709–23, 2008.

[12] E. M. Tunbridge, "Catechol-O-Methyltransferase Inhibition Improves Set-Shifting Performance and Elevates Stimulated Dopamine Release in the Rat Prefrontal Cortex,"

J. Neurosci., vol. 24, no. 23, pp. 5331–5335, 2004.

[13] M. Matsumoto *et al.*, “Catechol O-methyltransferase mRNA expression in human and rat brain: evidence for a role in cortical neuronal function.,” *Neuroscience*, vol. 116, no. 1, pp. 127–137, 2003.

[14] A. G. Nackley *et al.*, “Human Catechol-O-Methyltransferase Haplotypes Modulate Protein Expression by Altering mRNA Secondary Structure,” *Science (80-.).*, vol. 314, no. 5807, p. 1930 LP-1933, Dec. 2006.

[15] I. Dumontheil, B. Hassan, S. J. Gilbert, and S.-J. Blakemore, “Development of the Selection and Manipulation of Self-Generated Thoughts in Adolescence,” *J. Neurosci.*, vol. 30, no. 22, pp. 7664–7671, 2010.

[16] J. H. Barnett, P. B. Jones, T. W. Robbins, and U. Müller, “Effects of the catechol-O-methyltransferase Val158Met polymorphism on executive function: A meta-analysis of the Wisconsin Card Sort Test in schizophrenia and healthy controls,” *Mol. Psychiatry*, vol. 12, no. 5, pp. 502–509, 2007.

[17] A. Bertolino *et al.*, “Prefrontal-Hippocampal Coupling During Memory Processing Is Modulated by COMT Val158Met Genotype,” *Biol. Psychiatry*, vol. 60, no. 11, pp. 1250–1258, 2006.

[18] C. Rothe *et al.*, “Association of the Val158Met catechol O-methyltransferase genetic polymorphism with panic disorder,” *Neuropsychopharmacology*, vol. 31, no. 10, pp. 2237–2242, 2006.

[19] I. T. Kolassa, S. Kolassa, V. Ertl, A. Papassotiropoulos, and D. J. F. De Quervain, “The Risk of Posttraumatic Stress Disorder After Trauma Depends on Traumatic Load and the Catechol-O-Methyltransferase Val158Met Polymorphism,” *Biol. Psychiatry*, vol. 67, no. 4, pp. 304–308, 2010.

[20] J. A. Wojtalik and D. M. Barch, “An FMRI study of the influence of a history of substance abuse on working memory-related brain activation in schizophrenia,” *Front. psychiatry*, vol. 5, p. 1, Jan. 2014.

[21] M. Morena and P. Campolongo, “The endocannabinoid system: An emotional buffer in the modulation of memory function,” *Neurobiol. Learn. Mem.*, vol. 112, pp. 30–43, 2014.

[22] G. Marsicano *et al.*, “The endogenous cannabinoid system controls extinction of aversive memories,” *Nature*, vol. 418, no. 6897, pp. 530–534, 2002.

[23] A. Caspi *et al.*, “Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene:

Longitudinal evidence of a gene X environment interaction," *Biol. Psychiatry*, vol. 57, no. 10, pp. 1117–1127, 2005.

[24] P. W. Frankland, B. Bontempi, L. E. Talton, L. Kaczmarek, and A. J. Silva, "The Involvement of the Anterior Cingulate Cortex in Remote Contextual Fear Memory," *Science (80-.).*, vol. 304, no. 5672, p. 881 LP-883, May 2004.

[25] S. Tambaro, M. L. Tomasi, and M. Bortolato, "Long-term CB1receptor blockade enhances vulnerability to anxiogenic-like effects of cannabinoids," *Neuropharmacology*, vol. 70, pp. 268–277, 2013.

[26] F. Managò *et al.*, "Genetic Disruption of Arc/Arg3.1 in Mice Causes Alterations in Dopamine and Neurobehavioral Phenotypes Related to Schizophrenia," *Cell Rep.*, vol. 16, no. 8, pp. 2116–2128, 2016.

[27] G. Paxinos, K. B. J. Franklin, K. B. J. Paxinos, G and Franklin, G. Paxinos, and K. B. J. Franklin, *Mouse Brain in Stereotaxic Coordinates*. 2001.

[28] G. Astarita and D. Piomelli, "Lipidomic analysis of endocannabinoid metabolism in biological samples," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2009.

[29] E. Zamberletti, M. Gabaglio, P. Prini, T. Rubino, and D. Parolaro, "Cortical neuroinflammation contributes to long-term cognitive dysfunctions following adolescent delta-9-tetrahydrocannabinol treatment in female rats," *Eur. Neuropsychopharmacol.*, vol. 25, no. 12, pp. 2404–2415, 2015.

[30] S. Bolte and F. P. Cordelières, "A guided tour into subcellular colocalization analysis in light microscopy," *Journal of Microscopy*. 2006.

[31] A. K. Hall, U. Rutishauser, and D. Biol, "Modality-Specific Retrograde Amnesia of Fear Author (s): Jeansok J . Kim and Michael S . Fanselow Published by : American Association for the Advancement of Science Stable URL : https://www.jstor.org/stable/2876873 JSTOR is a not-for-profit service that," vol. 256, no. 5057, pp. 675–677, 2018.

[32] J. J. Quinn, Q. D. Ma, M. R. Tinsley, C. Koch, and M. S. Fanselow, "Inverse temporal contributions of the dorsal hippocampus and medial prefrontal cortex to the expression of long-term fear memories," *Learn. Mem.*, vol. 15, no. 5, pp. 368–372, 2008.

[33] L. Restivo, G. Vetere, B. Bontempi, and M. Ammassari-Teule, "The Formation of Recent and Remote Memory Is Associated with Time-Dependent Formation of Dendritic Spines in the Hippocampus and Anterior Cingulate Cortex," *J. Neurosci.*, vol. 29, no. 25, pp. 8206–8214, 2009.

[34] M. R. Milad and G. J. Quirk, “58005 51..106,” vol. 420, no. NOVEMBER, pp. 1–5, 2002.

[35] F. Papaleo *et al.*, “Genetic Dissection of the Role of Catechol- O - Methyltransferase in Cognition and Stress Reactivity in Mice,” vol. 28, no. 35, pp. 8709–8723, 2008.

[36] J. A. Gogos *et al.*, “Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior,” *Proc. Natl. Acad. Sci.*, vol. 95, no. 17, pp. 9991–9996, 1998.

[37] A. Gozzi *et al.*, “A neural switch for active and passive fear,” *Neuron*, vol. 67, no. 4, pp. 656–666, 2010.

[38] V. Di Marzo, N. Stella, and A. Zimmer, “Endocannabinoid signalling and the deteriorating brain,” *Nat. Rev. Neurosci.*, vol. 16, no. 1, pp. 30–42, 2015.

[39] J. Gräff *et al.*, “Epigenetic priming of memory updating during reconsolidation to attenuate remote fear memories,” *Cell*, vol. 156, no. 1–2, pp. 261–276, 2014.

[40] E. H. Simpson *et al.*, “Genetic variation in COMT activity impacts learning and dopamine release capacity in the striatum,” *Learn. Mem.*, vol. 21, no. 4, pp. 205–214, 2014.

[41] A. Contarino and F. Papaleo, “The corticotropin-releasing factor receptor-1 pathway mediates the negative affective states of opiate withdrawal,” *Proc. Natl. Acad. Sci.*, vol. 102, no. 51, pp. 18649–18654, 2005.

[42] B. H. Schott, “The Dopaminergic Midbrain Participates in Human Episodic Memory Formation: Evidence from Genetic Imaging,” *J. Neurosci.*, vol. 26, no. 5, pp. 1407–1417, 2006.

[43] M. Wimber *et al.*, “Prefrontal dopamine and the dynamic control of human long-term memory,” *Transl. Psychiatry*, vol. 1, no. June, pp. 1–7, 2011.

[44] F. Papaleo, S. Sannino, F. Piras, and G. Spalletta, “Sex-dichotomous effects of functional COMT genetic variations on cognitive functions disappear after menopause in both health and schizophrenia,” *Eur. Neuropsychopharmacol.*, vol. 25, no. 12, pp. 2349–2363, 2015.

[45] F. Papaleo, L. Erickson, G. Liu, J. Chen, and D. R. Weinberger, “Effects of sex and COMT genotype on environmentally modulated cognitive control in mice,” pp. 2–7, 2012.

[46] S. Sannino *et al.*, “COMT genetic reduction produces sexually divergent effects on cortical anatomy and working memory in mice and humans,” *Cereb. Cortex*, vol. 25, no. 9, pp. 2529–2541, 2015.

[47] E. Jacobs and M. D'Esposito, "Estrogen Shapes Dopamine-Dependent Cognitive Processes: Implications for Women's Health," *J. Neurosci.*, vol. 31, no. 14, pp. 5286–5293, 2011.

[48] T. P. White *et al.*, "Sex differences in COMT polymorphism effects on prefrontal inhibitory control in adolescence," *Neuropsychopharmacology*, vol. 39, no. 11, pp. 2560–2569, 2014.

[49] D. Scheggia, A. Bebensee, D. R. Weinberger, and F. Papaleo, "The ultimate intra-/extra-dimensional attentional set-shifting task for mice," *Biol. Psychiatry*, vol. 75, no. 8, pp. 660–670, 2014.

[50] G. E. Bruder *et al.*, "Catechol-O-methyltransferase (COMT) genotypes and working memory: Associations with differing cognitive operations," *Biol. Psychiatry*, vol. 58, no. 11, pp. 901–907, 2005.

[51] M. F. Egan *et al.*, "Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia," *Pnas*, vol. 98, no. 12, pp. 6917–6922, 2001.

[52] A. K. Malhotra, L. J. Kestler, C. Mazzanti, J. A. Bates, T. Goldberg, and D. Goldman, "A functional polymorphism in the COMT gene and performance on a test of prefrontal cognition," *Am. J. Psychiatry*, 2002.

[53] D. Durstewitz and J. K. Seamans, "The Dual-State Theory of Prefrontal Cortex Dopamine Function with Relevance to Catechol-O-Methyltransferase Genotypes and Schizophrenia," *Biol. Psychiatry*, vol. 64, no. 9, pp. 739–749, Sep. 2017.

[54] D. Mueller, J. T. Porter, and G. J. Quirk, "Noradrenergic Signaling in Infralimbic Cortex Increases Cell Excitability and Strengthens Memory for Fear Extinction," *J. Neurosci.*, vol. 28, no. 2, pp. 369–375, 2008.

[55] C. Y. Kao, G. Stalla, J. Stalla, C. T. Wotjak, and E. Anderzhanova, "Norepinephrine and corticosterone in the medial prefrontal cortex and hippocampus predict PTSD-like symptoms in mice," *Eur. J. Neurosci.*, vol. 41, no. 9, pp. 1139–1148, 2015.

[56] R. Clark *et al.*, "Predicting post-traumatic stress disorder in veterans: Interaction oftraumatic load with COMT gene variation," *J. Psychiatr. Res.*, vol. 47, no. 12, pp. 1849–1856, 2013.

[57] K. L. Humphreys, M. S. Scheeringa, and S. S. Drury, "Race Moderates the Association of Catechol- *O* -methyltransferase Genotype and Posttraumatic Stress Disorder in Preschool Children," *J. Child Adolesc. Psychopharmacol.*, 2014.

[58] E. A. Winkler *et al.*, "COMT Val158Met polymorphism is associated with post-

traumatic stress disorder and functional outcome following mild traumatic brain injury," *J. Clin. Neurosci.*, vol. 35, pp. 109–116, 2017.

[59] S. Sannino *et al.*, "Adolescence is the starting point of sex-dichotomous COMT genetic effects," *Transl. Psychiatry*, vol. 7, no. 5, 2017.

[60] C. O. Val *et al.*, "Val 158 Met Genotype and Neural Mechanisms Related to Affective Arousal and Regulation," *Imaging*, vol. 63, no. 12, pp. 1396–406, 2006.

[61] A. Heinz and M. N. Smolka, "The effects of catechol O-methyltransferase genotype on brain activation elicited by affective stimuli and cognitive tasks.," *Rev. Neurosci.*, vol. 17, no. 3, pp. 359–67, 2006.

[62] M. Koenigs *et al.*, "Focal brain damage protects against post-traumatic stress disorder in combat veterans," *Nat. Neurosci.*, vol. 11, no. 2, pp. 232–237, 2008.

[63] H. Y. Tan, J. H. Callicott, and D. R. Weinberger, "Dysfunctional and compensatory prefrontal cortical systems, genes and the pathogenesis of schizophrenia," *Cereb. Cortex*, vol. 17, no. SUPPL. 1, pp. 171–181, 2007.

[64] D. M. Barch and A. Ceaser, "Cognition in schizophrenia: Core psychological and neural mechanisms," *Trends Cogn. Sci.*, vol. 16, no. 1, pp. 27–34, 2012.

[65] B. Lutz, G. Marsicano, R. Maldonado, and C. J. Hillard, "The endocannabinoid system in guarding against fear, anxiety and stress," *Nat. Rev. Neurosci.*, vol. 16, no. 12, pp. 705–718, 2015.

[66] M. Morena *et al.*, "Endogenous cannabinoid release within prefrontal-limbic pathways affects memory consolidation of emotional training," *Proc. Natl. Acad. Sci.*, vol. 111, no. 51, pp. 18333–18338, 2014.

[67] J. F. Oliveira da Cruz, L. M. Robin, F. Drago, G. Marsicano, and M. Metna-Laurent, "Astroglial type-1 cannabinoid receptor (CB1): A new player in the tripartite synapse," *Neuroscience*, vol. 323, pp. 35–42, 2016.

