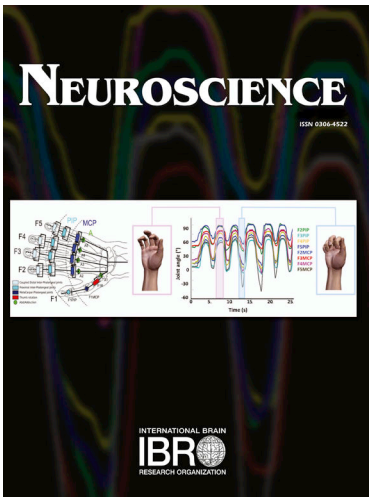
Journal Pre-proofs



Research Article

Motor transitions' peculiarity of heterozygous DAT rats when offspring of an unconventional KOxWT mating

Carbone Cristiana, Brancato Anna, Adinolfi Annalisa, Sara Lucia Maria Lo Russo, Alleva Enrico, Cannizzaro Carla, Adriani Walter

PII: S0306-4522(20)30148-2
DOI: https://doi.org/10.1016/j.neuroscience.2020.03.005
Reference: NSC 19563

To appear in: *Neuroscience*

Received Date: 15 November 2019
Accepted Date: 3 March 2020

Please cite this article as: C. Cristiana, B. Anna, A. Annalisa, S.L.M. Lo Russo, A. Enrico, C. Carla, A. Walter, Motor transitions' peculiarity of heterozygous DAT rats when offspring of an unconventional KOxWT mating, *Neuroscience* (2020), doi: https://doi.org/10.1016/j.neuroscience.2020.03.005

Motor transitions' peculiarity of heterozygous DAT rats when offspring of an unconventional KOxWT mating

CARBONE Cristiana $ (1); BRANCATO Anna, PhD $ (2); ADINOLFI Annalisa (1); LO RUSSO Sara Lucia Maria (1); ALLEVA Enrico, PhD (1); CANNIZZARO Carla, MD (2); & ADRIANI Walter, PhD (1)

1 - Center for Behavioral Sciences and Mental Health, Istituto Superiore di Sanità, Rome, Italy

2 - Dept Sciences of Health Promotion & Mother and Child Care, Internal Medicine and Medical Specialties, University of Palermo, Italy

$ = Contributed equally.

Running Title: The "maternal" heterozygous DAT rats.

***Correspondence:** Dr. Walter Adriani, PhD. Center for Behavioral Sciences and Mental Health, Istituto Superiore di Sanità (ISS), Building 19 floor D room 11, Viale Regina Elena 299, I-00161 Rome, Italy. Tel +39 06 4990 3285, Fax +39 06 495 7821, e-mail: walter.adriani@iss.it

Acknowledgements: The financial support from University of Palermo (2012-ATE-0167) is gratefully acknowledged. This source had in no way interference on the design and data analysis of the experiment. WA, CaC and CrC designed the study; CrC, SLR, AA carried out the behavioral experiment; AB carried out the ex-vivo experiment; AB and CrC wrote a first draft of the paper; EA, CaC, and WA contributed to its final version. All original data leading to this paper are stored on a computer located at ISS in the office of the corresponding author. All raw data can be made available upon request. We thank Raul Gainetdinov and Damiana Leo who developed and kindly provided the progenitors of the DAT rat colony; Stella Falsini for help with all management issues, and Antonio Di Virgilio for animal care and welfare. There is no conflict of interest to disclose.

Word Count Abstract: **250 --** Number of figures, **5 (five)** - no tables.

ABSTRACT: Causal factors of psychiatric diseases are unclear, due to gene×environment interactions. Evaluation of consequences, after a dopamine-transporter (DAT) gene knock-out (DAT-KO), has enhanced understanding the pathological dynamics of several brain disorders, such as Attention-Deficit/Hyperactivity and Bipolar-Affective disorders. Recently, our attention has shifted to DAT hypo-functional (heterozygous, HET) rodents: HET dams display less maternal care and HET females display marked hypo-locomotion if cared by HET dams (Mariano et al., 2019). We assessed phenotypes of male DAT-heterozygous rats as a function of their parents: we compared "maternal" origin (MAT-HET, obtained by breeding KO-male rats with WT-female dams) to "mixed" origin (MIX-HET, obtained by classical breeding, both heterozygous parents) of the allele. MAT-HET subjects had significantly longer rhythms of daily locomotor activity than MIX-HET and WT-control subjects. Furthermore, acute methylphenidate (MPH: 0, 1, 2 mg/kg) revealed elevated threshold for locomotor stimulation in MAT-HETs, with no response to the lower dose. Finally, by Porsolt-Test, MAT-HETs showed enhanced escape-seeking (diving) with more transitions towards behavioral despair (floating). When comparing both MAT- and MIX-HET to WT-control rats, decreased levels of DAT and HDAC4 were evident in the ventral-striatum; moreover, with respect to MIX-HET subjects, MAT-HET ones displayed increased DAT density in dorsal-striatum. MAT-HET rats displayed region-specific changes in DAT expression, compared to "classical" MIX-HET subjects: greater DAT availability may elevate threshold for dopamine action. Further behavioral and epigenetic characterizations of MAT-HETs, together with deeper characterization of maternal roles, could help to explore parent-of-origin mechanisms for such a peculiar phenotype.

Keywords: DAT heterozygous rat; depressive-like phenotype; histone-deacetylase; nucleus accumbens; parent-of-origin effect.

INTRODUCTION

Progress in understanding the etiology of psychiatric disorders is the basis for more effective and targeted treatment strategies. Several studies showed that genetic factors play an important role in the etiology of psychiatric traits (Khodayari et al., 2004; Franke et al., 2009; Psychiatric GWAS Consortium, 2009; Pettersson et al., 2018). Nevertheless, most psychiatric disorders have multifactorial origins and a number of other factors, including gene×environment interactions as well as parent-of-origin effects (POE), are potentially involved (Goose et al., 2007; Zayats et al., 2015). During gestation and early postnatal life, brain development is modulated by complex epigenetically-mediated processes, related to environmental stimuli. In particular, POE represent situations in which parents may differ in the relative quantity and/or quality of risk factors they transmit, providing a not equal contribution to a given phenotype in their offspring.

Several mechanisms are responsible for differential influence of one parent or the other, including chromosomal or hormonal factors, sex differences, or genomic imprinting (Kopsida et al., 2011). "Imprinting" is the term used to describe epigenetic effects, like the differential expression of paternal versus maternal alleles (Wilkins and Haig, 2003). Imprinting may involve differential epigenetic regulation and/or differential chromatin modification of the paternally vs. maternally inherited alleles (Delaval and Feil, 2004; Holmes and Soloway, 2006). In particular, recent work indicates that parental-specific histone acetylation is involved in regulation of imprinted gene expression (Svensson et al., 1998; Saitoh and Wada, 2000; Grandjean et al. 2001). Acetylation of histones is a dynamic process, able to affect chromatin structure in response to upstream cellular signaling: it can regulate multiple behavioral phenomena, including addiction, depression, age-related memory impairment, and memory recall (Fischer et al., 2010).

In this regard, manipulation of the dopamine-transporter (DAT) gene in animal models results in delayed clearance of dopamine (DA) and down-regulation of its receptors, contributing to several behavioral abnormalities including hyperactivity and cognitive deficits (Giros et al., 1996; Jones et al., 1998). The DAergic neurotransmitter system is being investigated because of involvement in critical psycho-physiological functions, including the modulation of motivated and habit-based behavioral processes (Kim et al., 2015; Owesson-White et al., 2016). Dysregulation in DAergic reuptake and signaling is implicated in pathogenesis of many psychiatric diseases, among which schizophrenia (SZ) (Barr et al., 2001), post-traumatic stress disorder (PTSD) (Drury et al., 2009); bipolar disorder (BD) (Hahn and Blakely, 2007), attention-deficit / hyper-activity disorder (ADHD) (Yang et al. 2007), as well as obsessive-compulsive disorder (OCD) (Kurian et al., 2011). Due to the

variety of symptoms in the listed diseases, it is not easy to select focused tasks for DAT-related phenotyping in a preclinical study; given the pilot nature of our approach, we reasoned that a basic role of DAT would be better explored by general locomotion, both as a (spontaneous) circadian rhythm and after a (drug-induced) psychostimulant challenge. Additionally, general tone of mood may be revealed by the Porsolt-Test: of note, increased depressive-like behavior was found in heterozygous mice for DAT (Perona et al. 2008).

Dopamine transporter (DAT) belongs to the solute carrier family 6 (SLC6) of plasma membrane transporters; it plays a key role in DA reuptake by transporting released DA from the synaptic cleft back into neurons. Hence, proper regulation of DAT expression modulates the DA signaling intensity by regulating concentration of extracellular DA and is critical to maintain homeostasis in the DAergic system (Giros and Caron, 1993; Bannon et al., 2001). Several genetic polymorphisms of the human DAT gene (DAT1, SLC6A3) have been currently identified (Mazei-Robinson and Blakely, 2005). Furthermore, human SLC6A3 gene shows a high sensitivity to epigenetic regulation (Shumay et al., 2010). Histone deacetylases (HDACs) have been indicated as the main epigenetic mechanism regulating the expression of DAT gene (Green et al., 2015; 2017). Notably, adolescent stress leads to deficits that recapitulate schizophrenia, whereas adult stress induces a depressive hypo-dopaminergic state. Enhanced neural plasticity, induced via histone deacetylase (HDAC) inhibition, may render adult stress able to produce schizophrenia-like rather than depression-like sequelae, similarly to adolescent stress (Gomes et al., 2019). As such, the HDAC activity seems to set the direction for deleterious effects, when adversities tap the DAT gene.

Genetically modified animals were proven to be a valid tool to elucidate the implication of DA reuptake abnormalities in the onset of psychiatric diseases. DAT-KO rats, generated by using zinc-finger nucleases (ZFN) technology (Geurts et al., 2009; Brown et al., 2013), have been phenotyped (Adinolfi et al., 2018, 2019) and represent the most recent example of DAT deficiency model (Leo et al., 2018; Cinque et al., 2018). In addition, N157K rats have been developed where ENU-driven mutagenesis generated a fully translated but functionally less active DAT protein (by a T/G transversion in DAT exon 3; asparagine into lysine at residue 157; Vengeliene et al., 2017). In order to explore the complex interaction between gene and environment, we consider the DAT-HET animals more informative than full KO ones (Tremolizzo et al., 2002): having just one functioning copy, they may be more sensitive to insults leading to epigenetic modulation of this allele.

