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A new "sudden fright paradigm" to explore the role of (epi) genetic modulations of the DAT gene in fear-induced avoidance behavior

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Abstract

Alterations in dopamine (DA) reuptake are involved in several psychiatric disorders whose symptoms can be investigated in knock out rats for the DA transporter (DAT-KO). Recent studies evidenced the role of epigenetic DAT modulation in depressive-like behavior. Accordingly, we used heterozygous (HET) rats born from both HET parents (termed MIX-HET), compared to HET rats born from WT-mother and KO-father (MAT-HET), implementing the role of maternal care on DAT modulation. We developed a "sudden fright" paradigm (based on dark-light test) to study reaction to fearful inputs in the DAT-KO, MAT-HET, MIX-HET, and WT groups. Rats could freely explore the whole 3-chambers apparatus; then, they were gently confined in one room where they experienced the fright; finally, they could freely move again. As expected, after the fearful stimulus only MAT-HET rats showed a different behavior consisting of avoidance towards the fear-associated chamber, compared to WT rats. Furthermore, ex-vivo immuno-fluorescence reveals higher prefrontal DAT levels in MAT-HET compared to MIX-HET and WT rats. Immuno-fluorescence shows also a different histone deacetylase (HDAC) enzymes concentration. Since HDAC concentration could modulate gene expression, within MAT-HET fore brain, the enhanced expression of DAT could well impair the corticostriatal-thalamic circuit, thus causing aberrant avoidance behavior (observed only in MAT-HET rats). DAT expression seems to be linked to a simply different breeding condition, which points to a reduced care by HET dams for epigenetic regulation. This could imply significant prefronto-cortical influences onto the emotional processes: hence an excessively frightful response, even to mild stressful agents, may draw developmental trajectories toward anxious and depressed-like behavior.

KEYWORDS

choice behavior, conditioned preference, DAT-KO rats, dopamine transporter, fear conditioning, prefrontal cortex

Silvia Zelli and Anna Brancato contributed equally to this work.

1 | INTRODUCTION

Thanks to genetic engineering, transgenic animal models have been generated to study determinants of many neuropsychiatric pathologies. In particular, recent studies focused on rats deprived of the gene coding for the dopamine transporter (DAT): namely, knockout (KO) rats for DAT were developed, starting from our first work,[1] in order to better understand behavioral disorders associated with high extracellular dopamine (DA) levels. To date, their basic phenotype has been investigated.[2-5] To compare such rats with the literature, the behavioral phenotype of KO mice for DAT is useful. In particular, the study by Perona and collegues[6] demonstrated that KO mice for DAT are easily stimulated and respond with a predominant hyperlocomotion both to a new environment and to a new stimulus.[7] This response depends on the phasic release of DA. However, this behavior should decrease when rodents become accustomed to the stimulus, at least in Wild-type (WT) subjects. Lack of habituation to stimuli, observed in KO mice for DAT, derives from the slower DA clearance,[8] indicating that their ability to adapt to new stimuli is disturbed.[9]

In addition, the establishment of a correct motivational state implies activation of meso-cortico-limbic DA neurons,[10] which could well be compromised in KO mice for DAT.[11] Symptoms of anxiety—and mood disorders—are thought to result from a disruption in the correct balance between emotional centers of the limbic brain and higher cognitive centers. In the healthy brain, prefronto-cortical regions regulate impulses, emotions, and behavior via inhibitory top-down control of limbic emotional structures[12]: medial prefrontal cortex (mPFC) is involved in processing both reward and the visceral response to emotions. This behavioral modulation is, at least in part, under the control of D1-like DA receptors, which comprise D1 and D5-specific subtypes (D1R and D5R, respectively). It was demonstrated[13] that the activation of D1/5R within the mPFC, during weak negative-valence experiences, induces aversive behaviors: thus, an unbalanced D5R subtype activation in the mPFC is suggested of provoking aberrant avoidance behaviors. This might have important implications on emotional processes and aversive learning, particularly within animal models where the DA system is clearly affected,[13-15] further revealing top-down control of mPFC over the striata.

We wanted hereby to study the reaction to a fearful input in the DAT-KO rat colony. We developed a new paradigm where a sudden fright was caused by unexpected turning on of a very bright light: we evaluated then if a subsequent avoidance to that environment, where such unescapable and highly aversive experience occurred, was subitaneously generated. In general, an acoustic stimulus is used in the startle test[16] to generate a freezing behavior in laboratory animals.[17] Other paradigms are broadly used to provide information on the neuro-biological basis of fear and anxiety. Pavlovian conditioning (with a light and\or a tone coupled with an electric shock) is classically used to evaluate fear, subsequently evoked by the light or tone alone: if the cue (light and\or tone) is repeatedly presented just before the footshock, the animal will be quickly conditioned to then perform freezing, a form of anxious behavior.[18,19] Present knowledge indicates that only DAT-KO rats are not sensitive to this cue-shock association[3] while other genotypes do not differ.