[68] C. D. Paspalas, "Presynaptic D1 Dopamine Receptors in Primate Prefrontal Cortex: Target-Specific Expression in the Glutamatergic Synapse," *J. Neurosci.*, vol. 25, no. 5, pp. 1260–1267, 2005.

[69] J. K. Seamans and C. R. Yang, "The principal features and mechanisms of dopamine modulation in the prefrontal cortex," *Prog. Neurobiol.*, vol. 74, no. 1, pp. 1–57, 2004.

[70] H. C. Bergstrom, "The neurocircuitry of remote cued fear memory," *Neurosci. Biobehav. Rev.*, vol. 71, pp. 409–417, 2016.

[71] S. Duvarci and D. Pare, "Amygdala microcircuits controlling learned fear," *Neuron*, vol. 82, no. 5, pp. 966–980, 2014.

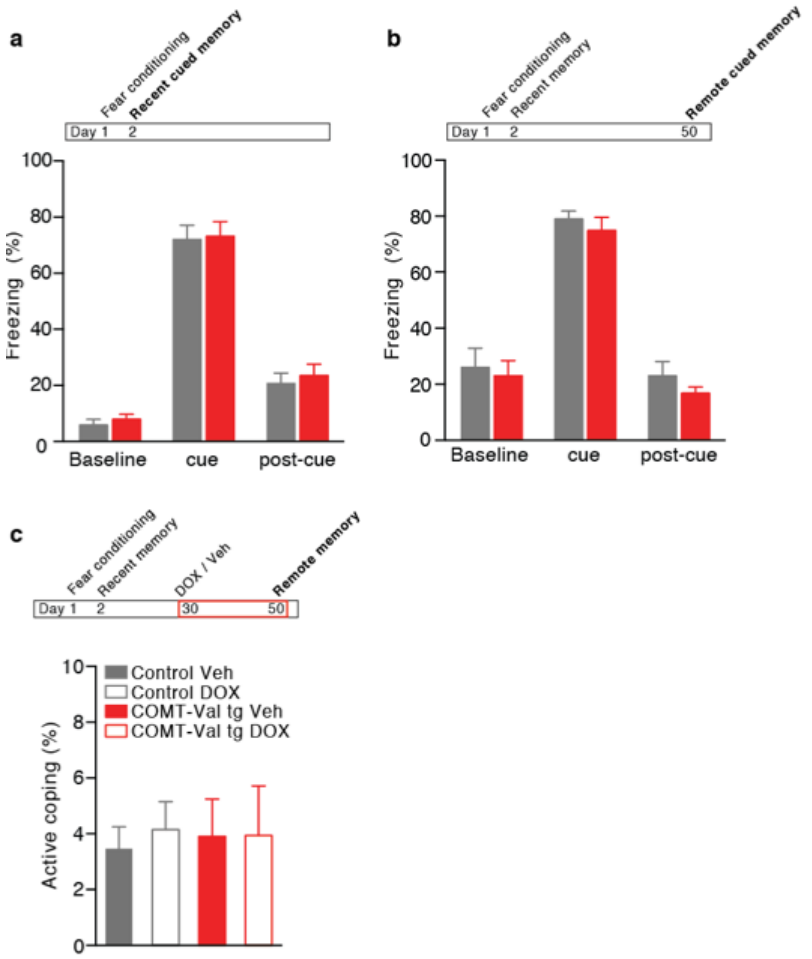
[72] S. Tanimoto, T. Nakagawa, Y. Yamauchi, M. Minami, and M. Satoh, “Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats,” *Eur. J. Neurosci.*, vol. 18, no. 8, pp. 2343–2350, 2003.

[73] M. N. Smolka, “Catechol-O-Methyltransferase val158met Genotype Affects Processing of Emotional Stimuli in the Amygdala and Prefrontal Cortex,” *J. Neurosci.*, vol. 25, no. 4, pp. 836–842, 2005.

[74] H. Tan, N. M. Lauzon, S. F. Bishop, N. Chi, M. Bechard, and S. R. Laviolette, “Cannabinoid Transmission in the Basolateral Amygdala Modulates Fear Memory Formation via Functional Inputs to the Prelimbic Cortex,” *J. Neurosci.*, vol. 31, no. 14, pp. 5300–5312, 2011.

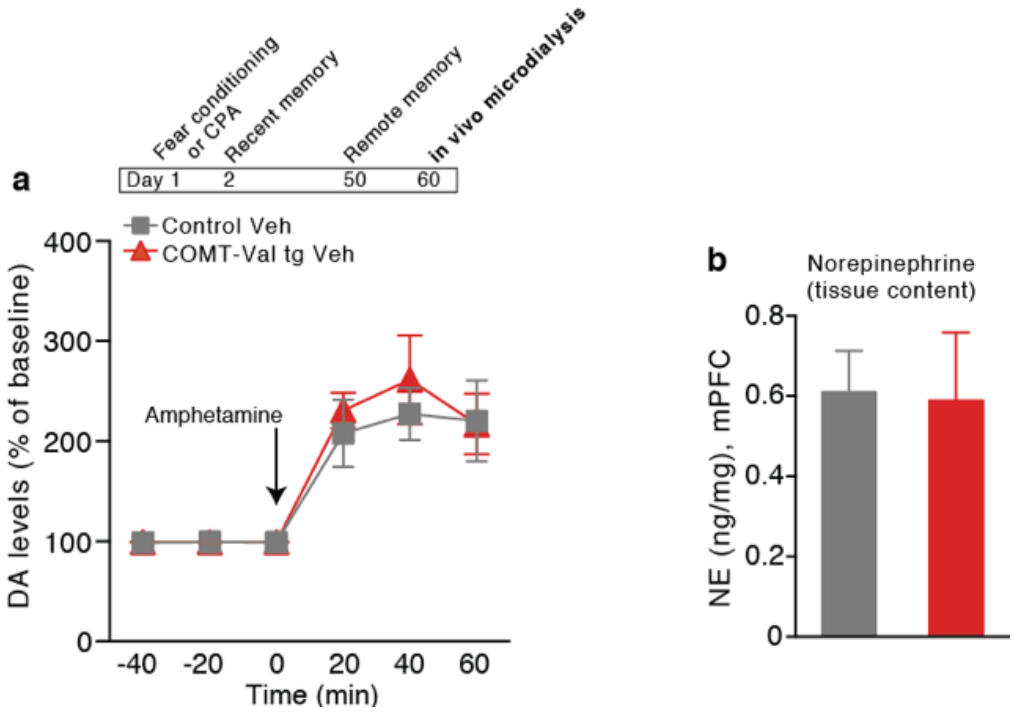
4.8. Supplementary figures

4.8.1 Supplementary figure 1



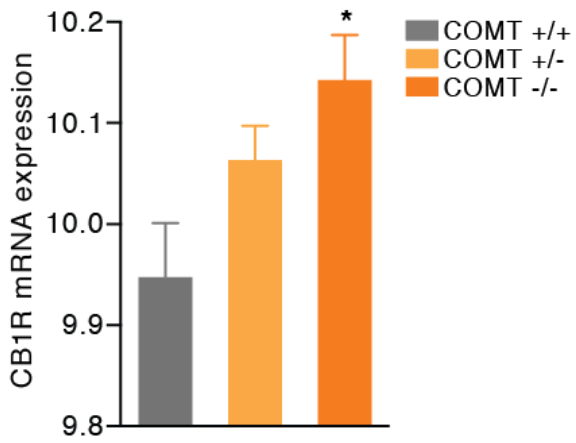
Supplementary Figure 1. COMT and CB1R blockade did not affect proactive behavior. (a), Cued fear memory recall 24 hours after fear conditioning in control and COMT-Val tg mice. We did not find differences in freezing behavior during baseline, presentation of the cue and post-cue period (two-way RM ANOVA: genotype x time, $F_{(2,34)}$= 0.02, p= 0.97; n=8-11 each group). (b), Cued fear memory recall 50 days after fear conditioning. Freezing behavior was similar during baseline, presentation of the cue and post-cue period in control and COMT-Val tg mice (two-way RM ANOVA: genotype x time, $F_{(2,34)}$= 0.06, p= 0.93; n=8-11 each group). (c), Proactive behavior on remote memory recall after Veh or DOX treatment (n=9-12 each group). No differences between controls and COMT-Val tg mice (two-way ANOVA: genotype x treatment, $F_{(1,38)}$= 0.05, p= 0.80).

4.8.2 Supplementary figure 2



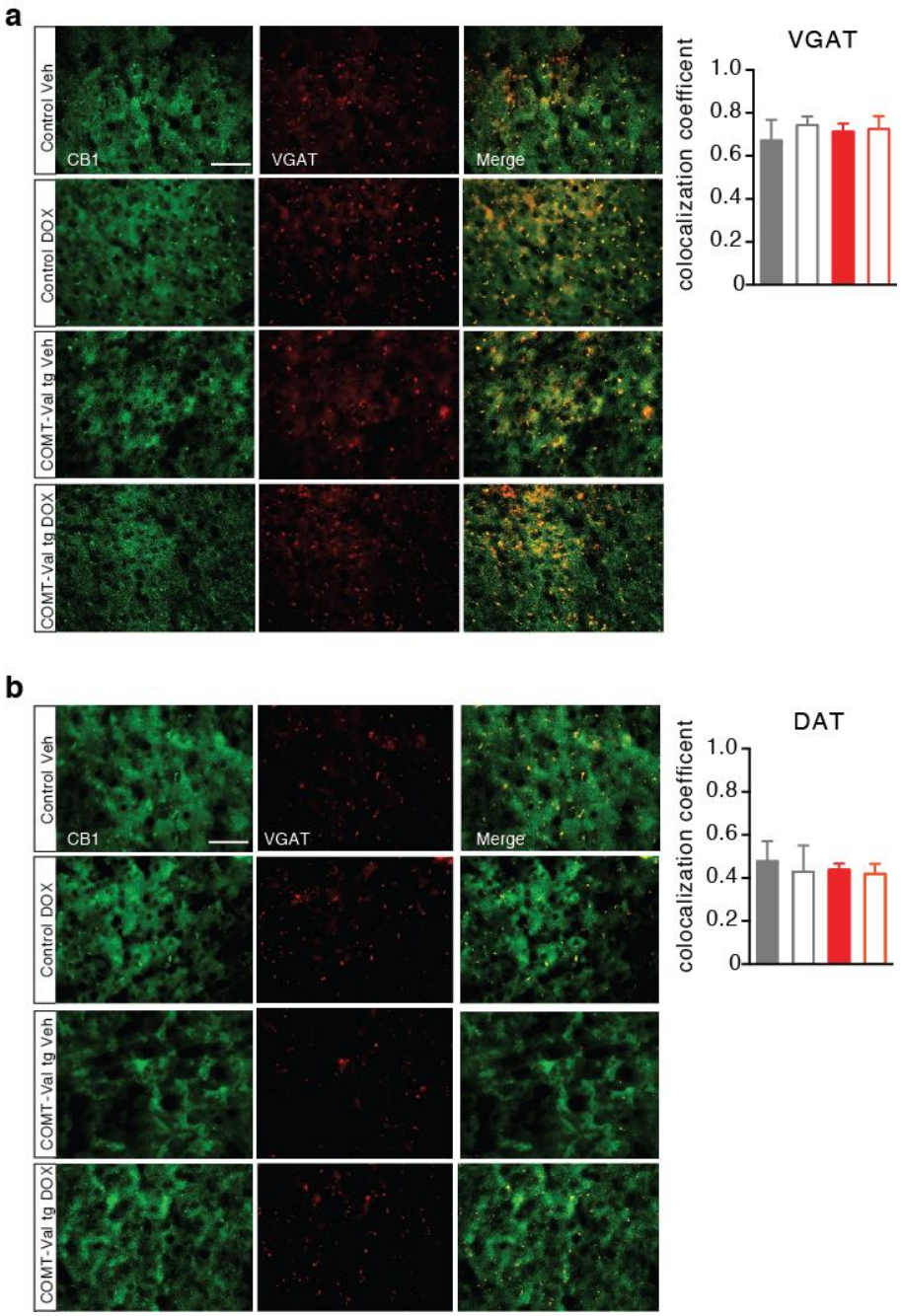
Supplementary Figure 2. Increased COMT enzymatic activity did not affect synaptic release of dopamine and norepinephrine levels in the mPFC. (a), Amphetamine-induced (1.5 mg/kg, i.p.) dopamine release in the mPFC of control (n=7) and COMT-Val-tg (n=7) mice. We found no differences between groups over a period of 60 minutes after amphetamine injection (two-way RM ANOVA: $F_{(1,10)}$= 0.19, p= 0.66). (b), Total mPFC content of norepinephrine in control (n=6) and COMT-Val-tg (n=8) mice (unpaired t-test: t=0.28, df=12, p=0.78).