Yet, the majority of studies have been conducted so far by the classical breeding of male and female DAT-HET subjects: this offspring includes “mixed” heterozygous subjects (MIX-HET), in which the functional DAT allele has mixed paternal/maternal origin (Adinolfi et al., 2019). To highlight the

potential involvement of a parent-of-origin effect, we recently (*footnote 1*) explored a rather unconventional DAT heterozygous rat (MAT-HET), obtained by the breeding of KO-male rats with WT-female dams, with functional DAT alleles always of maternal origin (Mariano et al., 2019). Since WT dams take care of homogeneous "maternal" HET pups, while HET dams take care of all pups with "mixed" DAT-HET, KO and WT genotypes, our rats also differed for postnatal environment (i.e. their dam, but also siblings). In recent studies, focused on drug intake by mothers during lactation, the additive / separate role of altered maternal cares was highlighted (see Brancato et al., 2016; Chirico et al., 2017).

In addition, different parental allele origin can have unsuspected effects. The male KO father could transmit to a MAT offspring unknown patterns of epigenetic signatures. Just as an example, cocaine is able to increase testicular and germ cell HDAC 3/4 and decrease expression of HDAC1/2, with the potential to transmit these to the offspring (González et al., 2018). It is tempting to hypothesize that similar change may be present in DAT KO males, chronically exposed to enhanced DA both centrally and peripherally, and influence their offspring. Presently we focused on two putative players of parental-specific epigenetics: the nuclear-specific HDAC2 (Fischer at al., 2010) and the synaptic HDAC4 (Darcy et al., 2010). Moreover, since regional expression of different HDAC isoforms are likely to contribute to refined and specific control over transcription and neural signaling, we investigated ventral and dorsal striata. Notably, for the sake of translation to clinics, HDAC inhibition is emerging as a possible approach against depression (Misztak et al., 2018) as well as BD (Konstantakopoulos et al., 2015). We propose that slight epigenetic changes, in specific areas of otherwise heterozygous subjects, could account for overlaps and divergences between phenotypes reminiscent of unipolar versus bipolar depression. Focus of the present study was to investigate male heterozygous subjects, cared by either HET or WT dams plus inheriting crucial epigenetic signature from either HET or KO fathers. The present pilot study reports differences in both behavior and these epigenetic markers.

MATERIALS AND METHODS

All experimental procedures have been approved by Italian Ministry of Health (formal license 937/2018-PR for project D9997.61, held by W. Adriani). Procedures were carried out in agreement with the Directive of the European Community Council (2010/63/EEC) and with the Italian Law

guidelines. All efforts have made to minimize the suffering of animals and to use as few animals as possible, according to the 3Rs principle.

SUBJECTS AND THEIR BREEDING CONDITIONS

The generation of Wistar-Han DAT knockout rats was previously described elsewhere (Leo et al., 2018). The colony was maintained in a heterozygous-heterozygous breeding fashion; these animals were intercrossed for >10 generations at Istituto Italiano di Tecnologia (IIT, Genoa, Italy). Some progenitors were shipped to Istituto Superiore di Sanità (ISS, Rome, Italy), where male DAT-KO rats were bred with Wistar-Han WT females (Charles River, Italy), to obtain a G0 of new founder heterozygous subjects. Present subjects are G2 of our ISS colony.

All experimental subjects were adult male rats (> 120 days old; average weight 500 g) born from this colony in our facility. In this experiment, we used a maximum of 3 male pups per dam: control subjects were 15 wild-type (WT) rats, out of 30 in total born and nurtured by 6 wild-type dams (bred with wild-type males); in parallel, experimental subjects were 15 "mixed" heterozygous rats (MIX-HET), out of 25 in total born and nurtured by 5 DAT heterozygous dams (classical breeding), and 15 "maternal" heterozygous rats (MAT-HET), out of 25 in total born and nurtured by 5 wild type dams: this latter group had DAT-KO male rats as fathers ("biased" unconventional breeding). Therefore, the functional DAT allele was always of maternal origin for MAT-HET and had a mixed paternal / maternal origin for MIX-HET offspring (hence justifying the use of the "maternal" vs "mixed" terms, respectively). Their siblings that did not take part to this study were assigned to another experiment (Zelli et al., *in preparation*). The exclusion of the KO subjects (siblings of MIX-HET) was mandatory, because of their abnormal activity pattern.

They were placed since weaning in group, within Plexiglas cages (33x13x14 cm), located in an air-conditioned room (T 21˚±1˚C, relative humidity 60±10%) with a 12h dark-light cycle (light turned-on at 7.00 PM). From weaning at post-natal day (PND) 21 to PND70 housing was of three non-sibling, same-genotype subjects (total 15 cages); at PND 100, housing was rearranged to two non-sibling, same-genotype rats (total 21 cages). One animal per group was sacrificed at this point.

Water and food (Altromin-R, A. Rieper S.p.A., Vandoies, Italy) were available ad libitum. All the animals (n=15 per group) underwent all the three protocols, listed below. These protocols were carried out sequentially, from the least invasive to the most invasive.

EXPERIMENTAL PROTOCOLS

All these experiments were conducted inside the animal facility room, to minimize the impact of transport to a novel testing room.

DAILY ACTIVITY CYCLE

Rats were divided into three runs with counterbalanced genotypes (one sibling per run). From PND 70 to 100, they were individually placed (one run per week) within fifteen homecage-like cages into the recording rack, with ad libitum water and food (Altromin-R, A. Rieper S.p.A, Vandoies, Italy). This recording rack unit is located in the same facility room as the colony housing racks.

As apparatus for continuous monitoring of locomotor activity, we used our automatic recording rack, with passive infrared sensors placed on top of each home-cage (ActiviScope system; TechnoSmart, Rome, Italy; www.newbehavior.com), hosted in the same air-conditioned room (temperature 21 ± 1° C, humidity 60 ± 10%) with a 12 h reverse dark-light cycle (light turned-on at 7.00 p.m.). Sensors detected rats' movement (sampling rate: 20 per second). Data were recorded by a computer with dedicated software; scores were automatically divided into 60-min intervals. The seven days of recording per individual rat were averaged, to obtain a single "mean" day; as such, each rat entered into analysis only once, with its own "mean" day, composed of 24 hourly points per rat (n=15 per group). Authorized personnel was allowed to access the animal room according to the routine schedule.

LOCOMOTION WITH METHYLPHENIDATE

To assess differential effect on rats' locomotor activity, related to their genotype, we injected all subjects with methylphenidate (i.v., through tail vein), a central nervous system stimulant used in the treatment of ADHD and narcolepsy. For each of the three main groups (WT, MIX-HET, MAT-HET), we formed three dosage groups (n=5 per group): Control subjects were injected with vehicle (saline solution i.v. 200 µl/kg); MPH1 subjects were injected with a dose of 1 mg/kg methyl-phenidate i.v. ; MPH2 subjects were injected with a dose of 2 mg/kg methyl-phenidate i.v. (through the tail vein).

The home-cages were carefully placed on a cart, the three home-mate animals were individually weighed, injected and gently placed individually in a Plexiglas cage, identical to the home-cage, which was immediately positioned in the recording rack. The experiment was designed so that each

home-mate out of a triplet was randomly assigned to one of the three planned doses (vehicle, MPH 1, MPH 2), and monitored for three hours. Then, the triplet was placed back into their home cage.

Apparatus: to assess the drug-induced activity we used the same recording rack (as above). Scores were automatically divided into 6-min intervals and further grouped five-by-five. As such, each rat entered into analysis only once, with its own dosage and data from its six 30-min bins. The access of authorized personnel to the animal room was not restricted and followed the routine schedule.

FORCED-SWIM TEST

A forced-swim test was carried out at PND > 100 (n=14 per genotype), during 4 working days. According to classical experimental design, during a first exposure day rats were subjected to a 15-min habituation. During a second exposure day, each subject underwent the actual test consisting in a 5-min trial. Each animal was gently placed into a 25 cm (d) x 65 cm (h) cylindrical container filled with slightly warmed water (24˚C ±1). Water level was 30 cm deep, so that animals were unable to touch the bottom of the tank neither with their paws nor with their tails. Water was changed every two subjects, which were wiped and dried for 1 min with a hair-phon.

Behavior was videotaped and scored for: floating (immobile), climbing, swimming, struggling and diving. Each videotape was viewed and scored two times (spaced one month apart) by a skilled researcher who was blinded to the genotype group. As such, each rat entered into analysis twice, with its own data from both views considered as a repeated measure (n=14 per group). Data were analyzed as latency, frequency and total duration of each behavior. The program in use was The Observer (Noldus, the Netherlands).

EX-VIVO IMMUNOFLUORESCENCE EXPERIMENTS

Dopamine transporter (DAT) and two HDAC enzymes (HDAC2 and HDAC4) were investigated by immuno-fluorescence in the nucleus accumbens (shell and core sub-regions) and dorsal striatum. Among class I HDACs, despite the high sequence homology between HDAC1 and HDAC2, only HDAC2 was reported to be decreased in SZ patients (Schroeder et al., 2017). HDAC4 has been localized in the dendritic shaft and spines (Darcy et al., 2010), suggesting that it may work outside the cell body to regulate synaptic activity and dendritic transport.

PERFUSION AND TISSUE PROCESSING

Half of all rats already used for behavior, one month after last protocol and two months after the acute drug injection, were given a lethal dose of 10% chloral hydrate i.p. and transcardially perfused with cold phosphate-buffered saline (PBS; pH 7.4) followed by fixation with cold 4% para-formaldehyde (PFA) in PBS. One rat per genotype was discarded at this point due to inaccurate perfusion (final n=6 per genotype group). The other half of all rats (21 subjects) were used for other endpoints. Brains were dissected and post-fixed in the same fixative at 4°C. Coronal sections were prepared on a vibratome at 35 μm thickness. Serial slices were collected through the rostral-caudal dimension of the brain (every 6th slice) and stored at 4°C in 0.05% sodium azide in PBS until immuno-fluorescence processing.