Recently, several studies have been carried out on the role of DAT epigenetic modulation in relation to social interactions, maternal care, as well as stress and depressive-like behaviors.[20] In addition to DAT-KO rats, we recently started to use DAT-HET rats with differential breeding conditions: in addition to classical colony settings with both HET parents (leading to HETs of "mixed" origin, termed MIX), and WT control rats, we started with an atypical breeding, obtaining HET rats with WT mother and KO father (termed MAT). We observed that the latter group had just small differences in the activity cycle compared to WT females, while most profound differences were shown by MIX-HET females.[21] As regards males, MAT-HET subjects have a higher locomotor activity and higher levels of DAT within the dorsal striatum, than MIX-HET subjects.[4] In both cases, DA-related neuroadaptations may be responsible for altered gene expression through epigenetic modifications.[22] Acetylation of histone tails by histone acetyltransferases and deacetylation by histone deacetylases (HDACs) are common epigenetic modifications thought to participate in the PFC functions.[23] Class I HDACs, particularly HDAC2, are found mostly within the nucleus of neurons and, amongst their functions, exert an endogenous restraint on memory formation.[24-26] On the other hand, class IIa HDACs, including HDAC4, shuttle between the nucleus and cytoplasm, in accordance to the phosphorylation by input-activated kinases, thus providing a mechanism for neuronal activity-regulating gene expression.[27]

In the present article, we further sought for (epi) genetic differences of DAT and HDACs expression within the PFC, in combination with the reaction to the fright. PFC alterations could well affect top-down control, hence, causing major consequences in social and emotional behaviors.[28] We formulated the hypothesis that our DAT-HET mutant rats, also as a function of the breeding scheme and maternal care, could have an impaired affective elaboration about the environmental context after a sudden fright\fearful input, developing and showing an exaggerated conditioned aversion to that place.

2 | MATERIALS AND METHODS

2.1 | Ethical note

All experimental procedures have been approved by the Istituto Superiore di Sanità animal welfare survey board, on behalf of Italian Ministry of Health (formal license 937/2018-PR for project D9997.61, delivered to W. Adriani; plus pending license application for project D9997.110, filed on 19 March 2019 and audited March 2020). Procedures were all carried out in close agreement with the Directive of the European Community Council (2010/63/EEC) and with the Italian law guidelines. All efforts have been made to minimize the suffering of animals and to use as few animals as possible, according to the 3Rs principle.

2.2 | Experimental subjects

The generation of Wistar-Han DAT knock-out rats was previously described elsewhere (see Reference 1). The colony was kept in a HET

breeding fashion; these animals were inter-crossed for >10 generation at Istituto Italiano di Tecnologia (IIT; Genoa, Italy). Some progenitors were then shipped to Istituto Superiore di Sanità (ISS; Rome, Italy); here, male DAT-KO rats were bred with Wistar-Han WT females (Charles River, Italy), obtaining a new G0 of founder HET subjects. Parents, used to conceive all present offspring groups, were G2 of our ISS colony.

The sample consists of adult male rats (>120 days old; average weight 500 g) born in our colony and culled at birth to six males and two females. In this experiment, control subjects were 18 WT Wistar-Han rats born and nurtured by six WT dams mated with WT males (three offspring pups per dam); in parallel, experimental subjects (two offspring pups per dam) were "mixed" HET rats (MIX-HET), born and nurtured by six DAT HET dams (classical breeding with a HET male), and "maternal" HET rats (MAT-HET), born and nurtured by 6 wild type dams (unconventional breeding with a KO male). Therefore, MAT-HET's functional DAT allele was always coming from the mother; instead, it had a mixed paternal / maternal origin for MIX-HET pups (hence, justifying the use of the "maternal" vs "mixed" terms, respectively). Subjects of former group were all HETs; subjects of latter group had 10 KO male sibling rats, which were used as well for behavior (1 or 2 pups per dam). For both kinds of HETs, group size was 12 (always out of six dams).

Since weaning they were placed in groups of two or three non-sibling, same-genotype subjects within Plexiglas cages (33 × 13 × 14 cm), located in an air-conditioned room (T 21° ± 1°C, relative humidity 60 ± 10%) with a 12 h dark–light cycle (light turned-on at 7.00 p.m. in winter and 8.00 p.m. in summer). Rats were given ad libitum access to food and water (Altromin-R, A. Rieper S.p.A., Vandoies, Italy).

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

2.3 | Apparatus

The experimental apparatus used for the sudden fright test is an opaque gray Plexiglas box composed of three rooms with different shapes and with smooth walls and floor (70 × 30 × 35 cm). The walls on the longer sides are hosting transversal panels, with doors, and eight photocell beams in total. The middle (starting) chamber (10 × 30 × 35 cm) gives access to the two end chambers (30 × 30 × 35 cm) that differ for shapes (D and L), as already used in Carbone et al.[29] The doors, which separate the rooms, can be opened or closed allowing or not the experimental subject to pass from one room to the other. The D shaped environment was the stimulus room where the rats experienced the fright, imposed by turning on a light (see procedure).