4.8.3 Supplementary figure 3



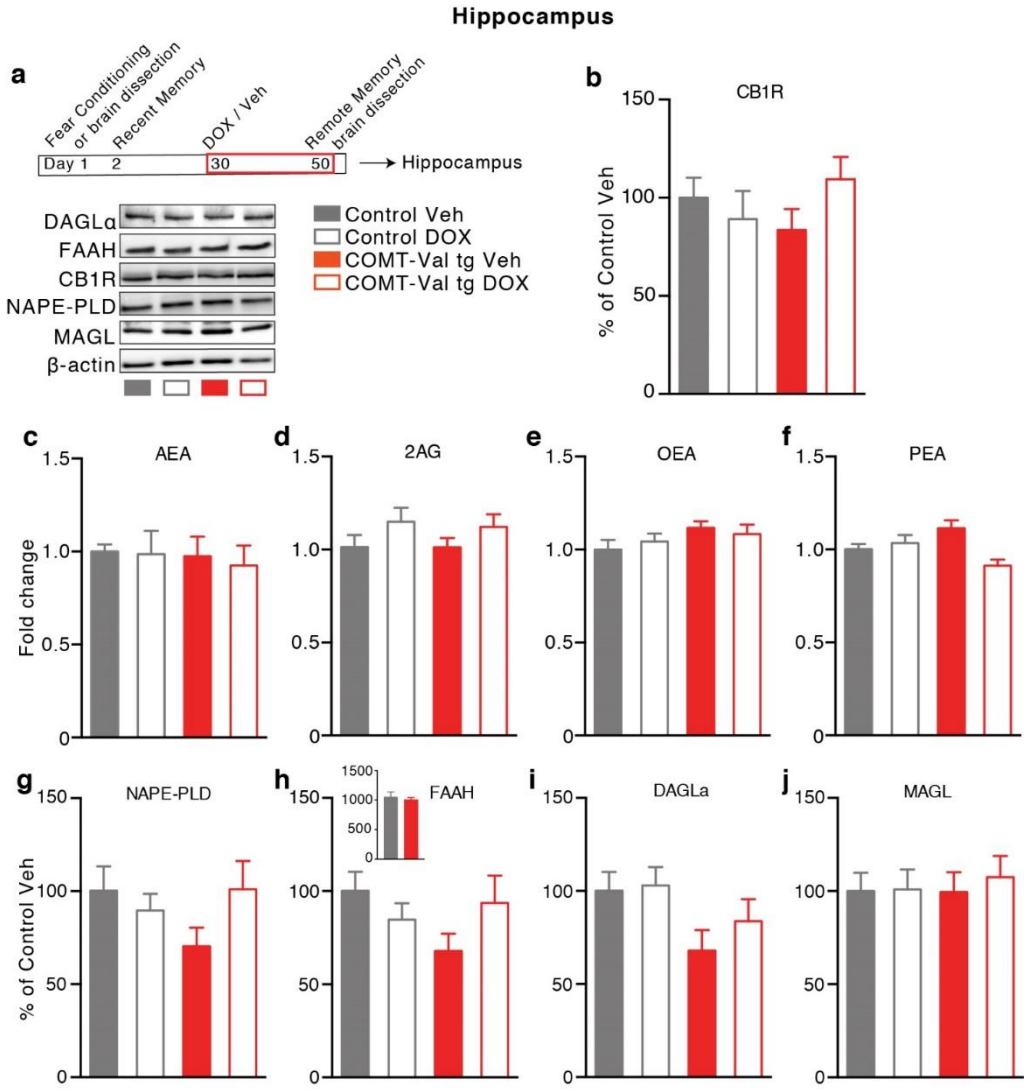
Supplementary Figure 3. CB1R were increased in COMT knock-out mice. Increase of CB1R gene expression in the mPFC of COMT knock-out mice (-/-, n=6) compared to wild-type controls (+/+, n=6; one-way ANOVA: $F_{(2,14)}$= 4.42, p<0.05). *p< 0.05 vs COMT +/+.

4.8.4 Supplementary figure 4



Supplementary Figure 4. CB1R reduction in COMT-Val tg mice did not involve dopaminergic and GABAergic terminals in the PFC. (a), Immunohistochemistry on sections of PFC for CB1 receptor (green) and vesicular GABA transporter (VGAT, red), and colocalization coefficient for controls and COMT-Val-tg mice after Veh or DOX treatment. (b), Immunohistochemistry on sections of PFC for CB1 receptor (green) and dopamine transporter (DAT, red), and colocalization coefficient for controls and COMT-Val-tg mice after Veh or DOX treatment.

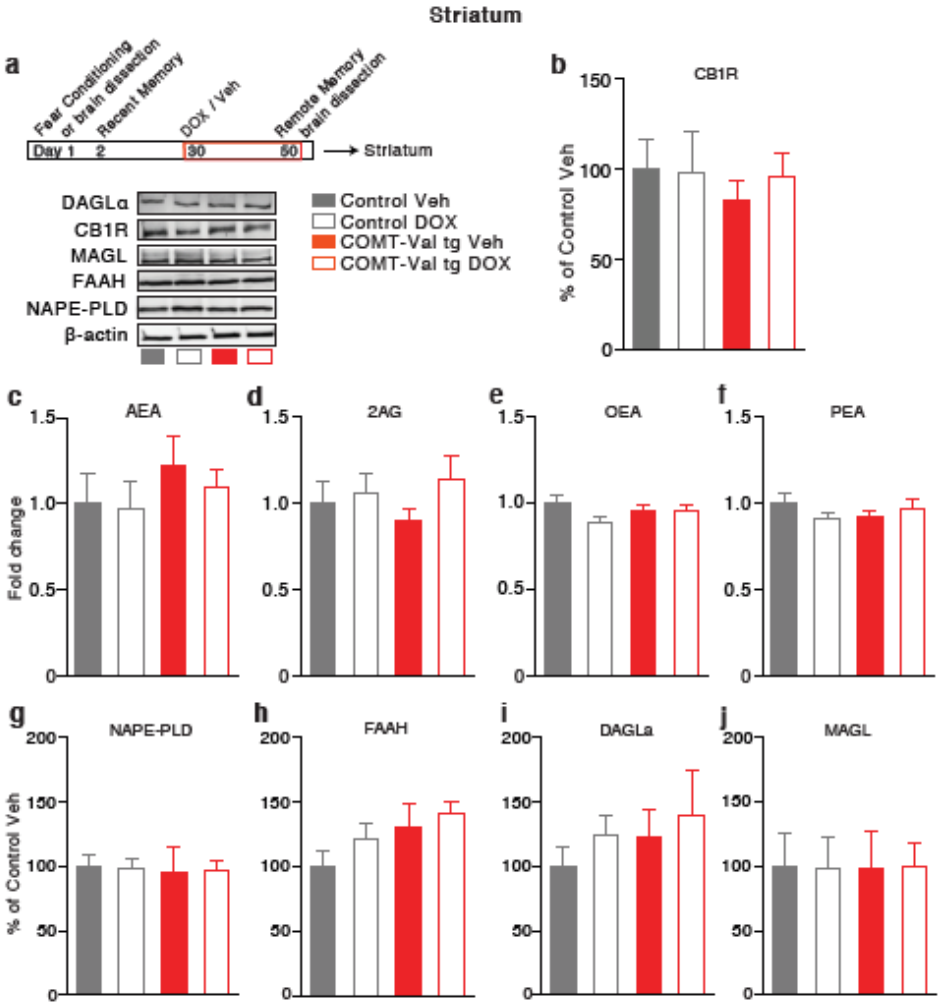
4.8.5 Supplementary figure 5



Supplementary Figure 5. Increased COMT activity did not alter the endocannabinoid system in the hippocampus. (a), Representative western blot images of DAGL-a, FAAH, CB1R, NAPE-PLD and MAGL in the Hippocampus of COMT-Val-tg and control mice after Veh or DOX treatment. (b), No change of CB1R (two-way ANOVA: genotype x treatment, $F_{(1,29)}$=2.34, p= 0.13) in the hippocampus of COMT-Val-tg (Veh n=7, DOX n=8) and controls (Veh n=9, DOX n=9) after the remote memory recall. (c), No change of AEA in the hippocampus of COMT-Val-tg (Veh n=10, DOX n=5) and controls (Veh n=9, DOX n=6) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,26)}$= 0.03, p= 0.85). (d), No change of 2AG in the hippocampus of COMT-Val-tg (Veh n=10, DOX n=5) and controls (Veh n=9, DOX n=6) after the remote memory recall 2AG (two-way ANOVA: genotype x treatment, $F_{(1,26)}$= 0.03, p= 0.84). (e), No change of OEA in the hippocampus of COMT-Val-tg (Veh n=10, DOX n=5) and controls (Veh n=9, DOX n=6) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,26)}$= 0.61, p= 0.43). (f), No change of PEA in the hippocampus of COMT-Val-tg (Veh n=10, DOX n=5) and controls (Veh n=9, DOX n=6) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,26)}$= 7.90, p= 0.91). (g), No change of NAPE-PLD in the hippocampus of COMT-Val-tg

(Veh n=6, DOX n=9) and controls (Veh n=9, DOX n=9) after the remote memory recall (two-way ANOVA: genotype x treatment , $F_{(1,29)}$= 2.59, p= 0.11). (h), No change of FAAH in the hippocampus of COMT-Val tg (Veh n=7, DOX n=9) and controls (Veh n=9, DOX n=9) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,30)}$=3.25, p=0.08). Inset, no change of FAAH enzymatic activity (expressed as pmol/min/mg; unpaired t-test: t=0.40, df=13, p=0.68). (i), No change of DAGL-a in the hippocampus of COMT-Val-tg (Veh n=7, DOX n=8) and controls (Veh n=9, DOX n=8) after the remote memory recall (two-way ANOVA: genotype x treatment , $F_{(1,28)}$= 0.35, p= 0.55). (j), No change MAGL in the hippocampus of COMT-Val-tg (Veh n=7, DOX n=9) and controls (Veh n=9, DOX n=9) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,30)}$= 0.10, p= 0.74).

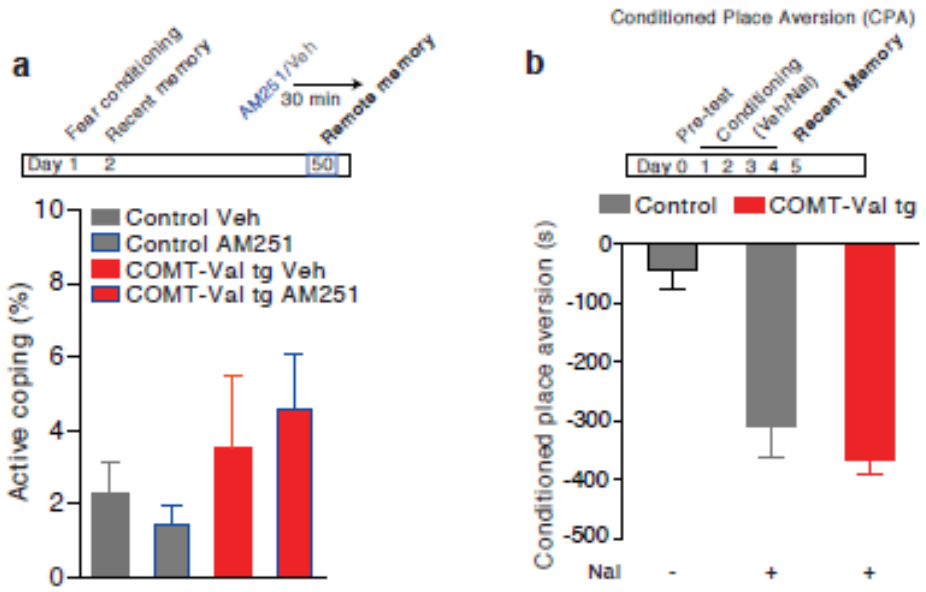
4.8.6 Supplementary figure 6



Supplementary Figure 6. Increased COMT activity did not alter the endocannabinoid system in the striatum. (a), Representative western blot images of DAGL-a, FAAH, CB1R, NAPE-PLD and MAGL in the striatum of COMT-Val-tg and control mice after Veh or DOX treatment. (b), No change of CB1R (two-way ANOVA: genotype x treatment, $F_{(1,14)}$= 0.19, p= 0.66) in the striatum of COMT-Val-tg (Veh n=4, DOX n=5) and control mice (Veh n=4, DOX n=5) after the remote memory recall. (c), No change of AEA in the hippocampus of COMT-Val-tg (Veh n=8, DOX n=5) and control mice (Veh n=6, DOX n=6) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,21)}$= 0.10, p= 0.75). (d), No change of 2AG in the striatum of COMT-Val-tg (Veh n=8, DOX n=5) and controls (Veh n=7, DOX n=6) after the remote memory recall 2AG (two-way ANOVA: genotype x treatment, $F_{(1,22)}$= 0.68, p= 0.41). (e), No change of OEA in the striatum of COMT-Val-tg (Veh n=8, DOX n=5) and controls (Veh n=7, DOX n=6) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,22)}$= 1.80, p= 0.19). (f), No change of PEA in the striatum of COMT-Val-tg (Veh n=8, DOX n=5) and controls (Veh n=7, DOX n=6) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,22)}$=2.00, p=0.17). (g), No change of NAPE-PLD in the striatum of COMT-Val-tg (Veh n=4, DOX n=5) and controls (Veh n=4, DOX n=5) after the remote memory recall (two-way ANOVA: genotype x treatment,

$F_{(1,14)}$=0.01, p=0.89). (h), No change of FAAH in the striatum of COMT-Val-tg (Veh n=4, DOX n=5) and controls (Veh n=4, DOX n=5) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,14)}$=0.12, p=0.72). (i), No change of DAGL-a in the striatum of COMT-Val-tg (Veh n=4, DOX n=5) and controls (Veh n=4, DOX n=5) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,14)}$=0.02, p=0.87). (j), No change of MAGL in the striatum of COMT-Val-tg (Veh n=4, DOX n=5) and controls (Veh n=4, DOX n=5) after the remote memory recall (two-way ANOVA: genotype x treatment, $F_{(1,14)}$=0.00, p=0.93).