IMMUNOFLUORESCENCE STAINING

Immuno-fluorescence was performed as previously described (Brancato et al., 2017), with a few modifications. Sections (six per animal) were washed in PBS for 30 min and incubated in blocking solution (3% normal goat serum (NGS), 0.3% Triton X-100 in PBS) for 2h at room temperature under gentle shaking. Sections were then incubated in primary antibody solution for 72h at 4°C under gentle shaking (3% NGS, 0.3% Tween-20 in PBS, with either anti-DAT, anti-HDAC2 or anti-HDAC4, 1:500, Santa Cruz Biotech). Sections were washed in PBS for 1h, incubated in secondary antibody for 2h under gentle shaking (goat anti-rat Alexa Fluor 488, 1:200; goat anti-mouse Alexa Fluor 594, 1:200; Jackson ImmunoResearch, West Grove, PA, USA). After 1h washing in PBS, slices were briefly incubated with DAPI (1 mg/ml). Sections were slide mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and cover slipped before imaging.

IMAGING FOR IMMUNOFLUORESCENCE

DAT-, HDAC2- and HDAC4 immuno-fluorescence were analyzed in the nucleus accumbens (shell and core sub-regions) and dorsal striatum, according to Paxinos and Watson (2013). Images (one per section) were acquired on a Meiji Techno fluorescence microscope at 40x magnification, by employing Deltapix Insight imaging software. Immuno-fluorescence was quantified by using ImageJ, measuring number of DAT-positive puncta or mean grey values for histone deacetylase-related images, and reported as relative immuno-reactivity values normalized to the average of WT controls.

STATISTICS

Data from experiments were analyzed by analysis of variance (ANOVA), when data distributions were normal and had equal variances (except in one instance, see below). The first assumption was confirmed by Shapiro-Wilk test (W = 0.9276, p = 0.1759; W = 0.9552, p = 0.5126; W = 0.9800, p = 0.9502 ; W = 0.9710, p = 0.8170; W = 0.9390, p = 0.2789; W = 0.8985, p = 0.0541; W = 0.9255, p = 0.1618); the second assumption was confirmed by the Levene test (W = lower than critical value) or the Brown-Forsythe test ($F_{2, 15}$ = lower than critical value). Statistical analyses were performed using Prism 6.0 (Graphpad Software Inc.) or StatView II (Abacus Concepts). Statistical significance was set at $p < 0.05$ and significant tendencies for $0.10 < p < 0.05$ were also explored.

BEHAVIORAL DATA

Analysis about daily activity cycles was carried out by a repeated-measure 3x24 model (n=15 per group). ANOVA had two independent variables: between-subjects genotype (3 levels: WT, MAT-HET, MIX-HET), and within-subject time (24 levels). Analysis concerning locomotor activity with methylphenidate was carried out by a split-plot 3x3x6 model (n=5 per group) with three independent variables: between-subjects genotype (3 levels: WT, MIX-HET, MAT-HET) and treatment (3 levels: VEH, MPH 1, MPH 2), plus within-subject time (6 levels: 5-min bins).

Regarding the forced-swim test, analysis was carried out by a 3x2 model (n=14 per group) with two independent variables: between-subjects genotype (3 levels: WT, MIX-HET, MAT-HET), and within-subject observer (2 levels: view 1, view 2). In order to reach the fifteen control subjects, one was estimated by exploiting a replacement dataset, with values of one median WT rat from another experiment. No rats were excluded from the study.

Multiple post-hoc comparisons were run with the Tukey HSD test, which is protected against false positives and may be used even on non significant ANOVA effects. Although the general advice is that individual post-hoc comparisons are inappropriate, when run without significant ANOVA effects or interactions, the logic behind multiple-comparison procedures does not require overall significance before making specific comparisons. These tests were designed, and their significance levels established, without regard to the overall F value (see Howell, 2010; pages 372 and 373).

EX-VIVO DATA

Immuno-fluorescence data from each brain region were tested for normality and equal variances. When data exhibited normality and equal variances, differences between groups were determined

using a one-way ANOVA (considering genotype as factor) followed by a Tukey's post-hoc test (when necessary). HDAC4 NAcc data did not display a normal distribution nor equal variances: these were analyzed by a non-parametric Kruskal–Wallis test, followed by a Dunn's post-hoc test (when necessary). Data are reported as mean±SD.

RESULTS

DAILY ACTIVITY CYCLE

We compared locomotor activity for MAT-HET, MIX-HET and WT subjects. The main effect of genotype was not significant; however, the interaction between time and genotype was significant (Time*Genotype, $F_{46, 920} = 1.902$, $p < 0.001$). The post-hoc analysis (Tukey threshold: $q = 467.8$; $df = 920$; $k = 9$) showed significant differences between activities of MAT-HET and WT subjects in waking hours. Furthermore, in their first part of sleep period, particularly at about 11 p.m., the MAT-HET rats showed a significantly higher locomotor activity also compared to MIX-HET group (see Fig. 1), which already reached WT-like rest levels.

LOCOMOTION WITH METHYLPHENIDATE

Treatment with methylphenidate showed a very clear and dose-related pharmacological effect on locomotor activity in all genotypes (Treatment $F_{2, 34} = 10.112$, $p < 0.001$) as well as the interaction with time (Time*Genotype, $F_{10, 170} = 3.646$, $p < 0.001$). Due to the elevated impact of the drug, dose-related variations of locomotor activity were almost but not entirely similar for the genotypes, with MPH1 usually about intermediate between MPH2 and vehicle (VEH).

The main effect of genotype as well as interaction between genotype and treatment did not show significance ($p >> 0.10$). Nevertheless, the post-hoc analysis (Tukey threshold: $q = 209.19$; $df = 34$; $k = 4$) displayed a strong and characteristic profile for the MAT-HET subjects. Specifically, whereas there was no difference at all between MPH1 and vehicle, so pharmacological effect was limited to MPH2, greatly elevated over both MPH1 and vehicle ($p < 0.01$). On the contrary, for both MIX-HET and WT subjects, locomotor activity did not show any significant difference between MPH2 and intermediate MPH1 levels, whereas activity after injection of MPH2 was significantly higher than vehicle group ($p < 0.01$). Since three comparisons emerged, we considered Bonferroni-corrected significance only for $p < 0.0165$; as a whole, the picture displayed that only MIX-HET and WT

subjects showed a robust and nearly significant difference between dose MPH1 and vehicle (Fig. 2), while MAT-HET rats did not.

DEPRESSIVE PHENOTYPE

There were no significant differences in latency, frequency and total duration of swimming and struggling behavior among the three groups. The diving behavior was shown only by MIX-HET and MAT-HET rats (genotype, $F_{2, 40} = 1.752$; $p = 0.1865$) while completely absent for WT controls (Fig. 3a). Total number of times for the start of a floating behavior was significantly higher for MAT-HET rats (genotype, $F_{2, 40} = 4.374$; $p = 0.0191$) compared to the remaining two genotype groups (Fig. 3b), whereas total duration and latency did not significantly differ. Finally, there was also a significant trend about climbing behavior (genotype, $F_{2, 40} = 2.604$; $p = 0.0865$), being the MIX-HET slightly more likely to climb the tank wall (Fig. 3c); the post-hoc analysis, however, just missed significance (Tukey threshold: $q = 41.87$; $df = 40$; $k = 3$).

EX-VIVO IMMUNOFLUORESCENCE

HISTONE DEACETYLASE 2 – HDAC2

Ventral and dorsal striata were investigated for the expression of HDAC isoforms, involved in epigenetic modifications in rats. One-way ANOVA on HDAC2 immuno-positivity data revealed no significant effect of genotype in nucleus accumbens shell and core ($F_{2, 15} = 0.3261$, $p = 0.7267$; $F_{2, 15} = 0.9568$, $p = 0.4064$) nor in dorsal striatum ($F_{2, 15} = 0.4331$, $p = 0.6564$).

HISTONE DEACETYLASE 4 – HDAC4

At last, HDAC4-positive immunofluorescence was assessed. When the nucleus accumbens was examined, data did not show normal distribution (Shapiro-Wilk test in shell, $W = 0.8626$, $p = 0.0135$; core, $W = 0.8668$, $p = 0.0158$); in addition, Brown-Forsythe test indicated that standard deviation among groups was statistically different in shell ($F_{2, 15} = 3.769$, $p = 0.0472$) and core ($F_{2, 15} = 3.786$, $p = 0.0467$). Thus, the non-parametric Kruskal–Wallis H test showed a significant effect of genotype on HDAC4 immuno-positivity in both shell and core ($H = 11.1$, $p < 0.001$; $H = 9.556$, $p < 0.01$). In particular, Dunn's post-hoc test indicated that HDAC4 immuno-positivity decreased (Fig. 4 a-d) in MAT-HET and MIX-HET rats with respect to WT controls in both shell ($p < 0.05$; $p < 0.01$) and core ($p < 0.05$; $p < 0.05$). No significant effects were observed in dorsal striatum ($F_{2, 15} = 0.3755$, $p = 0.6933$).

DOPAMINE TRANSPORTER - DAT

DAT-positive puncta were assessed in ventral and dorsal striata of WT, MAT-HET and MIX-HET rats. When the nucleus accumbens was examined, analysis showed a significant effect of genotype on DAT-positive puncta-density in both shell and core sub-regions ($F_{2, 15} = 7.618$, $p < 0.01$; $F_{2, 15} = 6.813$, $p < 0.01$). In particular, Tukey's post-hoc test highlighted that DAT immuno-positivity decreased in MAT-HET and MIX-HET rats with respect to WT rats in both shell ($q = 4.773$, $df = 15$, $p < 0.05$; $q = 4.788$, $df = 15$, $p < 0.05$) and core ($q = 3.736$, $df = 15$, $p < 0.05$; $q = 5.026$, $df = 15$, $p < 0.001$) sub-regions (Fig. 5a-e).

When dorsal striatum was investigated, one-way ANOVA unveiled a significant main effect of genotype on DAT-positive puncta-density ($F_{2, 15} = 40.81$, $p < 0.001$), with a significant decrease in both MAT-HET and MIX-HET rats when compared to WT rats ($q = 8.672$, $df = 15$, $p < 0.001$; $q = 12.46$, $df = 15$, $p<0.001$). Nevertheless, MAT-HET rats showed a higher DAT-positive puncta-density than MIX-HET rats ($q = 3.79$, $df = 15$, $p<0.05$) in the dorsal striatum (Fig. 5a, f-g).