4.8.7 Supplementary figure 7



Supplementary Figure 7. (a), Proactive behavior on remote memory recall after Veh or AM251 treatment (n=6-8 each group). No differences between control and COMT-Val-tg mice (two-way ANOVA: genotype x treatment, $F_{(1,24)}$= 0.38, p= 0.53). (b), Nal administration induced conditioned place aversion 24 hours after conditioning (one-way ANOVA: treatment, $F_{(2,23)}$= 1.79, p< 0.0005 vs Veh). No difference between COMT-Val-tg and control mice (p=0.38).

Chapter 5

Emotion Recognition in Mice: involvement of endocannabinoid system

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Abstract

Social cognition is a fundamental ability that influences development, survival and evolution of animals. Human social cognition is assessed measuring the ability to recognize others' emotions, a function that remains elusive for laboratory animals. Here, we revealed that mice are able to discriminate unfamiliar conspecifics based on negative- or positive-valence emotional states. This process was distinct from sociability or observational transfer of emotions and was differently dependent on olfactory and visual cues. We showcase the value of the mouse emotion recognition paradigm unraveling the role of endocannabinoid system in social cognition.

5.1. Introduction

Social interactions are fundamentally dependent on the ability to distinguish expression of emotions in others. This biologically innate process defined as "social cognition" has profound implications in everyone's life [1], [2]. Consistently, disturbances in social cognition are early and distinctive features of many neuropsychiatric, neurodevelopmental and neurodegenerative disorders [2]. Abnormalities in social cue identification define autism spectrum disorders [3], and patients with schizophrenia have marked impairments in processing non-verbal social affective information while showing normal affect sharing and emotion experience [4]. Notably, social cognitive impairments in these individuals have a more deleterious impact on daily functioning than non-social cognitive deficits [5]. Emotion recognition tasks are the most-extensively used paradigms to assess human social cognition [2], [4]. Indeed, the ability to "read" others' emotions is fundamental to achieve more complex and high-level social processes. In agreement, several training programs targeting facial emotion perception have been implemented for individuals with schizophrenia and autism [6], [7]. Social emotion recognition abilities are also evident in non-human primates, as well as in dogs and sheep [8]. Despite this, there is no evidence that mice, the most widely used laboratory animals, might be able to discriminate individuals on the base of their emotional state. Previous studies identified the existence of a transmission of pain/fear responses from one rodent to a familiar observer, a process possibly related to affect sharing and empathy [9]-[12], and thought to be an automatic response [13]. However, the cognitive processes by which rodents discriminate conspecifics emotional states are poorly evaluated. In this study, we developed a new method to investigate emotion discrimination abilities in mice. We reliably showed measurable indices of mice discrimination of negative and positive emotions evoked in unfamiliar conspecifics. These measures were disconnected from any possible sign of automatic mimicry, contagion or basic social exploration processes. The initial application of the mouse emotion recognition task established the role of endocannabinoid system in social cognition. In fact, starting from evidence from fMRI studies that showed deficits in emotion recognition task in cannabis users [14]-[16], we hypothesized an involvement of CB1 receptors in this set of abilities. In particular, CB1 receptors within the astrocytes have been indicated to have profound effects in behavioral outputs [30]. More generally, astrocytes play a central role in controlling synapse formation and maturation, but also in the modulation of many aspects of synapses physiology, network activity, and cognitive functions [17]. In the context of emotion recognition, a brain region of particular interest indicated by several human studies is the prefrontal cortex (PFC),

which is thought to regulate the top down control of emotion recognition abilities. Intriguingly, within the PFC, astrocytes have been recently reported to have a major and peculiar impact in dopaminergic homeostasis [18]. Thus, according with our data about the selective interaction between dopaminergic and endocannabinoid system within the PFC in the modulation of cognitive functions [19], we hypothesized that a CB1 dysfunction in prefrontal astrocytes might lead to 'social' deficits. In order to investigate the role of astrocytic CB1R we decided to remove the receptor selectively in prefrontal astrocytes using CB1 floxed mice. Altogether, our results demonstrate the potential of this method to increase our knowledge of the mechanisms underlying social cognition in rodents, with a translational perspective.

5.2. Materials and Methods

All procedures were approved by the Italian Ministry of Health (permits n. 230/2009-B and 107/2015-PR) and local Animal Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the European Community Council Directives. Males and females C57BL/6J mice, and CB1 floxed mice all of 3-6 month-old were used. Animals were housed two to four per cage in a climate-controlled (22±2 C) and specific pathogen free animal facility, with *ad libitum* access to food and water throughout, a standard environmental enrichment (material for nest and cardboard house), and with a 12-hour light/dark cycle (7pm/7am schedule). Experiments were run during the light phase (within 10am-5pm). All mice were handled on alternate days during the week preceding the first behavioral testing. Female mice were visually checked for estrus cycle immediately after the test and no correlation was found between estrus status and performance in the test. Behavioral scoring was performed *a posteriori* from videos by trained experimenters blind to the manipulations of both the observers and demonstrators. A sniffing event was considered when the observer touched with the nose the demonstrators' wire cup or when the observer's nose directly touched the demonstrator.

5.2.1. Emotion recognition task.

Habituation of the mice to the testing setting occurred on three consecutive days before the first experiment; each habituation session lasted 10 minutes. Test observer mice were habituated inside a Tecniplast cage (35.5x23.5x19 cm) to a separator and two cylindrical wire cups (10.5 cm in height, bottom diameter 10.2 cm, bars spaced 1 cm apart; Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH), around which they could freely move, as occurred during the test. A cup was placed on the top of the wire cups to prevent the observer mice from climbing and remaining on the top of them. The separator (11x14cm) between the two wire cups was wide enough to cover the reciprocal view of the demonstrators while leaving the observer mice free to move between the two sides of the cage. The wire cups, separators and experimental cages were replaced with clean copies after each subject to avoid scent carryover. Similarly, the rest of the apparatus was wiped down with water and dried with paper towels for each new subject. After each testing day, the wire cups, separators, and cubicles were wiped down with 70% ethanol and allowed to air-dry. Testing cages were autoclaved as standardly performed in our animal facility. Demonstrator mice – matched by age, sex and genotype to the observers – were habituated inside the same Tecniplast cage (35.5x23.5x19 cm), under the

wire cups for three consecutive times, ten minutes each. During both habituation and behavioral testing, the cages were placed inside soundproof cubicles (TSE Multi Conditioning Systems) homogeneously and dimly lit (6±1 lux) to minimize gradients in light, temperature, sound and other environmental conditions that could produce a side preference. Digital cameras (imaging Source DMK 22AUC03 monochrome) were placed facing the long side of the cage and on top of the cage to recorded from different angles the three consecutive two-minute trials, using the program Anymaze (Stoelting).

5.2.1.1.Observers.

Before the test, mice were habituated to the experimental setting as reported above. The third day of habituation, mice were also habituated to the tone cue (4 kHz, 80 dB sound pressure level, three times for 30 seconds each with an intertrial interval of 90 seconds) without any conditioning. One hour prior to behavioral testing, mice were placed in the testing cage, in experimental setting (i.e. separator and two wire cups), in a room adjacent to the testing room. Five minutes before the experiment, the testing cages containing the observer mice were gently moved in the testing cubicles. After having placed one emotionally 'neutral' and one "emotionally altered" demonstrator mice under the wire cups, the 6-minute experiment began. The order of insertion of the neutral or emotionally-altered demonstrator was randomly assigned.

5.2.1.2. 'Neutral' demonstrators.

In the days before the test, all neutral mice were habituated to the experimental setting as reported above. For the "relief" condition, neutral demonstrators underwent no manipulation the day before the test. For the "fear" condition, the day before the test, neutral demonstrators were habituated to the tone cue inside the cups as for the experimental setting and as done for the observer mice. The day of the test, neutral demonstrators were brought inside their home cages in the experimental room one hour before the experiment began. Demonstrators were test-naïve and used only once. In some cases, we re-used the same demonstrator for maximum two/three times, with always at least one week between each consecutive test. No differences were observed in the performance of the observer mice depending on the demonstrators' previous experience.

5.2.1.3. 'Relief' demonstrators.

The days before the test, mice were habituated to the experimental setting as reported above. 'Relief' demonstrators were then water deprived 23 hours before the experiment. One hour before the test *ad libitum* access to water was reestablished, and mice were brought inside the experimental room in their home cages. Food was *ad libitum* all the time and some extra pellets were put inside the home cage during the 1-hour water reinsertion.

5.2.1.4. 'Stressed' demonstartors

The days before the test, mice were habituated to the experimental setting as reported above. "Stress" demonstrators were put in the restrainer 15 minutes before the task. Stressed mice were put in the restrainer only once. In the rare case of a second exposure to the test, these demonstrators were re-exposed to the restrainer at least one week apart from the previous exposure.

5.2.1.5'Fear' demonstrators.

The days before the test, mice were habituated to the experimental setting as reported above. 'Fear' demonstrators were fear conditioned using the parameters and context previously described[23], and using the same tone delivered to the observers and neutral demonstrators during their habituation process. In particular, the conditioned stimulus was a tone (4 kHz, 80 dB sound pressure level, 30 sec) and the unconditioned stimulus were three scrambled shocks (0.7 mA, 2-s duration, 90-s intershock interval) delivered through the grid floor that terminated simultaneously with the tone (2 sec). The day of the test these mice were habituated, inside their home cages, in a room adjacent to the testing room for one hour prior to the test; they were consequently brought inside the experimental room one by one, before placing them under their designated wire cup. Fear mice were conditioned only once and in a separate room and apparatus (Ugo Basile SRL, Italy) respect to where the emotion recognition task would be performed. In the rare case of a second exposure to the test, these demonstrators were just re-exposed to the same conditioned tone, at least one week apart from the previous exposure and maximum 1 month from the initial conditioning.

5.2.1.6. Shock" demonstrators.

This manipulation was performed for direct comparison with a rat protocol and was performed as previously described [20]. In particular, these demonstrator mice were exposed to two

footshocks (1 mA, 5-s duration, 60-s intershock interval) immediately before the 6-minute test session. All other procedures were identical to the other demonstrators as described above.

5.2.1.7. Habituation- dishabituation task.

Social interaction in freely interacting mice was tested as previously reported [21]. Briefly, mice were individually placed in the testing cage 1 h prior to the testing. No previous single housing manipulation was adopted to avoid any instauration of home-cage territory and aggressive behaviors. Habituation – dishabituation task consisted of 5 trials (1 minute each), with 3 minutes interval lasted from each trial. Testing began when a stimulus mouse (S1), matched for sex and age, was introduced into the testing cage for the first 1-min period interaction. S1 mouse was showed to the test mouse for four time. A new stimulus mouse (S2), matched for sex and age, and was introduced during trial 5. During behavioral testing, the cages were placed inside soundproof cubicles (TSE Multi Conditioning Systems) homogeneously and dimly lit (5±1 lux) to minimize gradients in light, temperature, sound and other environmental conditions that could produce a side preference. Digital cameras (imaging Source DMK 22AUC03 monochrome) were placed facing the long side of the cage and on top of the cage to recorded from different angles the five 1 minute trials, using the program Anymaze (Stoelting).

5.2.1.8. One-on-one social exploration tests.

This test was similarly performed as previously described[3]. One hour prior to behavioral testing, each experimental subject was placed into a Tecniplast cage (35.5x23.5x19 cm) with shaved wood bedding and a wire lid, in a room adjacent to the testing room. Five minutes before the experiment, the testing cages containing the observer mice were gently moved in the testing sound proof cubicles. To begin the test a demonstrator mouse was introduced to the cage for 6 minutes (as for the emotion recognition task), and exploratory behaviors initiated by the test subject were timed by two independent experimenters blind to the manipulations. Demonstrators mice were used only once. Each observer was given tests on consecutive days: once with an unfamiliar naive conspecific, once with an unfamiliar fear conspecific (fear conditioning exactly as above), once with an unfamiliar relief conspecific (manipulated exactly as above), and once an unfamiliar stressed conspecific (stressed as previously explained). Test order was counterbalanced.

5.2.1.9. Sensory modality assessment.

In the "complete darkness" experiments, mice were tested as above, but eliminating all sources of light within the testing cage as well as in the entire testing room. Videos were recorded for successive scoring either with an infrared thermal camera (FLIR A315, FLIR Systems) or with Imaging Source DMK 22AUC03 monochrome camera (Ugo Basile). The two cameras setting gave the same experimental results. For acoustic stimuli experiments, ultrasonic vocalisations (USVs) were recorded during the test phases performed as above in two different experimental settings: 1) exactly as above with one observer mouse and two demonstrators, and 2) with only one demonstrator for each emotional condition present in the apparatus. This was done to make sure that the USVs recorded could be attributed to a single emotional state and/or to a communication between demonstrators and observer. The ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany), sensitive to frequencies between 10 and 180 kHz, was mounted 20 cm above the cage to record for subsequent scoring of USV parameters. Vocalisations were recorded using AVISOFT RECORDER software version 3.2. Settings included sampling rate at 250 kHz; format 16 bit. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (Version 4.40) and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated with an FFT-length of 1024 points and a time window overlap of 75% (100% Frame, Hamming window). The spectrogram was produced at a frequency resolution of 488 Hz and a time resolution of 1 ms. A lower cut-off frequency of 15 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. Call detection was provided by an automatic threshold-based algorithm and a hold-time mechanism (hold time: 0.01 s). An experienced user checked the accuracy of call detection, and obtained a 100% concordance between automated and observational detection. Parameters analysed for each test day included number of calls and duration of calls. Quantitative analyses of sound frequencies measured in terms of frequency and amplitude at the maximum of the spectrum were not performed because of the paucity of emitted USVs in all conditions performed. For odor stimuli experiments, observers were tested as described above, but presenting as "demonstrator" only cotton balls impregnated with the odor of demonstrators. Odors were separately collected from neutral, relief (after the 1 hour *ad libitum* access to water), fear (immediately after the delivery of the conditioned tone cue) and stressed (immediately after 15 minutes in the restrained) demonstrators by gently brushing the cotton ball all over the body of the mice (especially including the nose, body and anogenital parts). Odors were taken from one single mouse, which was not reused. Odors were used only once and always freshly taken.

5.2.2. Place conditioning.

The place conditioning paradigm was performed in a rectangular Plexiglas box (length, 42 cm; width, 21 cm; height, 21 cm) divided by a central partition into two chambers of equal size (21×21×21 cm) as previously described[22]. One compartment had black walls and a smooth Plexiglas floor, whereas the other one had vertical black and white striped (2 cm) walls and a slightly rough floor. During the test sessions, an aperture (4×4 cm) in the central partition allowed the mice to enter both sides of the apparatus, whereas during the conditioning sessions the individual compartments were closed off from each other. To measure time spent in each compartment a video tracking system (Anymaze) was used. The place conditioning experiment lasted 5 days and consisted of three phases: preconditioning test, conditioning phase and post conditioning test. On day 1, each mouse was allowed to freely explore the entire apparatus for 20 min, and time spent in each of the two compartments was measured (preconditioning test). Conditioning sessions took place on days 2 and 4. Mice were divided in two groups: neutral and relief. Mice of the same home- cage were assigned to the same group. As for the same manipulation in the emotion recognition test, the relief group was assigned to receive a 23-hour water deprivation period before the two conditioning sessions on the day 2 and 4, when they were confined with their cage mates in one of the two compartments for 1 hour with free access to water and food (conditioning). Food in the home cage was available all time. Other than the two 23-hr deprivation periods, water was available all time. The neutral group was exposed to the same procedure but without any water deprivation periods. Post conditioning test was performed on day 5 in the same condition of preconditioning test. For each mouse, a conditioning score was calculated as the post conditioning time minus the preconditioning time (in seconds) spent in the conditioning compartment of the apparatus.

5.2.3. Corticosterone assay

Corticosterone concentration was analyzed from mice plasma. Immediately after the behavioral test, each mouse was sacrificed by decapitation. The blood was quickly collected in EDTA(0,5M)-coated tubes and centrifuged at 2500 rpm for 10 min; the supernatant obtained was stored at -20°C until the assay. The corticosterone concentration was detected by a commercially available Detect X® corticosterone enzyme-linked immunoassay (ELISA) kit (Arbor Assays, MI, USA; Cat N K014-H1) following the manufacturer's protocol. The level of corticosterone was expressed as fold changes compared to the control group average.

5.2.4. Stereotaxic Injections.

Mice were anesthetized with 2% isoflurane in O2 by inhalation and mounted into a stereotaxic frame (Kopf) linked to a digital reader. Mice were maintained on 1.5 - 2% isoflurane during the surgery. Brain coordinates of injections were chosen in accordance to the mouse brain atlas (Paxinos and Watson, 1998): mPFC (AP: +1.9 mm; L: ± 0.25 mm; DV: -2.5 mm). Mice had been injected with AAV-GFAP-mCherry-egfp (control) or AAV-GFAP-mCherry-Cre virus and were allowed 1 month to recover and for the viral transgenes to adequately express before undergoing behavioral experiments. The injected volume virus was 60 nl volume. Only mice with appropriate placements were included in the reported data.

5.2.5. Statistical analyses.

Results are expressed as mean+standard error of the mean (s.e.m.) throughout. Each observer behavior towards the two different demonstrator mice were analyzed using a within-groups Repeated Measures ANOVA. The behaviors of the two demonstrators were analyzed by Two-Way ANOVAs with emotional state as between-subjects factors, and the within-session 2-min consecutive intervals as a repeated measure within-subject factor. The behaviors of the observer mice in the one-on-one setting were analyzed by Two-Way ANOVAs with the emotional state of the demonstrator as between-subjects factors, and the within-session 2-min consecutive intervals as a repeated measure within-subject factor. Two or One-Way ANOVAs were used for social interactions when different genotypes and treatments were involved. Newman–Keul's post-hoc test with multiple comparisons corrections was used for making comparisons within groups when the overall ANOVA showed statistically significant differences. The accepted value for significance was $p<0.05$. Statistical analyses were performed using Statistica 13.2 (StatSoft).

5.3. Results

5.3.1. Mice are able to discriminate unfamiliar conspecifics based on negative-valence emotional states.

We hypothesized that mice could discriminate altered emotional states of unfamiliar conspecifics, showing selective behavioral responses. To test this hypothesis, we placed an "observer" mouse in a cage containing two age- and sex-matched unfamiliar conspecifics ("demonstrators"), in a wire cup that allowed visual, tactile, auditory and olfactory communication (Fig. 1a). This setting focused the task on social approach initiated by the observer mouse, similar to emotion recognition tests performed in humans [2], avoiding potential confounders resulting from aggressive or sexual interactions. In the first condition, we measured observer's ability to discriminate between a "Neutral" demonstrator and a demonstrator that was previously fear-conditioned to a tone cue, or stressed ("Fear"; Fig. 1b; "Stress; Fig 2b), enabling the induction of a negative-valence state upon the tone presentation or restrained condition. In particular, in order to compare observers' behaviors before, during and after the induction of this negative emotion, we used a 3-consecutive 2-minute trials in which the tone cue was delivered only during the second 2-minute trial (Fig. 1b). As expected, freezing response, and associated reduced rearing, were present only in the "fear" demonstrator and exclusively during the 2-minute tone presentation (Fig. 1c). No other behavioral parameters differed between the two demonstrators during the whole 6-minute test session (Fig. 1c). Thus, this design allowed to tightly link any detectable observers' behavior with the triggered alteration of the emotional state in one of the demonstrators. We found that both male and female observers displayed increased sniffing towards the fear demonstrator compared to the neutral one (Fig. 1d-e). In particular, the discriminatory sniffing was observed specifically in the last 2-minute trial, thus only after the altered emotion was triggered, but not during the tone presentation when the fear demonstrator was freezing. Observer discrimination during the last trial did not correlate with the quantity of freezing showed by the fear demonstrator, but a significant inverse correlation was evident during the 2-minute tone presentation (Fig. 1f-g). This indicate that freezing observation inhibited the observer approach during trial 2, but did not influence successive discriminatory behaviors. In light of previous evidence [10], [11], [20], [22] we searched for signs of potential fear-transfer or emotional contagion from the demonstrator to the observer, and we did not detect any. In particular, observers showed no freezing behavior, escape attempts or stress-related behaviors, no changes in rearing and

grooming (Fig. 1h) during the whole test. Similarly, no altered behaviors were evident when the observer was exposed to two neutral demonstrators. However, as expected, observers did not discriminate between two neutral demonstrators (Fig. S2). Observers' corticosterone levels did not differ when exposed to two neutral or to a neutral and a fear demonstrators (Fig. S3).

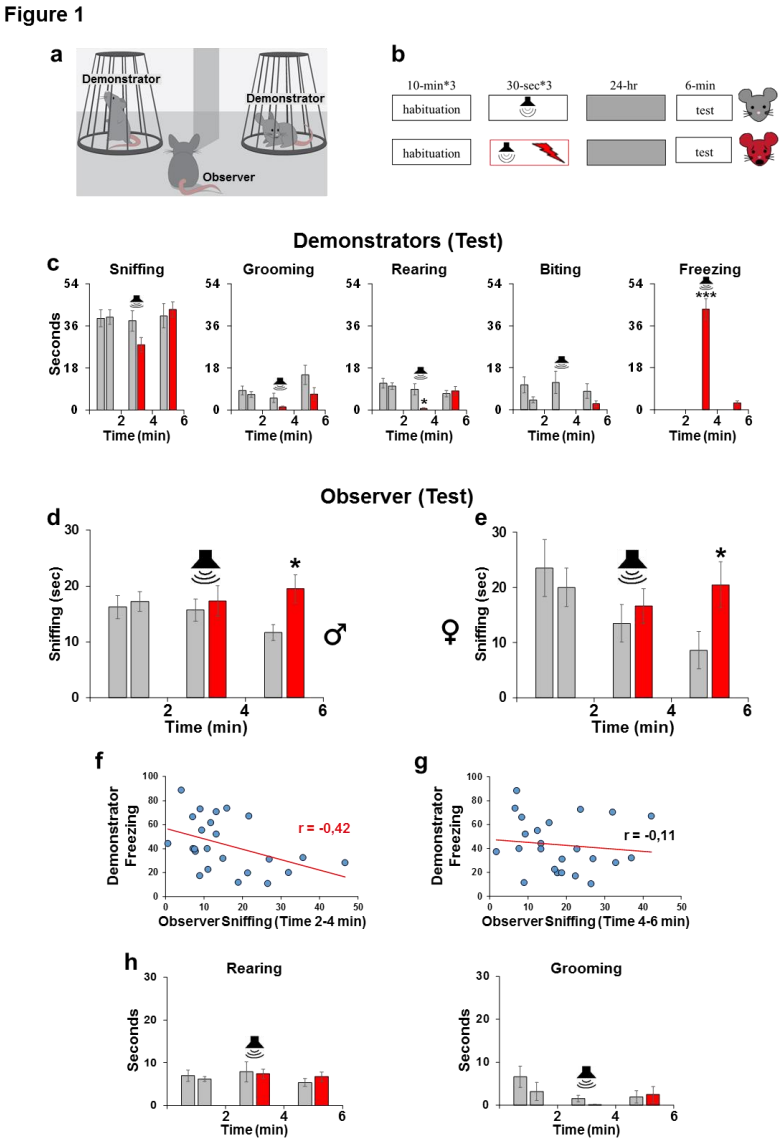


Figure 1. Mouse emotion recognition task for negative emotions. (a) Schematic drawing of the task setting. (b) Timeline of Pre-Test and Test procedures to trigger in one of the demonstrator a "fear" emotional states during the middle of the test phase. (c) Observable behaviors displayed by the neutral and fear demonstrator mice during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean. No demonstrator defecated or urinated during the whole test session. Emotion-by-time statistical interaction for sniffing ($F_{2,36}$= 2,72, p= 0.08), grooming ($F_{2,36}$=1,07, p=0.35), rearing ($F_{2,36}$=5,09, p=0.01), biting ($F_{2,36}$=1,28, p=0.29), and freezing ($F_{2,36}$=48,82, p<0.0001). *p<0.05, and ***p<0,0001 versus all other points. N=10 demonstrators per group. (d, e) Time (in seconds) spent sniffing demonstrators in neutral (grey bars) or tone-induced fearful states

(red bars) displayed by (d) male and (e) female observer mice during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean. Conditioned tone delivered between 2-4 minutes of the test (last 2-min RM ANOVA for males $F_{1,15}$=6,51, p=0.022, and females $F_{1,11}$=10,98, p=0.006). *p<0.05 versus the exploration of the neutral demonstrator. N=8-15 observers per group. (f, g) Correlation analyses between (in y axis) the time spent freezing by the fear-conditioned demonstrator and (in x axis) time spent by the observer mouse sniffing the fear-conditioned demonstrator (f) in the time 2-4 or (g) in the time 4-6 of the emotion recognition test. (h,) Other observable behaviors displayed by the same observer mice during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean.

These findings suggest that an observer mouse can evaluate and discriminate unfamiliar conspecifics based on a negative emotional state. Thus, we then investigated whether discriminatory behaviors could be detected even when negative-valence states was induced before the task. In fact, we exposed observer to a neutral demonstrator and to another that was put in the restrainer 15- minutes before the task ("Stress"; Fig. 2a-b). As expected, increased grooming response was found the "stress" demonstrators during all the 3- consecutive trials (Fig. 2c). No other behavioral differences were noticed between the two demonstrators during the whole 6-minutes test session (Fig. 2c). We found that both male and female observers showed increased sniffing towards the stress demonstrator compared to the neutral one (Fig. 2d-e). In particular, the discriminatory sniffing was observed specifically in the first 2-minute trial. Nevertheless, observer discrimination during the first trial did not correlate with the quantity of grooming showed by the fear demonstrator (Fig. 2f). Moreover, the observers did not show any other discriminatory behaviors such as changes in rearing and grooming patterns towards the demonstrators (Fig. 2g).