DISCUSSION

The present study allowed us to highlight substantial differences between two subtypes of the same genotype, the "maternal" and "mixed" DAT-HET rats; these were born respectively from breeding a WT-female with a KO-male or a HET-female with a HET-male: of note, the former had longer rhythms of daily locomotor activity, elevated threshold for MPH-induced locomotor stimulation and enhanced escape-seeking (diving) with more transitions towards behavioral despair (floating). We assumed that (at least some of the) changes, observed in MAT vs MIX heterozygous subjects, were due to differential maternal care (further detailed in Mariano et al., 2019). In order to get further insights into the neurobiological underpinnings, we evaluated brain regions relevant to decision making, motivation, mood regulation and behavioral reactivity: in parallel, we assessed differences in DAT-positive fibers and in the chromatin-remodeling epigenetic enzymes (class I and class II HDACs, i.e. HDAC2 and HDAC4).

As for daily activity, MAT-HET subjects displayed significantly enhanced profiles, while MIX-HET subjects' locomotor profile was not significantly increased compared to WT control rats. Furthermore, during the transition to the rest phase, MIX-HET rats underwent a rapid activity decrease, reaching the same levels of control subjects. In contrast, the hyper-locomotion of MAT-

HET rats persisted during the first hours of their rest period, thus resulting significantly higher even compared to MIX-HET subjects. This made MAT-HET rats quite more similar to DAT-KOs (see Leo et al., 2018).

According to our previous study, administration of MPH had a paradoxical calming effect in hyperactive DAT-KO animals, while inducing a strong increase in locomotor activity in DAT-HET rats as in WT controls (Leo et al., 2018). However, present MAT-HET subjects displayed a somewhat sharper pharmacological response than the classical "mixed" heterozygous rats. MPH, administered at doses of 1 and 2 mg/kg i.v., caused a similar dose-related increase of locomotor activity in both WT and MIX-HET subjects. Conversely, MAT-HET rats did not show response at the lower dose, whereas only the 2 mg/kg one caused a significant increase in locomotor activity compared to both the lower dose and vehicle.

Finally, we assessed the depressive phenotype through the Forced-Swim Test (FST, Porsolt et al., 1978; Bogdanova et al., 2013; Brancato et al., 2018), one of the most commonly used protocols to assess depressive-like behavior in animal models. No significant differences resulted between animals as regards the immobility time and the latency to the initial immobility period; yet, MAT-HET subjects exhibited significantly more frequent initiation of the floating behavior, compared to MIX-HET and control groups. Notably, compared to WT ones, only both heterozygous subjects exhibited the diving behavior, consistently with a risk-prone phenotype. Furthermore, there was a marginal significance in the tendency for MIX-HET subjects to climb the tank walls. Hence, the two types of heterozygous rats manifested differential profiles for pro-active behaviors in the FST; only MIX-HET rats sought to escape by climbing, whereas only MAT-HET subjects were more often displaying transitions towards a state of behavioral despair.

The immuno-fluorescent assessment of DAT-positive puncta showed, for MAT- and MIX-HET rats, a decrease of DAT in both ventral and dorsal striatum, when compared to WT rats. A delayed DA uptake from the synaptic cleft, related to decreased DAT levels, may be responsible for the hyper-locomotion observed in the two HET experimental groups. In particular, in MIX-HET rats, the presynaptic store may presumably get exhausted earlier, as a consequence of the reduced DA uptake: this may lead to the very rapid decrease in locomotor activity observed at onset of the rest period (see Figure 1). On the contrary, the increased DAT density observed in the dorsal striatum of MAT-HET rats, with respect to MIX-HET subjects, may help preventing a functional spoiling of DA signaling and thus promoted the long-lasting hyper-locomotion. In addition, relatively higher levels of striatal DAT density can help explaining the marked change in behavioral response to MPH displayed by MAT-HET rats. Since MPH increased DA release by de-activating DAT in the striatum (Kodama et

al., 2017), much more dosage may be needed to de-activate an enhanced striatal DAT. In other words, MPH effects may be dependent on striatal DAT expression, so that a MPH1 dose was not sufficient to lower DA uptake, in the case of MAT-HET rats, as conversely observed in both MIX-HET and WT rats.

To date, few studies investigated epigenetic mechanisms associated with alterations of DAT expression. Since acetylation of histones is a dynamic process that regulates gene expression, we evaluated one class I HDAC, HDAC2, and one class II HDAC, HDAC4. Although decreased levels of HDAC2 were highlighted in the pre-frontal cortex of SZ patients (Schroeder et al., 2017) and may be responsible for reduced histone deacetylation and lack of gene-transcription repression, our data showed no differences on HDAC2, in the striatum. On the other hand, for HDAC4, we observed decreased expression in both core and shell of the nucleus accumbens, for both MAT- and MIX-HET rats with respect to WT rats. HDAC4 presents heterogeneous regional and subcellular distribution in the brain. It shuttles between cytoplasm and nucleus in response to physiological cues, and its regulation may depend on neural activity (not only within a specific region, but in a cell type-specific manner as well: see Darcy et al., 2010). In our experimental conditions, the HDAC4 expression pattern overlapped with DAT expression.

More studies are needed to assess whether there is a causal relationship between DA signaling alterations and HDAC4 regulation. Interestingly, drugs of abuse inhibited nuclear class II HDAC activity, resulting in increased histone acetylation in the nucleus accumbens and creating a permissive environment for drug-induced gene expression (Kumar et al., 2005; Host et al., 2011; Griffin et al., 2017). On the other hand, the repressive properties of class II HDACs on drug-related plasticity and behavior have been described: over-expression of HDAC4 in the nucleus accumbens inhibited conditioned place preference and motivation for cocaine self-administration (Kumar et al., 2005; Wang et al., 2010). It is tempting to speculate that this reduced HDAC4 and DAT levels may increase availability of DA in the ventral striatum of all our HET rats.

THE EPIGENETIC MARK ON A GENETICALLY-VULNERABLE GENOTYPE

Here, we present data about long-term sequelae of being cared by either a WT or a DAT-HET mother. The former (unconventional) situation increases vulnerability to behavioral transitions: the offspring of WT dams (and of KO males) manifested a rather complex phenotype, consisting of 1) hyper-locomotion and escape-seeking behavior; 2) a sharp increase in the slope of MPH dose-response curves; 3) higher transitions towards states of behavioral despair.

The causal factors of most psychiatric diseases are so far unclear. Nevertheless, human studies involving twins or national registers and, more recently, genome-wide association studies (GWAS) showed that genetic factors play an important role (Franke et al., 2009; Polderman et al., 2015; Pettersson et al., 2016). In a recent study, Petterson et al. (2018) found that ADHD, ASD, BD, and SZ showed heritability between 51 and 80%. The remaining variance indicated that non-genetic factors, perhaps including epigenetically-mediated processes, played important roles. Furthermore, human studies demonstrated a systematic over-transmission of paternal alleles at candidate genes associated with ADHD, including DAT1 (Hawi et al., 2005; Hawi et al., 2010). Such data denoted a parent-of-origin effect (POE), not necessarily due to an imprinting process. To mimic a paternal over-transmission of susceptibility alleles, we run the present experiment with KO fathers bred with WT mothers, therefore producing HET subjects -- but differently from classical HET × HET breeding.

Differences between MAT- and MIX-HET phenotypes, being both DAT-heterozygous (+/-), can be ascribed to mechanisms including: 1) parental imprinting, i.e. parent-specific gene expression; 2) maternal effect, i.e. hormonal or environmental factors, including the intrauterine environment and early post-natal life; 3) a combination of the above. Note that the functional allele is always maternal in MAT-HET rats, as paternal allele expression would render these subjects similar to DAT-KOs. Conversely, MIX-HET animals are more similar to WTs.

In MAT-HET rats, the overlap of internalizing (behavioral despair) and externalizing (escape seeking) symptoms is combined with hyper-locomotion, a difficulty to get asleep, and a quantal rather than graded dose-response curve. This, notably, provided a complex behavioral framework that may well represent a model of BD (Meier et al., 2018). Notably, polymorphisms of DAT gene have been linked with BD (Greenwood et al, 2006; Pinsonneault et al, 2011): reduced DAT levels were found in unmedicated BD patients (Anand et al, 2011). Furthermore, DAT hypomorphic mice (DAT-HY: 50% expression) exhibited consistent hyper-sensitivity to summer-like and winter-like photo-periods, including more mania-relevant and depression-relevant behaviors (Young et al., 2018). Further studies following e.g. lithium administration should be carried out to specifically test the presently suggested notion.

Recently, multiple studies have observed that psychiatric disorders such as SZ, ADHD, ASD and BD showed common etiological risk factors (Chen et al., 2018; Wen et al., 2018; Sengupta et al., 2018), including the rare, yet functional, DAT coding variant Val559 (Thal et al., 2018). These disorders could be considered as extremes on a spectrum of overlapping traits (Thapar et al., 2017). In particular, ADHD and depression, including BD, often co-occur in the same patient (Meier et al., 2018; Aedo et al., 2018; Pinna et al., 2019). There are also some evidences of POE in depression:

children of fathers with a history of ADHD rated themselves as more depressed than did those of mothers with a history of ADHD (Goos et al., 2007). Consistently with the aforementioned results, present DAT-heterozygous rats displayed significantly different epigenetic-machinery patterns in relation to the parent carrier of the functional allele.

FUTURE PERSPECTIVES

We recently described some behavioral anomalies of DAT-HET rats, which however were born from a classical HET×HET breeding (Adinolfi et al., 2018, 2019). The phenotypic characterization of MAT-HET subjects, born from a KO father bred with a WT dam, could help to explore both genetic and epigenetic mechanism of complex psychiatric diseases. We underline that obtaining a DAT-heterozygous offspring under a classical breeding regimen leads to some limitations: 1) all pups including WTs are raised by a HET dam, hence hampering their validity as controls; and 2) for MIX-HET rats, the parent transmitting the (non) functional allele is entirely unknown. The MAT-HET rats are more adherent to real world, since the offspring is: 1) raised by a WT dam, hence allowing a more tight comparison with full WT controls; and 2) the father is transmitting the susceptibility gene, allowing to model such inheritance. Future work is warranted by exploiting the various breeding possibilities further. In line of principle, a lineage (namely, next generations coming from these epigenetically-differing HET subjects) may well be explored: offspring of MAT-HET and MIX-HET females could - in turn - differ as well. In that case, the question would be what exactly is inherited through generations. One possibility may be a different style of maternal care, in turn coming from their own mothers (namely, offspring would have a WT compared to a HET grand-dam). As for the exact phenotypes, it would be worthy to explore traits related to inhibitory control, for instance with tasks for impulsive and/or compulsive behavior.

FOOTNOTE

(1) Of note, a specular breeding of WT male rats with KO females was also tried but success has been hampered because none of the KO females ever got pregnant.

CAPTIONS

Figure 1. Daily activity cycle (counts per hour, Mean ± SEM): in waking hours, the locomotor activity of MAT-HET rats (within home-cages) was significantly higher than WT group, with MIX-HET subjects being intermediate (n = 15; * P < 0.001); furthermore, in their first part of sleeping period, MAT-HET rats showed a significantly higher locomotor activity compared also to the MIX-HET group ($ P < 0.001).

Figure 2. Locomotor activity (counts per bin, Mean ± SEM) with acute methylphenidate (0, 1, 2 mg/kg i.v.): original 6-min bins were collapsed five-by-five; shown is average over three hours of observation. We considered Bonferroni-corrected significance only for $p < 0.0165$; a significant difference emerged between pharmacological effect of MPH2 and basal levels for both MPH1 and vehicle in MAT-HET subjects. Conversely, there was a similar profile for both WT and MIX-HET groups, where MPH1 levels were about intermediate between MPH2 effect and vehicle baseline (n = 5 per dosage group; ** $p < 0.01$).

Figure 3. Behavior (Mean ± SEM) in the Porsolt's Forced-Swimming Test. a) Diving (total duration): during the second test exposure, diving was shown only by MIX-HET and MAT-HET rats (n = 14; p = 0.1865). **b) Floating (total number per 5-min session):** MAT-HET rats exhibited floating more frequently, and significantly if compared to MIX-HET and WT genotype groups (n = 14; * p = 0.0191). **c) Climbing (total duration):** MIX-HET rats exhibited slightly more climbing, compared to MAT-HET and WT subjects (n = 14; p = 0.0865).

Figure 4. HDAC4 immuno-fluorescence in the striatum. MAT-HET and MIX-HET rats displayed decreased HDAC4 immuno-fluorescence in the shell (a, b) and core (c, d) subregions of the nucleus accumbens with respect to WT rats. No significant differences were observed in the dorsal striatum. * $p<0.05$ versus WT, scale bar 100 μm.

Figure 5. DAT immuno-fluorescence in the striatum. DAT-positive puncta-density was evaluated in dorsal and ventral striatum, both shell and core subregions (a). MAT-HET and MIX-HET rats displayed decreased DAT-positive puncta-density in the shell (b, c) and core (d, e) subregions of the nucleus accumbens, and in the dorsal striatum (f, g), with respect to WT rats. Increased DAT-positive puncta-density was observed in the dorsal striatum of MAT-HET with respect to MIX-HET rats (f, g). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus WT; ^ $p<0.05$ versus MIX-HET, scale bar 100 μm.

REFERENCES

Adinolfi, A., Carbone, C., Leo, D., Gainetdinov, R.R., Laviola, G., Adriani, W. (2018). Novelty-related behavior of young and adult dopamine transporter knockout rats: Implication for cognitive and emotional phenotypic patterns. *Genes Brain Behav.* **17**, e12463.

Adinolfi, A., Zelli, S., Leo, D., Carbone, C., Mus, L., Illiano, P., Alleva, E., Gainetdinov, R.R., Adriani, W. (2019). Behavioral characterization of DAT-KO rats and evidence of asocial-like phenotypes in DAT-HET rats: The potential involvement of norepinephrine system. *Behav Brain Res.* **359**, 516-527.

Aedo, A., Murru, A., Sanchez, R., Grande, I., Vieta, E., Undurraga, J. (2018). Clinical characterization of rapid cycling bipolar disorder: Association with attention deficit hyperactivity disorder. *J Affect Disord.* **240**, 187-192.

Anand, A., Barkay, G., Dzemidzic, M., Albrecht, D., Karne, H., Zheng, Q.H., Hutchins, G.D., Normandin, M.D., Yoder, K.K. (2011). Striatal dopamine transporter availability in unmedicated bipolar disorder. *Bipolar Disord.* **13**, 406-13.

Bannon, M.J., Michelhaugh, S.K., Wang, J., Sacchetti, P. (2001). The human dopamine transporter gene: gene organization, transcriptional regulation, and potential involvement in neuropsychiatric disorders. *Eur Neuropsychopharmacol.* **11**, 449-55.

Barr, W.B. (2001). Schizophrenia and attention deficit disorder. Two complex disorders of attention. *Ann N Y Acad Sci.* **931**, 239-50.

Bogdanova, O.V., Kanekar, S., D'Anci, K.E., Renshaw, P.F. (2013). Factors influencing behavior in the forced swim test. *Physiol Behav.* **118**, 227-39.

Brancato, A., Bregman, D., Ahn, H.F., Pfau, M.L., Menard, C., Cannizzaro, C., Russo, S.J., Hodes, G.E. (2017). Sub-chronic variable stress induces sex-specific effects on glutamatergic synapses in the nucleus accumbens. *Neuroscience* ***350***,180-189.

Brancato, A., Plescia, F., Lavanco, G., Cavallaro, A., Cannizzaro, C. (2016). Continuous and intermittent alcohol free-choice from pre-gestational time to lactation: Focus on drinking trajectories and maternal behavior. *Front Behav Neurosci.* **10**, 31.

Brown, A.J., Fisher, D.A., Kouranova, E., McCoy, A., Forbes, K.,Wu, Y., Henry, R., Ji, D., Chambers, A., Warren, J., Shu, W., Weinstein, E.J., Cui, X. (2013). Whole-rat conditional gene knockout via genome editing. *Nat Methods.* **10**, 638–640.

Burke, A.R., Watt, M.J., Forster, G.L. (2011). Adolescent social defeat increases adult amphetamine conditioned place preference and alters D2 dopamine receptor expression. *Neuroscience.* **197**, 269-79.

Carboni, E., Tanda, G.L., Frau, R., Di Chiara, G. (1990). Blockade of the noradrenaline carrier increases extracellular dopamine concentrations in the prefrontal cortex: evidence that dopamine is taken up in vivo by noradrenergic terminals. *J Neurochem.* **55**, 1067-70.

Chen, Q., Hartman, C.A., Haavik, J., Harro, J., Klungsøyr, K., Hegvik, T.A., Wanders, R., Ottosen, C., Dalsgaard, S., Faraone, S.V., Larsson, H. (2018). Common psychiatric and metabolic comorbidity of adult attention-deficit/hyperactivity disorder: A population-based cross-sectional study. *PLoS One.* **13**, e0204516.

Chirico, D., Romano E., Famele M., Draisci R., Mancinelli R., Pascucci T., Adriani W. (2017). Forced but not free-choice nicotine during lactation alters maternal behavior and noradrenergic system of pups: Impact on social behavior of adolescent isolated male rats. *Neuroscience* **361**, 6–18.

Cinque, S., Zoratto, F., Poleggi, A., Leo, D., Cerniglia, L., Cimino, S., Tambelli, R., Alleva, E., Gainetdinov, R.R., Laviola, G., Adriani, W. (2018). Behavioral phenotyping of dopamine transporter knockout Rats: Compulsive traits, motor stereotypies, and anhedonia. *Front Psychiatry.* **9**, 43.

Cole, B.J., Robbins, T.W. (1989). Effects of 6-hydroxydopamine lesions of the nucleus accumbens septi on performance of a 5-choice serial reaction time task in rats: implications for theories of selective attention and arousal. *Behav Brain Res.* **33**, 165-79.

Darcy, M.J., Calvin, K., Cavnar, K., Ouimet, C.C. (2010). Regional and subcellular distribution of HDAC4 in mouse brain. *J Comp Neurol.* **518**, 722-40.

Delaval, K., Feil, R. (2004). Epigenetic regulation of mammalian genomic imprinting. *Curr Opin Genet Dev.* **14**, 188-95.

Drury, S.S., Theall, K.P., Keats, B.J., Scheeringa, M. (2009). The role of the dopamine transporter (DAT) in the development of PTSD in preschool children. *J Trauma Stress.* **22**, 534-9. doi: 10.1002/jts.20475.

Fischer, A., Sananbenesi, F., Mungenast, A., Tsai, L.H. (2010). Targeting the correct HDAC(s) to treat cognitive disorders. *Trends Pharmacol Sci.* **31**, 605-17.

Franke, B., Neale, B.M., Faraone, S.V. (2009). Genome-wide association studies in ADHD. *Hum Genet.* **126**, 13-50.

Geurts, A.M., Cost, G.J., Freyvert, Y., Zeitler, B., Miller, J.C., Choi, V.M., Jenkins, S.S., Wood, A., Cui, X., Meng, X., Vincent, A., ... Buelow, R. (2009). Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* **325**, 433.

Giros, B., Caron, M.G. (1993). Molecular characterization of the dopamine transporter. *Trends Pharmacol Sci.* **14**, 43-9.

Giros, B., Jaber, M., Jones, S.R., Wightman, R.M., Caron, M.G. (1996). Hyper-locomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379**, 606-12.

Gomes, F.V., Zhu, X., Grace, A.A. (2019). The pathophysiological impact of stress on the dopamine system is dependent on the state of the critical period of vulnerability. *Mol Psychiatry.* [Epub ahead of print] doi: 10.1038/s41380-019-0514-1.

González, B., Pantoja, C.R.G., Sosa, M.H., Vitullo, A.D., Bisagno, V., González, C.R. (2018). Cocaine alters the mouse testicular epigenome with direct impact on histone acetylation and DNA methylation marks. *Reprod Biomed Online.* **37**, 269-278. doi: 10.1016/j.rbmo.2018.05.014. Epub 2018 Aug 17.

Goos, L.M., Ezzatian, P., Schachar, R. (2007). Parent-of-origin effects in attention-deficit hyperactivity disorder. *Psychiatry Res.* **149**, 1-9.

Grandjean, V., O'Neill, L., Sado, T., Turner, B., Ferguson-Smith, A. (2001). Relationship between DNA methylation, histone H4 acetylation and gene expression in the mouse imprinted Igf2-H19 domain. *FEBS Lett.* **488**, 165-9.

Green, A.L., Hossain, M.M., Tee, S.C., Zarbl, H., Guo, G.L., Richardson, J.R. (2015). Epigenetic Regulation of Dopamine Transporter mRNA Expression in Human Neuro-blastoma Cells. *Neurochem Res.* **40**, 1372-8.

Green, A.L., Zhan, L., Eid, A., Zarbl, H., Guo, G.L., Richardson, J.R. (2017). Valproate increases dopamine transporter expression through histone acetylation and enhanced promoter binding of Nurr1. *Neuropharmacology* **125**, 189-196.