Figure 2

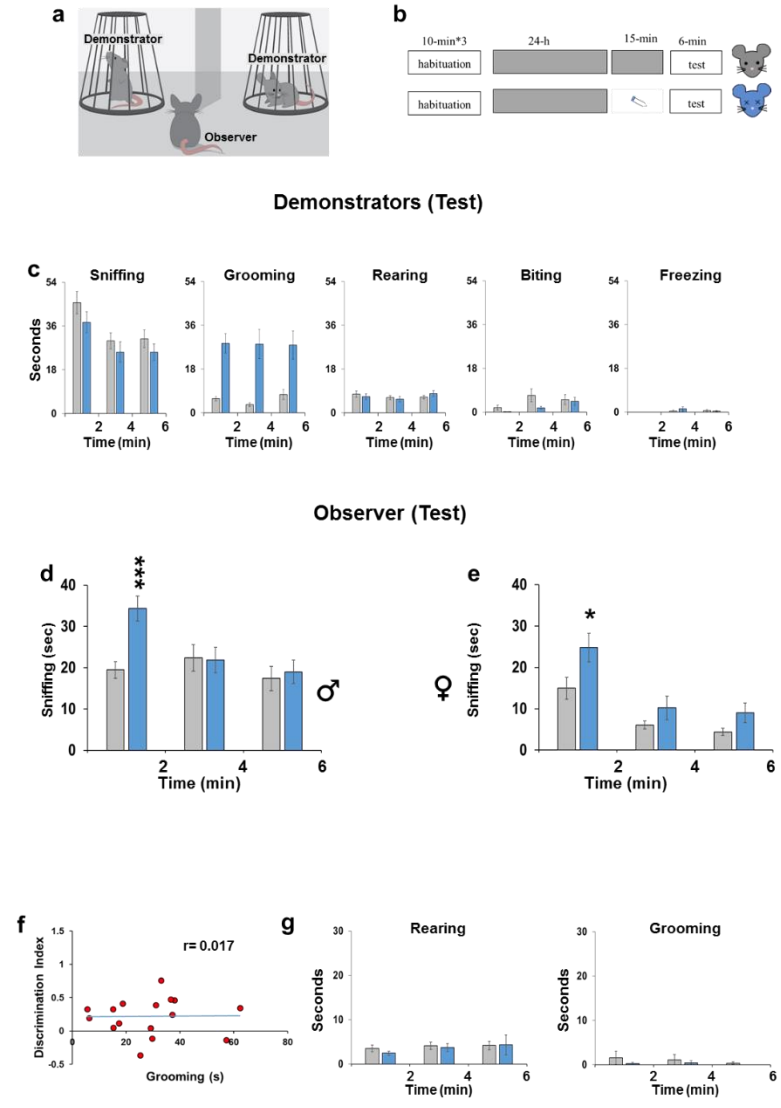


Figure 2. Mouse emotion recognition task for negative (stress) emotions. (a) Schematic drawing of the task setting. (b) Timeline of Pre-Test and Test procedures to induce in one of the demonstrator "stress" emotional states during the first phase of the task. (c) Behaviors showed by the neutral and stress demonstrator mice during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean. No demonstrator defecated or urinated during the whole test session. Emotion-by-time statistical interaction for sniffing (0-2 min: F1,15= 115.77; p= 0.09), grooming ($F_{1,15}$=74.04, p= 0.0002); 2-4 minutes: $F_{1,15}$= 27.413, p=0.001; 4-6 minutes ($F_{1.15}$=32.89 p=0.005), rearing 0-2 min: ($F_{1,15}$=70.73, p=0.57), biting 0-2 min: ($F_{1,15}$= 4.17, p=0.11), and freezing. *p<0.05, and ***p<0,0005 versus all other points. N=16 demonstrators per group. (d, e) Time (in seconds) spent sniffing demonstrators in neutral (grey bars) or restrainer induced stressful state (blue bars) displayed by (d) male and (e) female observer mice during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean (0- 2-min ANOVA for males $F_{1,17}$= 19.64, p=0.0004, and females $F_{1,12}$ = 5.76, p=0.03). *p<0.05 , ***p<0.0005 versus the exploration of the neutral demonstrator. N=13-18 observers per group. (f) Correlation analyses between (in y axis) the time spent grooming by the stress demonstrator and (in x axis) by the observer mouse sniffing the stress demonstrator (f) in the time 0-2 of the emotion recognition test. (g) Other

observable behaviors displayed by the same observer mice during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean.

5.3.2. Mice are able to discriminate unfamiliar conspecifics based on positive-valence emotional states.

Emotion recognition paradigms to assess human social cognition include the presentation of positive-valence emotions [2]. Thus, we then investigated whether discriminatory behaviors could be detected even towards positive-valence states. Observers were exposed to a neutral demonstrator and to another that received a 1-hour *ad libitum* access to water following 23-hour water deprivation (Fig. 3a-b). Water was selected as a rewarding factor to avoid odor-related cues that could differentiate the two demonstrators. We assumed that the relief from the latter distressful situation would result in a positive-valence emotional state ("relief"). Supporting this, we found that the 1-hour ad libitum access to water resulted in a conditional place preference only in mice that experienced the 23-hour water deprivation, but not in mice in the ad libitum water condition (Fig. 3c-d). We found no measurable behavioral difference between relief and neutral demonstrators (Fig. 3e). However, observers of both sexes equally showed more sniffing towards the relief demonstrator compared to the neutral, only in the first two minutes of the task (Fig. 3f-g and Fig. S1). The observers did not show any other discriminatory behaviors such as changes in rearing and grooming patterns towards the demonstrators (Fig. 3h). Moreover, observers did not show freezing behavior, escape attempts or stress-related behaviors during the entire test session. Observers' corticosterone levels did not differ when exposed to two neutral or to a neutral and a relief demonstrators (Fig. S2). These data indicate that mice are able to discriminate emotions with a positive valence in unfamiliar conspecifics.

Figure 3

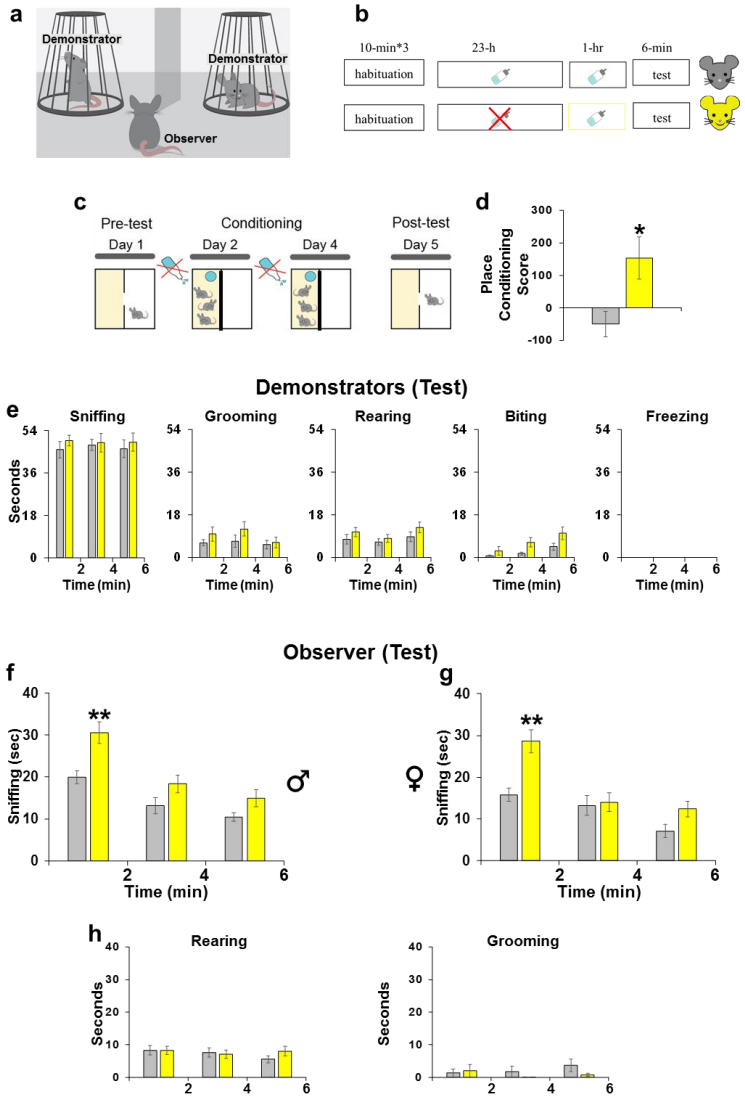


Figure 3. Mouse emotion recognition task for positive emotions. (a) Schematic drawing of the task setting. (b) Timeline of Pre-Test and Test procedures to trigger in one of the demonstrator a "relief" emotional states during the test phase. (c) Timeline of the Place Conditioning procedures used to assess if the "relief" manipulation was associated with a negative-, neutral- or positive-valence affective state. (d) Place conditioning scores (in seconds) displayed by mice conditioned during a neutral (grey bar) or relief (yellow bar) emotional state. For each mouse, a place conditioning score was calculated as the post- minus the preconditioning time spent in the conditioning-paired compartment of the apparatus. A positive score indicate place preference, a negative score a place aversion, 0 no place conditioning. N=7 per group. *p<0.05 versus the neutral group. (e) Observable behaviors displayed by the neutral and relief demonstrator mice during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean. No demonstrator defecated or urinated during the whole test session. No significant emotion-by-time statistical interaction was evident for sniffing, grooming, rearing, biting, and freezing. N=10 demonstrators per group. (f, g) Time (in seconds) spent sniffing demonstrators in neutral (grey bars) or water-induced relief state (yellow bars) displayed by (f) male and (g) female observer mice during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean (first 2-min RM ANOVA for

males $F_{1,14}$=15.07, p=0.001, and females $F_{1,14}$=14,60, p=0.001). **p<0.05 versus the exploration of the neutral demonstrator. N=15 observers per group. (h,) Other observable behaviors displayed by the same observer mice during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean.

5.3.3. Emotion discrimination abilities are a stable trait, not overlapping with sociability.

Human emotion recognition paradigms present strong test-retest reliability [23], which is a critical feature for longitudinal, drug response, psychobiological and clinical trials studies. In agreement with this human evidence, the ability to distinguish unfamiliar conspecifics based on emotional states remained unchanged when the same observer mouse was re-exposed to the same paradigm or even if the same mouse was tested in the two different paradigms (Fig. 4a). This indicated that emotion discrimination in mice is a stable trait, and that this setting is well-suited to be used for mechanicist pharmacological manipulations. Human emotion recognition tasks measure the ability to label or discriminate emotions in others, which is distinct from sociability (defined as the time spent interacting with others). Thus, we also evaluated the sociability of a mouse towards a neutral, fear, relief or stress conspecific measuring their free social interaction in one-on-one setting (Fig. 4b). We found that the levels of social exploration of the test mouse toward the subjects in neutral, negative or positive emotional states did not differ (Fig. 4b). In contrast, a recently published protocol in rats showed equivalent social interaction data in one-on-one and one-on-two settings [20]. The discrepancy might rely on the scalability feature of emotions [23]. Indeed, exposing the demonstrator immediately after the shock (Fig. 4c and [20]), generated a general aversion during the whole test session (Fig. 4c). Taken together, these data suggest that the changes in exploratory behavior we found in our setting (Fig. 1-3) indicate a specific discriminatory behavior between two different emotional states rather than a generalized index of sociability.

Figure 4

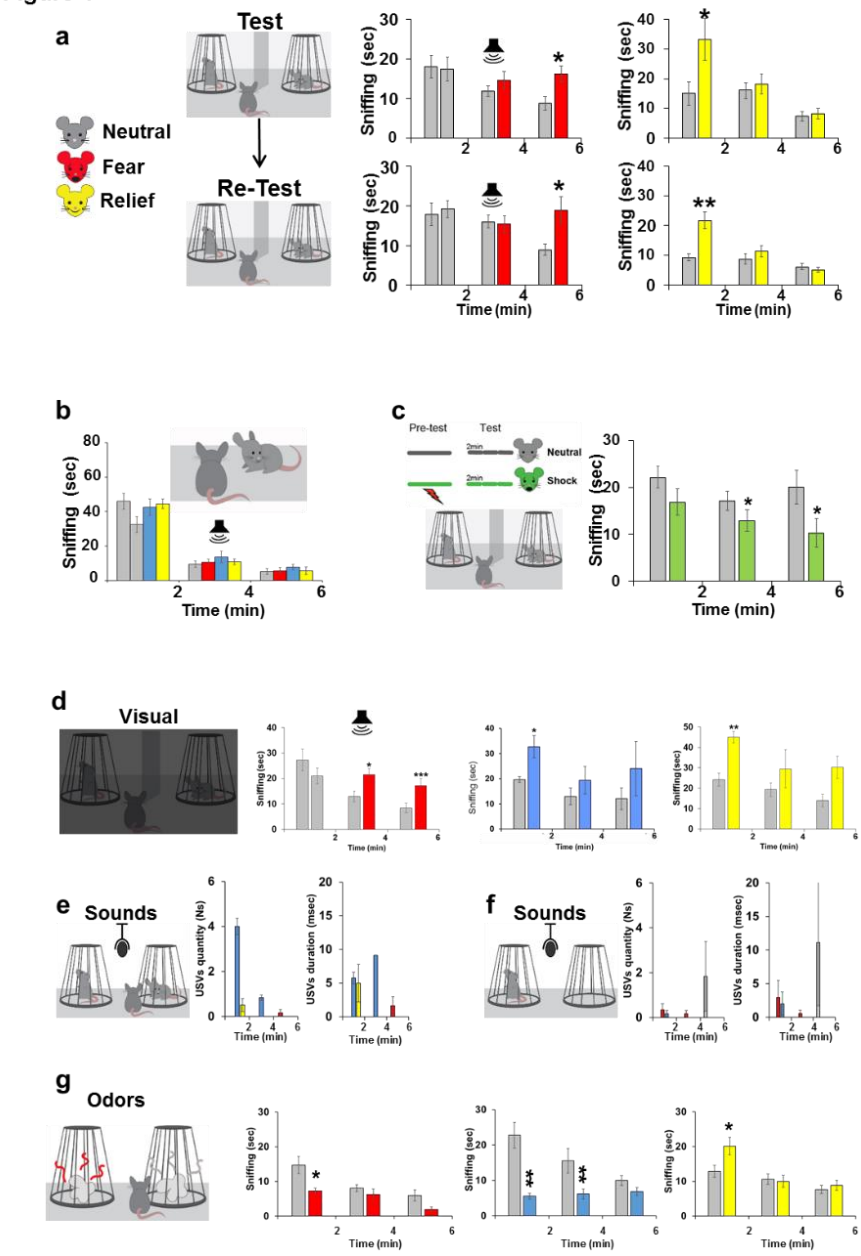


Figure 4. Reliability, distinction from sociability, and sensory modalities implication in the mouse emotion recognition task. (**a**) Schematic drawing of the test-retest reliability validation and time (in seconds) spent by observer mice sniffing the two demonstrators in the fear and relief paradigms during their first and second exposure to the 6-minute emotion recognition test. Time spent sniffing neutral demonstrators are depicted in grey, fear in red and relief in yellow. RM ANOVA for the "fear" manipulation, last 2-minute session, Test: $F_{1,13}$=6,10, p=0.028; Re-Test: $F_{1,13}$=8,59, p=0.012. RM ANOVA for the "relief" manipulation, first 2-minute session, Test: $F_{1,10}$=5,15, p=0.046; Re-Test: $F_{1,10}$=22,88, p=0.0008. *p<0.05, and **p<0,005 versus the exploration of the neutral demonstrator. N=11-14 observers per group. (**b**) Schematic drawing of the one-on-one test setting and time (in seconds) spent by observer mice sniffing a single demonstrator in a neutral, fear, stress or relief state during a 6-minute free interaction test. Time spent sniffing neutral demonstrators are depicted in grey, fear in red, stress in blue and relief in yellow. The tone for which only the fear demonstrator was fear-conditioned, as reported in the