Greenwood, T.A., Schork, N.J., Eskin, E., Kelsoe, J.R. (2006). 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* **11**, 125-33.

Griffin, E.A. Jr, Melas, P.A., Zhou, R., Li, Y., Mercado, P., Kempadoo, K.A., Stephenson, S., Colnaghi, L., Taylor, K., Hu, M.C., Kandel, E.R., Kandel, D.B. (2017). Prior alcohol use enhances vulnerability to compulsive cocaine self-administration by promoting degradation of HDAC4 and HDAC5. *Sci Adv.* **3**, e1701682.

Hahn, M.K., Blakely, R.D. (2007). The functional impact of SLC6 transporter genetic variation. *Annu Rev Pharmacol Toxicol.* **47**, 401-41.

Hawi, Z., Kent, L., Hill, M., Anney, R.J., Brookes, K.J., Barry, E., Franke, B., Banaschewski, T., Buitelaar, J., Ebstein, R., Miranda, A., Oades, R.D., ... Gill, M. (2010). ADHD and DAT1: further evidence of paternal over-transmission of risk alleles and haplotype. *Am J Med Genet B Neuropsychiatr Genet.* **153B,** 97-102.

Hawi, Z., Segurado, R., Conroy, J., Sheehan, K., Lowe, N., Kirley, A., Shields, D., Fitzgerald, M., Gallagher, L., Gill, M. (2005). Preferential transmission of paternal alleles at risk genes in attention-deficit/hyperactivity disorder. *Am J Hum Genet.* **77**, 958-65.

Holmes, R., Soloway, P.D. (2006). Regulation of imprinted DNA methylation. *Cytogenet Genome Res.* **113**, 122-9.

Host, L., Dietrich, J.B., Carouge, D., Aunis, D., Zwiller, J. (2011). Cocaine self-administration alters the expression of chromatin-remodelling proteins; modulation by histone deacetylase inhibition. *J Psychopharmacol.* **25**, 222-9.

Howell, D.C. (2010). *Statistical methods for psychology* (8th ed.). Belmont, CA: Cengage Wadsworth. ISBN-13: 978-1-111-83548-4

Jones, S.R., Gainetdinov, R.R., Jaber, M., Giros, B., Wightman, R.M., Caron, M.G. (1998). Profound neuronal plasticity in response to inactivation of the dopamine transporter. *Proc Natl Acad Sci U S A.* **95**, 4029-34.

Käenmäki, M., Tammimäki, A., Myöhänen, T., Pakarinen, K., Amberg, C., Karayiorgou, M., Gogos, J.A., Männistö, P.T. (2010). Quantitative role of COMT in dopamine clearance in the prefrontal cortex of freely moving mice. *J Neurochem.* **114**, 1745-55. doi: 10.1111/ j.1471-4159.2010.06889.x.

Khodayari, N., Garshasbi, M., Fadai, F., Rahimi, A., Hafizi, L., Ebrahimi, A., Najmabadi, H., Ohadi, M. (2004). Association of the dopamine transporter gene (DAT1) core promoter polymorphism -67T variant with schizophrenia. *Am J Med Genet B Neuropsychiatr Genet.* **129B**, 10-2.

Kim, H.F., Ghazizadeh, A., Hikosaka, O. (2015). Dopamine neurons encoding Long-Term Memory of object value for habitual behavior. *Cell.* **163**, 1165-1175.

Kodama, T., Kojima, T., Honda, Y., Hosokawa, T., Tsutsui, K.I., Watanabe, M. (2017). Oral administration of methylphenidate (Ritalin) affects dopamine release differentially between the prefrontal cortex and striatum: A Microdialysis Study in the Monkey. *J Neurosci.* **37**, 2387-2394.

Konstantakopoulos, G., Dimitrakopoulos, S., Michalopoulou, P.G. (2015). Drugs under early investigation for the treatment of bipolar disorder*. Expert Opin Investig Drugs.* **24**, 477-90. doi: 10.1517/13543784.2015.1019061.

Kopsida, E., Mikaelsson, M.A., Davies, W. (2011). The role of imprinted genes in mediating susceptibility to neuropsychiatric disorders. *Horm Behav.* **59**, 375-82.

Kumar, A., Choi, K.H., Renthal, W., Tsankova, N.M., Theobald, D.E., Truong, H.T., Russo, S.J., Laplant, Q., Sasaki, T.S., Whistler, K.N., Neve, R.L., Self, D.W., Nestler, E.J. (2005). Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. *Neuron.* **48**, 303-14.

Kurian, M.A., Gissen, P., Smith, M., Heales, S. Jr, Clayton, P.T. (2011). The monoamine neurotransmitter disorders: an expanding range of neurological syndromes. *Lancet Neurol.* **10**, 721-33.

Leo, D., Adriani, W., Cavaliere, C., Cirillo, G., Marco, E.M., Romano, E., di Porzio, U., Papa, M., Perrone-Capano, C., Laviola, G. (2009). Methylphenidate to adolescent rats drives enduring changes of accumbal Htr7 expression: implications for impulsive behavior and neuronal morphology. *Genes Brain Behav.* **8**, 356-68.

Leo, D., Sukhanov, I., Zoratto, F., Illiano, P., Caffino, L., Sanna, F., Messa, G., Emanuele, M., Esposito, A., Dorofeikova, M., ... Adriani, W., Gainetdinov, R.R. (2018). Pronounced Hyperactivity, Cognitive Dysfunctions, and BDNF Dysregulation in Dopamine Transporter Knock-out Rats. *J Neurosci.* **38**, 1959-1972.

Mariano, S., Pardo, M., Illiano, P., Buccheri, C., Adinolfi, A., Lo Russo, S.L.M., Alleva, E., Carbone, C., Adriani, W. (2019). Own or dam's genotype? The classical colony breeding may bias spontaneous and stress-challenged activity in DAT mutant rats. *Devl Psychobiol,* (in press).

Mazei-Robison, M.S., Blakely, R.D. (2005). Expression studies of naturally occurring human dopamine transporter variants identifies a novel state of transporter inactivation associated with Val-382-Ala. *Neuropharmacology* **49**, 737–749.

Meier, S.M., Pavlova, B., Dalsgaard, S., Nordentoft, M., Mors, O., Mortensen, P.B., Uher, R. (2018). Attention-deficit hyperactivity disorder and anxiety disorders as precursors of bipolar disorder onset in adulthood. *Br J Psychiatry* **213**, 555-560.

Misztak, P., Pańczyszyn-Trzewik, P., Sowa-Kućma M. (2018). Histone de-acetylases (HDACs) as therapeutic target for depressive disorders. *Pharmacol Rep.* **70**, 398-408. doi: 10.1016/j.pharep.2017.08.001. Epub 2017 Aug 12.

Novick, A.M., Forster, G.L., Hassell, J.E., Davies, D.R., Scholl, J.L., Renner, K.J., Watt, M.J. (2015). Increased dopamine transporter function as a mechanism for dopamine hypoactivity in the adult infralimbic medial prefrontal cortex following adolescent social stress. *Neuropharmacology* **97**, 194-200.

Owesson-White, C., Belle, A.M., Herr, N.R., Peele, J.L., Gowrishankar, P., Carelli, R.M., Wightman, R.M. (2016). Cue-evoked dopamine release rapidly modulates D2 neurons in the Nucleus Accumbens during motivated behavior. *J Neurosci.* **36**, 6011-21.

Paloyelis, Y., Asherson, P., Mehta, M.A., Faraone, S.V., Kuntsi, J. (2010). DAT1 and COMT effects on delay discounting and trait impulsivity in male adolescents with attention deficit/hyperactivity disorder and healthy controls. *Neuropsychopharmacology.* **35**, 2414-26.

Pattij, T., Janssen, M.C., Vanderschuren, L.J., Schoffelmeer, A.N., van Gaalen, M.M. (2007). Involvement of dopamine D1 and D2 receptors in the nucleus accumbens core and shell in inhibitory response control. *Psychopharmacology (Berl).* **191**, 587-98.

Paxinos, G., Watson, C. (2013). *The Rat Brain in Stereotaxic Coordinates 7th Edition.* Academic Press.

Perona, M.T., Waters, S., Hall, F.S., Sora, I., Lesch, K.P., Murphy, D.L., Caron, M., Uhl, G.R. (2008). Animal models of depression in dopamine, serotonin, and norepinephrine transporter knockout mice: prominent effects of dopamine transporter deletions. *Behav Pharmacol.* **19**, 566-74. doi: 10.1097/FBP.0b013e32830cd80f.

Pettersson, E., Larsson, H., Lichtenstein, P. (2016). Common psychiatric disorders share the same genetic origin: a multivariate sibling study of the Swedish population. *Mol Psychiatry* **21**, 717-21.

Pettersson, E., Lichtenstein, P., Larsson, H., Song, J.; ... Stahl E, Walters JTR, Walters R, Sullivan PF, Posthuma D, Polderman TJC. (2018). Genetic influences on eight psychiatric disorders based on family data of 4 408 646 full and half-siblings, and genetic data of 333 748 cases and controls. *Psychol Med.* **17**, 1-8.

Pinna, M., Visioli, C., Rago, C.M., Manchia, M., Tondo, L., Baldessarini, R.J. (2019). Attention deficit-hyperactivity disorder in adult bipolar disorder patients. *J Affect Disord.* **243**, 391-396.

Pinsonneault, J.K., Han, D.D., Burdick, K.E., Kataki, M., Bertolino, A., Malhotra, A.K., Gu, H.H., Sadee, W. (2011). Dopamine transporter gene variant affecting expression in human brain is associated with bipolar disorder. *Neuropsychopharmacology* **36**, 1644-55.

Polderman, T.J., Benyamin, B., de Leeuw, C.A., Sullivan, P.F., van Bochoven, A., Visscher, P.M., Posthuma, D. (2015). Meta-analysis of the heritability of human traits based on fifty years of twin studies. *Nat Genet.* **47**, 702-9.

Porsolt, R.D., Anton, G., Blavet, N., Jalfre, M. (1978). Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur J Pharmacol.* **47**, 379–91.

Pozzi, L., Invernizzi, R., Cervo, L., Vallebuona, F., Samanin, R. (1994). Evidence that extracellular concentrations of dopamine are regulated by noradrenergic neurons in the frontal cortex of rats. *J Neurochem.* **63**, 195-200.