methods section, was delivered between 2-4 minutes of the test. ANOVAs revealed only a time effect with normal decreased exploration throughout the 6 minutes ($F_{2,56}$=132.01, p<0.0001). N=12 observers. (**c**) Schematic drawing of the task setting and timeline of Pre-Test and Test procedures to trigger in one of the demonstrator a "shock" emotional state. The bar graph show the time (in seconds) spent by the observer mice sniffing demonstrators in neutral (grey bars) or shocked emotional state (green bars) during the 6 minutes of the emotion recognition test, divided by three consecutive 2-minute time bean (RM ANOVA, 0-2 min: $F_{1,6}$=2.40, p=0.17; 2-4-min: $F_{1,6}$=5.43, p=0.05; 4-6 min: $F_{1,6}$= 8.11, p= 0.02). *p<0.05 versus the exploration of the neutral demonstrator. N=7 observers. (**d**) Schematic drawing of the test setting performed in complete darkness, and time (in seconds) spent by observer mice sniffing neutral (grey), fear (red), stress (blue) or relief demonstrators (yellow) during the 6 minutes of the negative and positive valence versions of the emotion recognition test. RM ANOVA for the fear manipulation, 2-4 minutes: $F_{1,8}$=5.63, p=0.04; 4-6 minutes: $F_{1,8}$= 28.08, p= 0.0007; for the stress manipulation, 0-2 minutes: $F_{1,5}$= 10.43, p= 0.02 for the relief manipulation, 0-2 minutes: $F_{1,5}$= 33.32, p= 0.002. **p< 0.005 and *p< 0.05 versus the exploration of the neutral demonstrator. N=6/9 observers per group. (**e**) Schematic drawing of the test setting to record USVs, mean number of USV calls per minute, and mean duration of USVs in milliseconds emitted by mice during the fear, stress and relief emotion recognition tasks. N=6 observers per group. (**f**) Schematic drawing of the test setting to record USVs, mean number of USV calls per minute, and mean duration of USVs in milliseconds emitted by a single demonstrator mouse in neutral, fear, stress or relief emotional state. N=6 demonstrators per group. (**g**) Schematic drawing of the test setting performed only with demonstrators odors, and time (in seconds) spent by observer mice sniffing the odors from neutral (grey), fear (red), stress (blue) or relief demonstrators (in yellow) during the 6 minutes of the negative and positive valence versions of the emotion recognition test. RM ANOVA for the fear manipulation, 4-6 minutes: $F_{1,6}$= 9.15, p= 0.02; for the stress manipulation, 0-2 min: $F_{1,6}$= 33.64, p= 0.001; for relief manipulation, 0-2 min: $F_{1.,20}$= 4.25, p= 0.05 *p<0.05 , **p< 0.005 versus the exploration of the neutral odor. N=7/10 observers per group.

5.3.4. Visual and olfactory cues differently influence measures of emotion recognition.

Human emotion discrimination paradigms mostly rely on visual detection of facial and body expressions. The visual system in rodents is developed enough to acquire information as evident from observational transfer of fear/pain paradigms[11], [25]. However, in our setting the ability of mice to discriminate others' emotions might also implicate information conveyed by auditory and olfactory signals. Thus, we addressed the impact of visual, acoustic and olfactory stimuli in the ability of the observer mice to discriminate emotions in unfamiliar conspecifics. To test the value of visual cues, we performed the task in complete darkness (Fig. 4d). Observer mice showed increased sniffing towards the negative and positive emotionally altered demonstrators compared to neutral demonstrators (Fig. 4d), as found with lights on (Fig. 1-2). However, the lack of visual information extended the discrimination period. In fact, in the "fear" condition, the observer anticipated the discriminatory behavior at the presentation of the tone, while the discriminatory behavior in the "relief" paradigm tended to last for the

whole session. This evidence suggests a potential inhibitory control of visual cues in mouse emotion discrimination. To investigate the role of auditory information, we recorded ultrasonic vocalizations (USVs) in the fear, stress and relief manipulations. We used two different conditions: the standard setting with two demonstrators and the observer (Fig. 4e), and the isolated demonstrators (Fig. 4f). We found very few vocalizations, and no differences among the different conditions or emotional states (Fig. 4e-f). In agreement with previous evidence [28], our data indicated that USVs in mice do not communicate negative or positive emotions, and that auditory information are not necessary for emotion discrimination. To test the impact of olfactory cues on observer performance, we used a modified version of our setting in which the observer was presented to cotton balls enriched with the odor from neutral, fear, stress or relief demonstrators (Fig. 4g), instead of the demonstrators themselves. However, the relief odor induced in the observer the same pattern of discriminatory behavior as that found in the presence of the relief demonstrator. This suggests that odor cues are involved in emotion recognition, but the impact on the observer's discriminatory behavior is linked to the presence of the demonstrators. Overall, this set of data indicates distinct implications of both visual and olfactory social cues in the expression of mouse emotion discrimination.

5.4.5. Astrocytic CB1 receptor in PFC modulates emotion recognition in mice.

We then asked whether this newly identified social cognitive ability in mice might be mediated by conserved neurobiological mechanisms consistent with those implicated in humans. The endocannabinoid system plays a crucial role in facial emotion identification in human, in fact, deficits in both identification and discrimination of facial emotions in cannabis dependent patients have been demonstrated [15], [16]. However, the exact mechanisms by which endocannabinoid system is involved in social cognition are poorly understood. In order to understand the role of CB1 receptor in PFC in social cognition we decided to switch off the CB1 receptor selectively in astrocytes (Fig.4a). After the surgery, we left the animal recovery for one months and then they were tested in the classical habituation- dishabituation task and in the emotion recognition task. While CB1 floxed mice injected with AAV-GFAP-mCherry-Cre did not show an impairment in sociability and social memory abilities in the classic habituation- dishabituation task, we found that CB1 floxed mice injected with AAV-GFAP-mCherry-Cre spent significantly less time to interact with familiar mice during trial 2 and trial 3 in the social habituation- dishabituation task (Fig.4b).

Figure 5

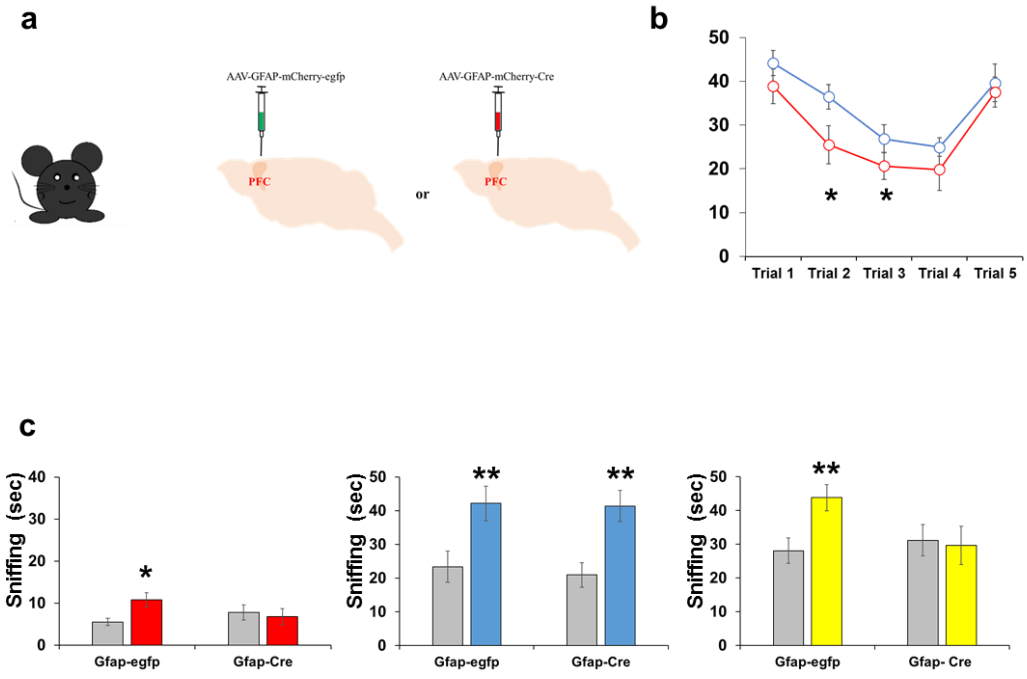


Figure 5. Astrocytic CB1 receptor in PFC modulates emotion recognition in mice. (a) Schematic drawing of viral vector used to infect PFC of CB1 floxed mice. (b)Time spent sniffing (in seconds) during the habituation-dishabituation task by CB1 floxed mice injected with control AAV-GFAP-mCherry- egfp (blue) or AVV-GFAP-mCherry-Cre (red). t test for trial *p < 0.05 vs Gfap- egfp mice. (c-e) Time (in seconds) spent sniffing each wire cage containing two stimulus mice during the emotion recognition test, and shown separately for each emotion by CB1 floxed mice injected with control AAV-GFAP-m Cherry- egfp or AVV-GFAP-mCherry-Cre. Time spent sniffing stimulus mice with neutral emotional state are depicted in grey and represented by grey bars. Time spent sniffing stimulus mice with (c) tone- induced fearful (last 2-min) or restrainer- induced stress (first 2-min) or water-induced happy state (first 2-min) are depicted in red, blue and yellow respectively. RM ANOVA Fear last 2-min, CB1 floxed mice injected with AVV-GFAP- egfp $F_{1,3}$= 13.43 p= 0.0035, CB1 floxed mice injected by AVV- GFAP-mCherry- Cre $F_{1,3}$= 39.24, p= 0.91; stress first 2-min CB1 floxed mice injected with AVV- GFAP-egfp: $F_{1.6}$ = 34.699, p= 0.001; CB1 injected by AVV- GFAP-mCherry- Cre $F_{1,11}$= 10.541, p= 0.007 ; relief first 2-min, CB1 floxed mice injected with AVV- GFAP-mCherry- egfp: $F_{1,5=}$ 10.098, p= 0.025; CB1 injected by AVV-GFAP-mCherry- Cre $F_{1,10}$= 0.026, p= 0.875. N=4-12 . observers per group. *p<0.05; **p<0.005 versus the exploration of the neutral demonstrator.

Relevantly, in CB1 floxed mice injected with AAV-GFAP-mCherry-Cre we identified emotion recognition deficits in both the fear and relief conditions (Fig. 4c-e), but not in stress condition (Fig.4 d). These data unravel a clinically-relevant genetic variation which concurrently produced deficits in emotion recognition abilities.

5.4. Discussion

The mouse emotion recognition test, inspired by human paradigms, extends the opportunity to study in mice the ability to discriminate negative and positive emotional states in conspecifics. We characterized the sensory modalities involved in emotion recognition and identified specific features, which differentiate this ability from basal sociability and other higher-order social processes such as emotional contagion. Furthermore, we provided initial proofs that the endocannabinoid system may modulate social cognition and emotion recognition in mice. The developed paradigm revealed detectable and measurable mice' ability to discriminate unfamiliar conspecifics based on either negative or positive emotions. Notably, observers' emotion discrimination was evident after (and not during) the induction of the altered emotional state in the demonstrators. This is in line with theories that emotional states are differentiated from simple reflex responses in their persistence after the disappearance of the triggering stimuli[24]. The manipulations performed in demonstrator mice were designed to alter emotional states in conditions of minimal physical distress. These features are distinct from previous settings designed to study the phenomena of observational transfer of pain/fear responses from a demonstrator to an observer[9]- [12], [16]. Indeed, transfer of fear/pain responses requires the direct observation of the demonstrator under pain or a foot shock physical challenge[10]. In agreement, no transfer of freezing, state-matching, escape behaviors or altered corticosterone levels were observed in our setting in the observer mice. This is also in line with the effort we made to design a paradigm which is centered on measuring innate social abilities in the observer mouse. In particular, the fact that the observers' discriminatory behaviors were different depending on the intensity of manipulations (e.g. fear *versus* shock), on visual cues (e.g. seeing or not freezing), or on olfactory cues (e.g. with or without the demonstrator) suggest some internal social cognitive appreciation of the context by the observer. This is distinct from previous evidence on emotional contagion processes, such as observational transfer of fear/pain which implies automatic responses to negative-emotional states[13] or from reported consolatory behaviors which require a strict reciprocal connection and familiarity between the observer and the demonstrator[9-12]. Altogether, this evidence indicates that our paradigm mimics what is commonly measured in humans with emotion recognition tasks and defined as "social cognition" [2]-[4].