Psychiatric GWAS Consortium Coordinating Committee, Cichon, S., Craddock, N., Daly, M., Faraone, S.V., Gejman, P.V., Kelsoe, J., Lehner, T., Levinson, D.F., Moran, A., Sklar, P., Sullivan, P.F. (2009). Genome-wide association studies: history, rationale, and prospects for psychiatric disorders. *Am J Psychiatry. 166*: 540-56.

Renthal, W., Maze, I., Krishnan, V., Covington, H.E. 3rd, Xiao, G., Kumar, A., Russo, S.J., Graham, A., Tsankova, N., Kippin, T.E., ... Nestler, E.J. (2007). Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron.* **56**, 517-29.

Saitoh, S., Wada, T. (2000). Parent-of-origin specific histone acetylation and reactivation of a key imprinted gene locus in Prader-Willi syndrome. *Am J Hum Genet.* **66**, 1958-62.

Schroeder, F.A., Gilbert, T.M., Feng, N., Taillon, B.D., Volkow, N.D., Innis, R.B., Hooker, J.M., Lipska, B.K. (2017). Expression of HDAC2 but Not HDAC1 Transcript Is Reduced in Dorsolateral Prefrontal Cortex of Patients with Schizophrenia. *ACS Chem Neurosci.* **8**, 662-668.

Sengupta, S.M., Fotopoulos, N., Devenyi, G.A., Fortier, M.È., Ter-Stepanian, M., Sagliker, S., Karama, S., Mallar Chakravarty, M., Labbe, A., Grizenko, N., Joober, R. (2018). Dissecting genetic cross-talk between ADHD and other neurodevelopmental disorders: Evidence from behavioural, pharmacological and brain imaging investigations. *Psychiatry Res.* **269**, 652-657.

Shumay, E., Fowler, J.S., Volkow, N.D. (2010). Genomic features of the human dopamine transporter gene and its potential epigenetic States: implications for phenotypic diversity. *PLoS One.* **5**, e11067.

Sokolowski, J.D., Salamone, J.D. (1994). Effects of dopamine depletions in the medial prefrontal cortex on DRL performance and motor activity in the rat. *Brain Res.* **642**, 20-8.

Svensson, K., Mattsson, R., James, T.C., Wentzel, P., Pilartz, M., MacLaughlin, J., Miller, S.J., Olsson, T., Eriksson, U.J., Ohlsson, R. (1998). The paternal allele of the H19 gene is progressively silenced during early mouse development: the acetylation status of histones may be involved in the generation of variegated expression patterns. *Development.* **125**, 61-9.

Thal, L.B., Tomlinson, I.D., Quinlan, M.A., Kovtun, O., Blakely, R.D., Rosenthal, S.J. (2019). Single quantum dot Imaging reveals PKCβ-Dependent alterations in membrane diffusion and clustering of an Attention-Deficit Hyperactivity Disorder / Autism / Bipolar Disorder-associated Dopamine Transporter variant. *ACS Chem Neurosci.* **10**, 460-471

Thapar, A., Cooper, M., Rutter, M. (2017). Neurodevelopmental disorders. *Lancet Psychiatry* **4**, 339-346.

Tremolizzo, L., Carboni, G., Ruzicka, W.B., Mitchell, C.P., Sugaya, I., Tueting, P., Sharma, R., Grayson, D.R., Costa, E., Guidotti, A. (2002). An epigenetic mouse model for molecular and behavioral neuropathologies related to schizophrenia vulnerability. *Proc Natl Acad Sci U S A* **99**, 17095-17100.

Tsutsui-Kimura, I., Ohmura, Y., Izumi, T., Kumamoto, H., Yamaguchi, T., Yoshida, T., Yoshioka, M. (2013). Milnacipran enhances the control of impulsive action by activating D_1-like receptors in the infralimbic cortex. *Psychopharmacology (Berl).* **225**, 495-504.

Vengeliene, V., Bespalov, A., Roßmanith, M., Horschitz, S., Berger, S., Relo, A.L., Noori, H.R., Schneider, P., Enkel, T., Bartsch, D., Schneider, M., Behl, B., Hansson, A.C., Schloss, P., Spanagel, R. (2017) Towards trans-diagnostic mechanisms in psychiatry: neurobehavioral profile of rats with a loss-of-function point mutation in the dopamine transporter gene. *Dis Model Mech,* **10**, 451-461.

Volkow, N.D., Wang, G.J., Fowler, J.S., Gatley, S.J., Logan, J., Ding, Y.S., Hitzemann, R., Pappas, N. (1998). Dopamine transporter occupancies in the human brain induced by therapeutic doses of oral methylphenidate. *Am J Psychiatry.* **155**, 1325-31.

Waldman, I.D., Rowe, D.C., Abramowitz, A., Kozel, S.T., Mohr, J.H., Sherman, S.L., Cleveland, H.H., Sanders, M.L., Gard, J.M., Stever, C. (1998). Association and linkage of the dopamine transporter gene and attention-deficit hyperactivity disorder in children: heterogeneity owing to diagnostic subtype and severity. *Am J Hum Genet.* **63**, 1767-76.

Wang, L., Lv, Z., Hu, Z., Sheng, J., Hui, B., Sun, J., Ma, L. (2010). Chronic cocaine-induced H3 acetylation and transcriptional activation of CaMKII-alpha in the nucleus accumbens is critical for motivation for drug reinforcement. *Neuro-psycho-pharmacology.* **35**, 913-28.

Watt, M.J., Roberts, C.L., Scholl, J.L., Meyer, D.L., Miiller, L.C., Barr, J.L., Novick, A.M., Renner, K.J., Forster, G.L. (2014). Decreased prefrontal cortex dopamine activity following adolescent social defeat in male rats: role of dopamine D2 receptors. *Psycho-pharmacology (Berl).* **231**, 1627-36.

Wen, Y., Zhang, F., Ma, X., Fan, Q., Wang, W., Xu, J., Zhu, F., Hao, J., He, A., Liu, L., Liang, X., Du, Y., Li, P., Wu, C., Wang, S., Wang, X., Ning, Y., Guo, X. (2018). eQTLs weighted genetic correlation analysis detected brain region differences in genetic correlations for complex psychiatric disorders. *Schizophr Bull*. (in press).

Wilkins, J.F., Haig, D. (2003). What good is genomic imprinting: the function of parent-specific gene expression. *Nat Rev Genet.* **4**, 359-68.

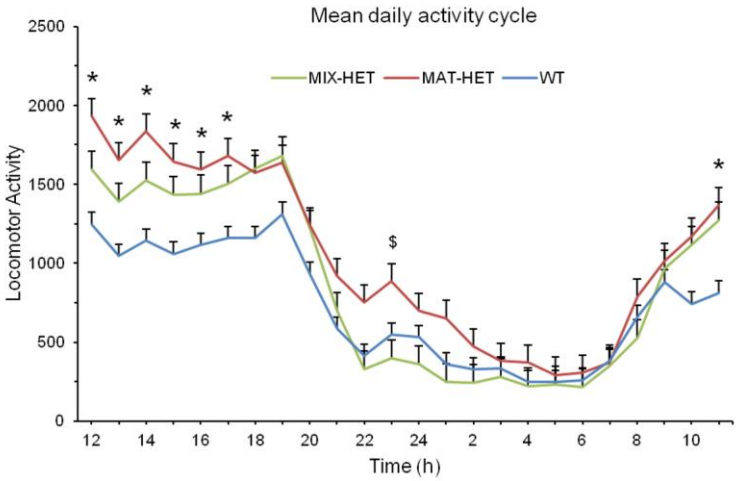
Wright, L.D., Hébert, K.E., Perrot-Sinal, T.S. (2008). Periadolescent stress exposure exerts long-term effects on adult stress responding and expression of prefrontal dopamine receptors in male and female rats. *Psychoneuroendocrinology* **33**, 130-42.

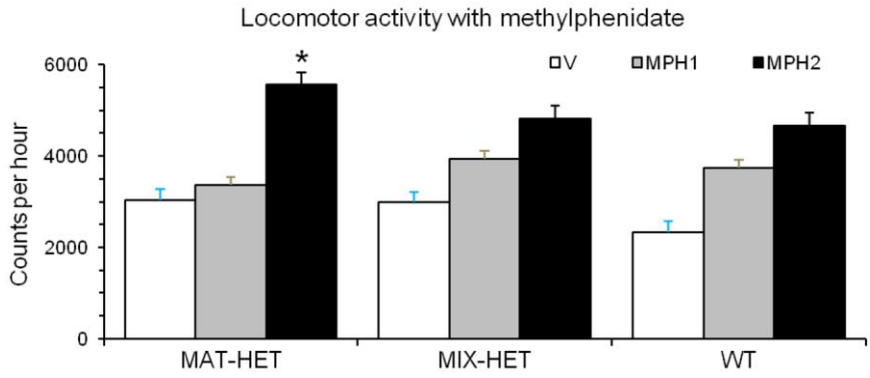
Yamamoto, B.K., Novotney, S. (1998). Regulation of extracellular dopamine by the norepinephrine transporter. *J Neurochem.* **71**, 274-80.

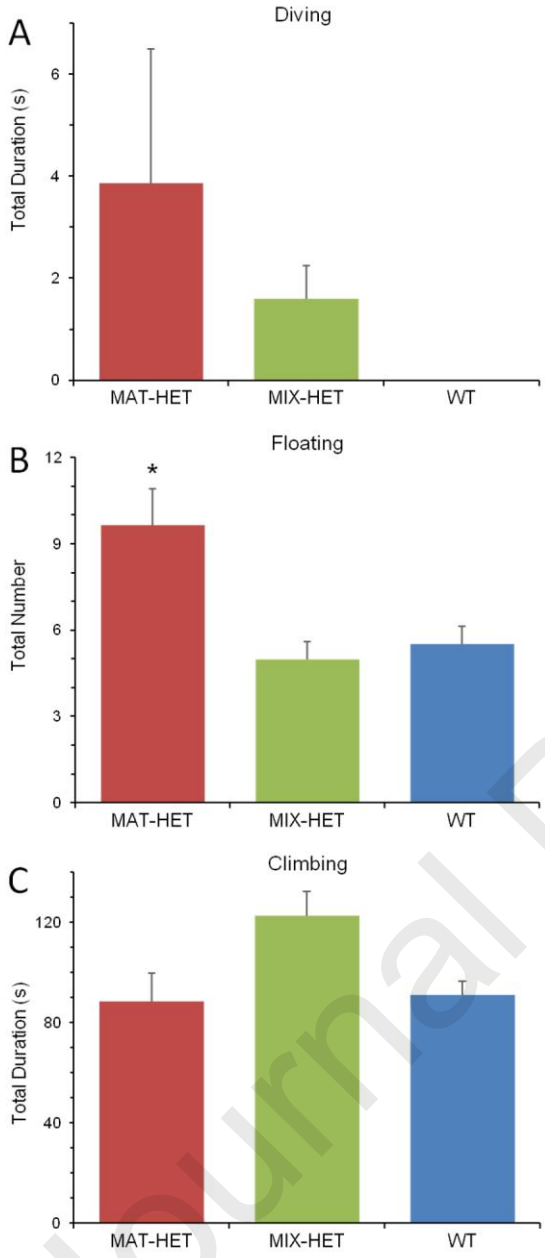
Yang, B., Chan, R.C., Jing, J., Li, T., Sham, P., Chen, R.Y. (2007). A meta-analysis of association studies between the 10-repeat allele of a VNTR polymorphism in the 3'-UTR of dopamine transporter gene and attention deficit hyperactivity disorder. *Am J Med Genet B Neuropsychiatr Genet.* **144B,** 541-50.

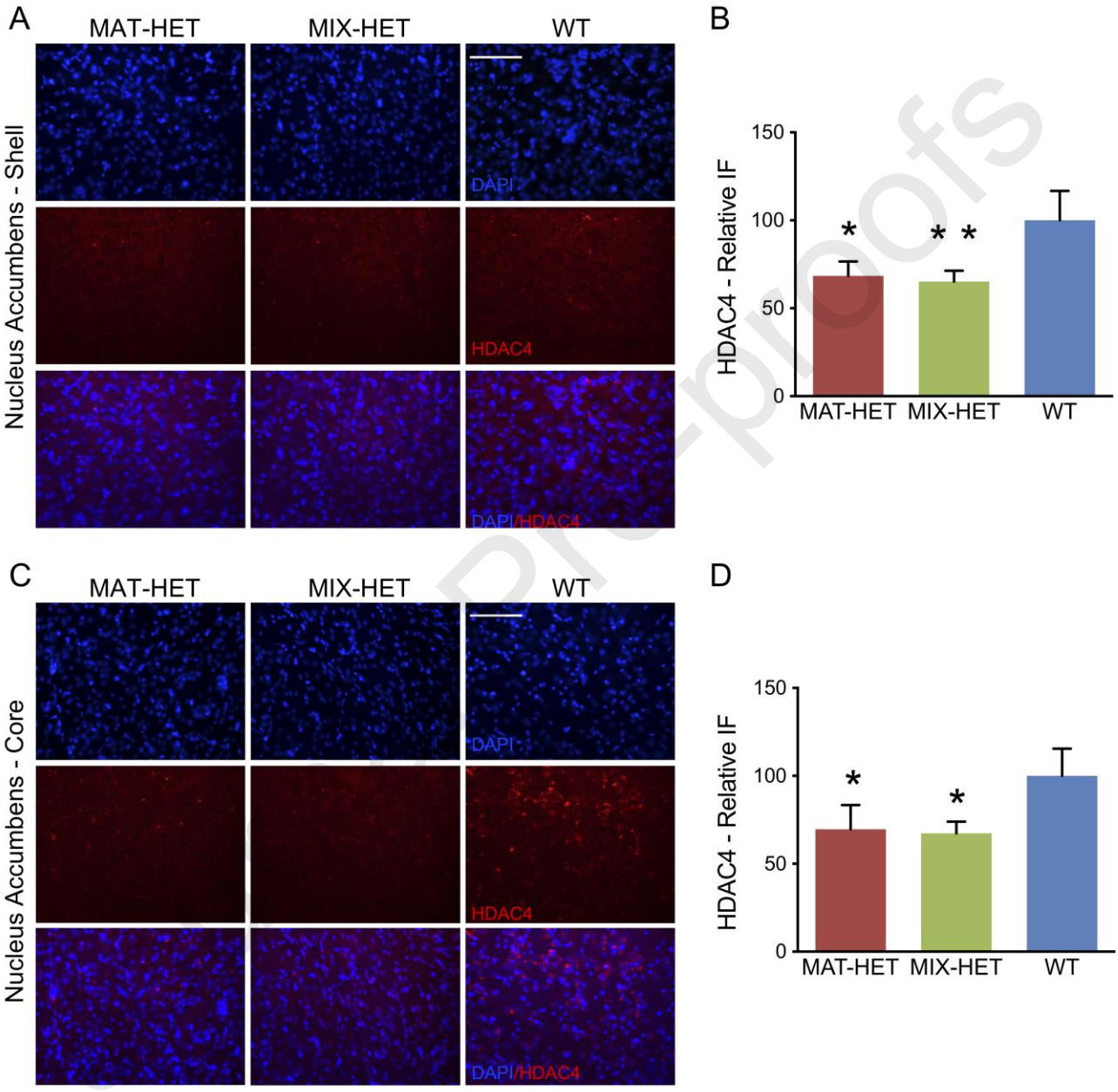
Young, J.W., Cope, Z.A., Romoli, B., Schrurs, E., Aniek, J., van Enkhuizen, J., Sharp, R.F., Dulcis, D. (2018). Mice with reduced DAT levels recreate seasonal-induced switching between states in bipolar disorder. *Neuropsychopharmacology* **43**, 1721-1731.

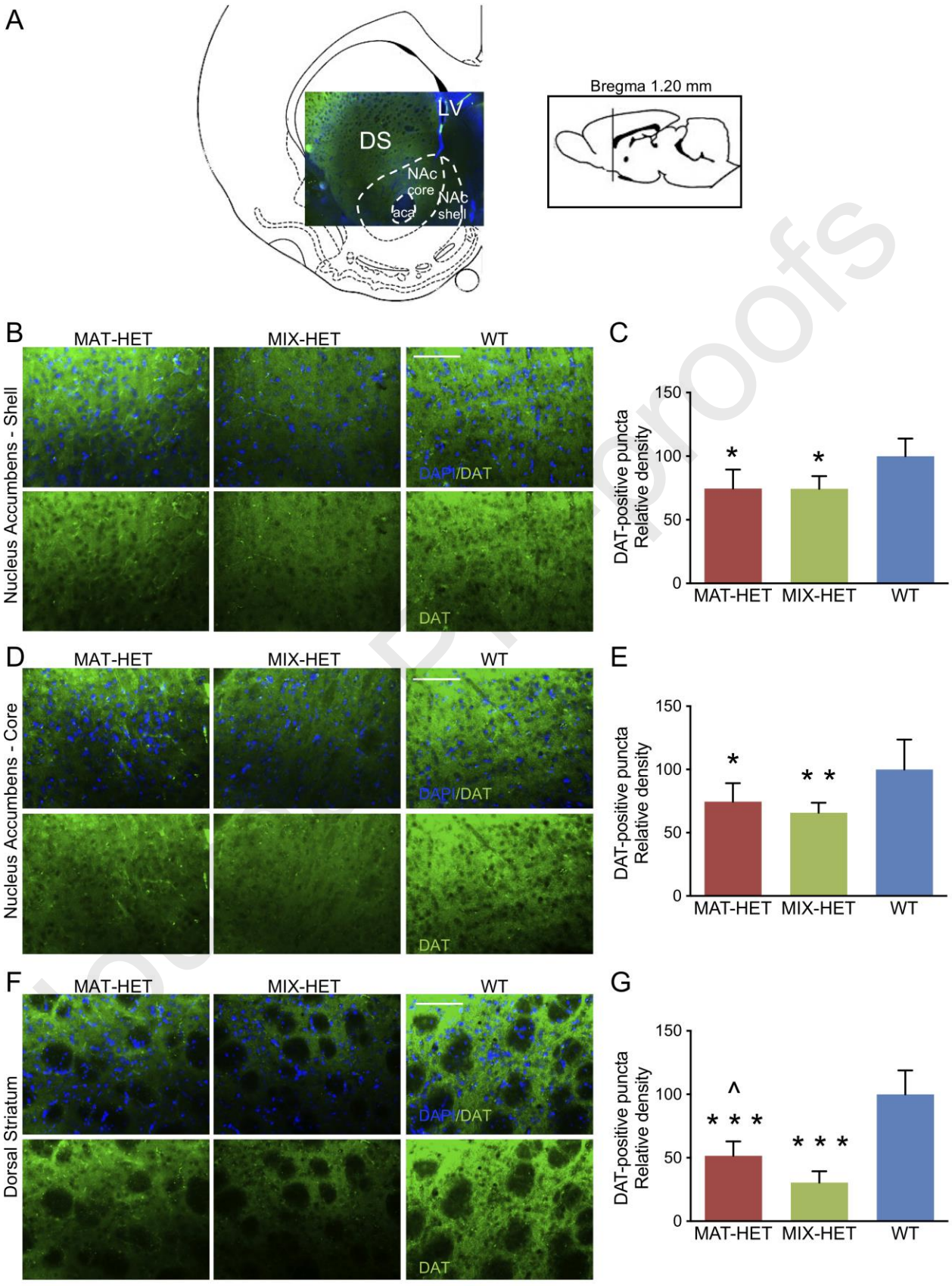
Zayats, T., Johansson, S., Haavik, J. (2015). Expanding the toolbox of ADHD genetics. How can we make sense of parent of origin effects in ADHD and related behavioral phenotypes? *Behav Brain Funct.* **11**, 33.











Motor transitions' peculiarity of heterozygous DAT rats when offspring of an unconventional KOxWT mating

CARBONE Cristiana $ (1); BRANCATO Anna, PhD $ (2); ADINOLFI Annalisa (1); LO RUSSO Sara Lucia Maria (1); ALLEVA Enrico, PhD (1); CANNIZZARO Carla, PhD (2); & ADRIANI Walter, PhD (1)

$ = Contributed equally.

DAT hypo-functional (heterozygous, HET) rat dams are known to display less maternal care.

We compared MAT-HET (KO-male x WT-female) to MIX-HET (heterozygous parents) rats.

MAT-HETs had more transitions between escape (diving) and despair (floating) on Porsolt.

When comparing to MIX-HETs, MAT-HETs displayed increased DAT in the dorsal-striatum.

MAT-HETs could help exploring parent-of-origin mechanisms in peculiar bipolar phenotype.