To detect emotion discrimination abilities, observer mice were simultaneously exposed to different conspecifics in different emotional states. Intriguingly, observer mice showed similar increased sniffing towards demonstrators in negative- or positive-valence emotional states. The

study of the sensory modalities involved in the processing of negative and positive emotions, however, provided some insight on possibly different ethological meaning of the three paradigms. In particular, exposing an observer to a relief mouse, with or without visual cues, or only to the odor of a relief mouse led to a similar approaching behavior, possibly implying general "attracting" signals. In contrast, while the exposure of an observer to a fear or stressed mouse induced an approach behavior, isolated fear and stress odor cues as well as exposure to a shocked mouse determined an aversion. Rodents have been reported to actively escape from intense aversive stimuli[11], from aversive USVs calls induced by heavy distress [25], [27], and from odors emitted by a shocked, heavily stressed, defeated, or sick conspecific[28]- [30]. Thus, the different directions and tunable nature of the discriminatory behaviors we observed point to the possibility to study with this novel setting different social cognitive processes possibly related to "attract", "alarm" or "help" social information.

Social discriminatory data in our emotion recognition paradigm were not predictive of exploratory sociability as assessed in one-on-one. This distinction indicates a true discriminatory social behavior dissociated from general sociability or automatic responses. In contrast, previous research on higher-order social functions is based on a one-on-one setting [9], [11] or reproduce similar exploratory results in a one-on-one and a one-or-two setting [20]. This provide further evidence that our paradigm addresses a different aspect of rodents' social abilities, complementing previous available tools. The first direct application of the new paradigm developed led us to reveal the role of astrocytic CB1 receptor in PFC, an essential brain area mediating emotion recognition [4]. Increasing studies showed that massive use of cannabis produced an impairment in social cognition in human [14]- [16]. However, heavy cannabis users usually take several kind of drugs at the same time, including alcohol. For this reason, it could be difficult to understand the exact mechanisms regulating these functions. For the first time, we provided a proof that astrocytic CB1 receptor regulate emotion recognition in mice. In fact, we removed receptor exclusively in the prefrontal astrocytes showing that this procedure cause deficits. This important result could be used for feature research that aim to discover new pharmacological treatment to rescue social deficits in psychiatric disorders. In conclusion, this set of findings provides a new method to address still scarcely explored aspects of rodents social cognition. This could support more translational approaches between rodent and human social cognitive studies, with relevance to circuits, genetics and neurochemical systems involved in different psychiatric disorders.

5.5. References:

[1] Dunbar, R.I. The social brain hypothesis and its implications for social evolution. *Annals of human biology* **36**, 562-572 (2009).

[2] Henry, J.D., von Hippel, W., Molenberghs, P., Lee, T. & Sachdev, P.S. Clinical assessment of social cognitive function in neurological disorders. *Nature reviews. Neurology* **12**, 28-39 (2016).

[3] Kennedy, D.P. & Adolphs, R. Perception of emotions from facial expressions in high-functioning adults with autism. *Neuropsychologia* **50**, 3313-3319 (2012).

[4] Green, M.F., Horan, W.P. & Lee, J. Social cognition in schizophrenia. *Nature reviews. Neuroscience* **16**, 620-631 (2015).

[5] Fett, A.K. et al. The relationship between neurocognition and social cognition with functional outcomes in schizophrenia: a meta-analysis. *Neuroscience and biobehavioral reviews* **35**, 573-588 (2011).

[6] Kurtz, M.M. & Richardson, C.L. Social cognitive training for schizophrenia: a meta-analytic investigation of controlled research. *Schizophr Bull* **38**, 1092-1104 (2012).

[7] Fletcher-Watson, S., McConnell, F., Manola, E. & McConachie, H. Interventions based on the Theory of Mind cognitive model for autism spectrum disorder (ASD). *The Cochrane database of systematic reviews*, CD008785 (2014).

[8] Tate, A.J., Fischer, H., Leigh, A.E. & Kendrick, K.M. Behavioural and neurophysiologicalevidence for face identity and face emotion processing in animals. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **361**, 2155-2172 (2006).

[9] Burkett, J.P. et al. Oxytocin-dependent consolation behavior in rodents. *Science* **351**, 375-378 (2016).

[10] Langford, D.J. et al. Social modulation of pain as evidence for empathy in mice. *Science* **312**, 1967-1970 (2006).

[11] Pisansky, M.T., Hanson, L.R., Gottesman, II & Gewirtz, J.C. Oxytocin enhances observational fear in mice. *Nature communications* **8**, 2102 (2017).

[12] Sterley, T.L. et al. Social transmission and buffering of synaptic changes after stress. *Nature neuroscience* (2018).

[13] Prochazkova, E. & Kret, M.E. Connecting minds and sharing emotions through mimicry: A neurocognitive model of emotional contagion. *Neuroscience and biobehavioral reviews* **80**, 99-114 (2017).

[14] Bossong MG, vanHella HH, Jagera G, Hahnd RS, Ramseya NF, Jansmaa JM (2013) The endocannabinoid system and emotional processing: a pharmacological fMRI study with Δ9-tetrahydrocannabinol. European Neuropsychopharmacology (2013) 23, 1687–1697

[15] Hindocha C, Wollenberg O, Carter Leno V, Alvarez BO, Curran HV and Freeman TP Emotional processing deficits in chronic cannabis use: A replication and extension. J Psychopharmacol, doi: 10.1177/0269881114527359 (2013)

[16] Bayrakçıa A, Sertb E, Zorlua N, Erola A, Sarıçiçeka A, Metea L. Facial emotion recognition deficits in abstinent cannabis dependent patients. Comprehensive Psychiatry 58 (2015) 160–164

[17] de Almeida V, Martins- de- Sousa (2018) Cannabinoids and glial cells: possible mechanism to understand schizophrenia. *European Archives of Psychiatry and Clinical Neuroscienc*e
https://doi.org/10.1007/s00406-018-0874-6

[18] Petrelli, F et al. Dysfunction of homeostatic control of dopamine by astrocytes in the developing prefrontal cortex leads to cognitive impairments. *Molecular psychiatry* (2018)

[19] Scheggia, D. et al. Remote memories are enhanced by COMT activity through dysregulation of the endocannabinoid system in the prefrontal cortex. *Molecular psychiatry* (2018).

[20] Rogers-Carter, M.M. et al. Insular cortex mediates approach and avoidance responses to social affective stimuli. Nature neuroscience 21, 404-414 (2018).

[21] Huang, H. et al. Chronic and acute intranasal oxytocin produce divergent social effects in mice. Neuropsychopharmacology : official publication of the American College of

[22] Contarino, A., Kitchener, P., Vallee, M., Papaleo, F. & Piazza, P.V. CRF1 receptor-deficiency increases cocaine reward. *Neuropharmacology* **117**, 41-48 (2017).

[23] Cecilione, J.L. et al. Test-retest reliability of the facial expression labeling task. *Psychol Assess* **29**, 1537-1542 (2017).

[24] Anderson, D.J. & Adolphs, R. A framework for studying emotions across species. *Cell* **157**, 187-200 (2014).

[25] Portfors, C.V. Types and functions of ultrasonic vocalizations in laboratory rats and mice. *J Am Assoc Lab Anim Sci* **46**, 28-34 (2007).

[26] Blanchard, R.J. & Blanchard, D.C. Attack and defense in rodents as ethoexperimental models for the study of emotion. *Prog Neuropsychopharmacol Biol Psychiatry* **13 Suppl**, S3-14 (1989).

[27] Sadananda, M., Wohr, M. & Schwarting, R.K. Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. *Neurosci Lett* **435**, 17-23 (2008).

[28] Zalaquett, C. & Thiessen, D. The effects of odors from stressed mice on conspecific behavior. *Physiol Behav* **50**, 221-227 (1991).

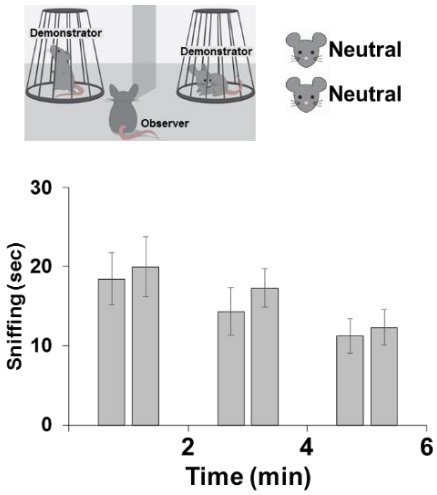
[28] Sawyer, T.F. Androgen effects on responsiveness to aggression and stress-related odors of male mice. *Physiol Behav* **25**, 183-187 (1980).

[29] Boillat, M. et al. The vomeronasal system mediates sick conspecific avoidance. *Curr Biol* **25**, 251-255 (2015).

[30] Metna-Laurent M. & Marsicano G. Rising stars: modulation of brain functions by astroglial type-1 cannabinoid receptors.*Glia* 63:353–364 (2015).

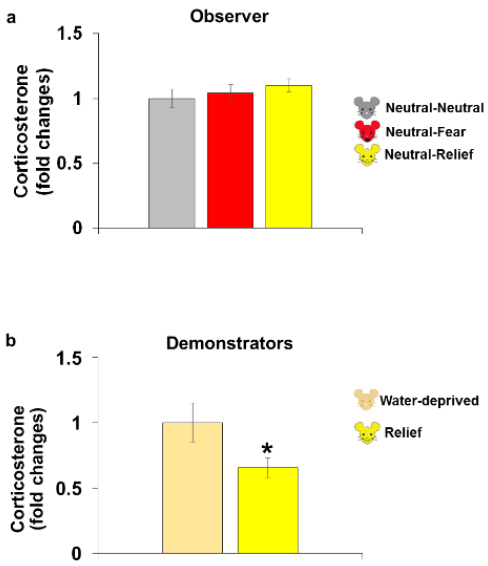
5.6 Supplementary figures

5.6.1 Mice did not discriminate unfamiliar two neutral conspecifics.



Supplementary figure 1. No discrimination towards two neutral demonstrators. Schematic drawing of the task setting, and time (in seconds) spent sniffing two demonstrators in neutral (grey bars) states displayed by observer mice during the 6 minutes of the emotion recognition test, divided into three consecutive 2-minute time beans. RM ANOVAs reveled no significant differences for the 0-2, 2-4, and 4-6 minutes test periods.

5.6.2 Corticosterone levels



Supplementary figure 2. Corticosterone levels. (a) Blood corticosterone levels displayed by observer mice immediately after being tested in the emotional recognition task with two neutral demonstrators (grey bar), one neutral and one fear demonstrators (red bar), and one neutral and one relief demonstrators (yellow bar). Data are expressed as fold changes compared to observers exposed to two neutral demonstrators (One-Way ANOVA: $F_{2,13}$=0.90, p=0.43). N=5/6 observers per group. (b) Blood corticosterone levels displayed by demonstrator mice immediately after a period of 24-hour water deprivation (grey bar) or after a period of 1-hour ad libitum access to water following 23-hour water deprivation (yellow bar). (T-test: df: 19; p=0.05). *p=0.05 versus water deprived mice.

Chapter 6

6.1 General discussion and conclusions

In the first part of my work, we demonstrated that genetic variations resulting in DAT hypofunction produced several core behavioral alterations analogous to those reported in patients with ADHD, but not schizophrenia. In order to get closer to the clinical setting, we specifically addressed two different time points: adolescence and adulthood. Indeed, adolescence is a critical transitional period of development from childhood to adulthood in which neurochemical and hormonal changes occur. In particular, higher order cognitive functions develop and mature during this time period. We provided a modified 5CSRTT to investigate behavioral changes in adolescent mice. We demonstrated that even within the short duration of mice adolescence, it is possible to evaluate different attentional control aspects using our modified 5CSRTT paradigm. Moreover, thanks to this new protocol we were able to asses cognitive abnormalities in both adolescent and adult DAT +/- mice throughout the lifespan. Furthermore, consistent with DAT +/- phenotypes, patients with ADHD may show, starting from childhood, hyperactivity and cognitive impairments that persists during adulthood. Taken together, these results could have a potential translational validity concerning human studies, applicable to genetic and pharmacological studies in mouse models relevant to cognitive abnormalities and psychiatric disorders.

In the second part of my studies, we started to characterize endocannabinoid system in COMT-Val tg mice. We found that increased COMT activity enhanced aversive remote memories and starting from this evidence, we proposed that the effect of COMT on dopamine metabolism in this cognitive balance might help to understand in mechanistic terms the dysfunction of cognitive and emotional functions in nervous system diseases like PTSD and SZ. In order to investigate the role of endocannabinoid system in emotional processes we assessed a new method to investigate emotion recognition in mice. In addition, our results could be important for translational approaches between rodent and human social cognitive studies. In fact, thanks to “emotion recognition task” we provided a proof about the role of astrocytic CB1 receptor. This is the first step to find new pharmacological treatments to recover deficits in social cognition in psychiatric disorders. In conclusion, our findings provided new methods to study different aspects of cognition in mice, giving new translational approaches between rodents and humans that could be used to better investigate biological mechanisms and neuronal circuits implicate in psychiatric disorders.

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