A custom-made software (PRS Italia, Rome, Italy) allowed scoring each subject's activity rate (beam interruptions per second) and time spent in every compartment. Data are automatically divided into 300-s bins.

2.4 | Procedure

For the first 15 min of each session, the door between all rooms remained open: subjects were gently placed in the middle starting chamber and were free to move between end rooms, for an initial exposure of at least 15 min (three 5-min bins). Their spontaneous behavior and room preference were measured, by automatic detection of locomotor activity rate and time spent in either end room.

Then, by gently closing the door to the middle chamber when rats were spontaneously exploring within the D shaped one, rats were confined inside the D shaped chamber and received the fright. A light (100 lux) located just above the chamber was turned on for a 5 minutes-long unescapable fright.

After that exposure, the light was turned off and the door reopened, allowing free access to all three chambers. The suddenly conditioned locomotor behavior and chamber preference were automatically measured for further 15 min (three 5-min bins). When the rat was removed, fecal boli were also counted.

2.5 | Ex-vivo markers of epigenetic singularity

DAT and two HDACs enzymes (HDAC2 and HDAC4) were then investigated by immuno-fluorescence in the prefrontal cortex (n = 6 non-sibling rats per group). All rats but KO ones, already used for behavior 1 month earlier, 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% paraformaldehyde (PFA) in PBS. Brains were dissected and postfixed 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.

Immuno-fluorescence was performed as previously described,[30] 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 2 h at room temperature under gentle shaking. Sections were then incubated in primary antibody for 72 h 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 1 h, incubated in secondary antibody for 2 h 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 1 h 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.

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 density of DAT-positive puncta or mean gray values for HDAC-related images, and reported as relative values normalized to the average of WT controls.

2.6 | Statistics

2.6.1 | Behavioral "sudden fright" test

On our experimental data, we used ANOVA with a four-level "genotype" x two-level "fear effect" (before vs after) x three-level "time" (5-min bins) design: the first was a *between* and the latter two *within* factors. Statistical analyses were performed using StatView II (Abacus Concepts, USA). Statistical significance was set at $p < 0.05$ and significant tendencies for $0.10 < p < 0.05$ were also explored. Multiple post-hoc comparisons were run with the Tukey HSD test, which is protected against false positives and may be used even on nonsignificant ANOVA effects (see Reference 31). Although the general advice is that individual posthoc 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 Reference 31).

We also compared each single genotype for difference in the number of fecal boli measured in either room at the end of the session. As regards fecal boli, we used ANOVA with a four-level "genotype" × two-level "chamber" (D vs L shape) design: the former was a *between* and the latter was a *within* factor.

2.6.2 | Ex-vivo data including epigenetic markers

Statistical analysis of immuno-fluorescence data was performed using Prism 6.0 (Graphpad Software Inc., USA). Data were assessed for normality and equal variances and analyzed by using one-way ANOVA, considering genotype as factor, followed by Tukey's post hoc test when necessary. Grubbs' test was performed to identify outliers, and one subject from MAT HET group was not included in DAT and HDAC4 data analysis. Statistical significance was set at $p < 0.05$ and data are reported as mean ± SEM.

3 | RESULTS

3.1 | Time spent in D shaped chamber

Time spent in D shaped chamber was evaluated for all experimental subjects (DAT KO, MAT-HET, MIX-HET, and WT) before and after the fearful input, there given by means of the 5-min lighting up while subjects were confined and could not escape (Figure 1).

The results show a change of preference after the fright, as witnessed by a significant trend for interaction *fear effect * time* ($F_{2,94} = 2.409$; $p = 0.0954$). In the pre-fear phase, all subjects somewhat spent higher time in D shaped chamber if compared to chance level (120 s). In the absence of ANOVA interaction with genotype, Tukey threshold was $q = 48.66$ ($df = 47$, $k = 3$). Both MIX-HET and WT rats resulted to spend equal time in D shaped room, before and after the 5-min switching on of the light. When comparing after this fearful input to before, while KO rats spent more time in D shaped chamber, MAT-HET rats spent much less time therein, showing a significant decrease in preference ($p < 0.05$). In other words, only for MAT-HET rats there was a sudden aversive conditioning to the room in which such light-induced fear was experienced.

3.2 | Locomotor activity rate

The results show a significant interaction *fear effect * genotype* ($F_{3,47} = 14.689$; $p < 0.0001$).

During the pre-fear phase, locomotor activity of MAT-HET rats was significantly higher ($ $p < 0.01$) compared to both KO and MIX-HET subjects as well as to control group (WT). During the post-fear phase, all subjects but KO ones displayed a significantly reduced locomotor activity (**$p < 0.01$), compared to before such frightening experience. Within KO group, locomotor activity was significantly higher (*$p < 0.05$) during the post-fear phase compared to the pre-fear one.

This piece of results (Figure 2) is consistent with the profile of time preference.

3.3 | Fecal boli

Both genotype and chamber showed significant effects or interactions in response to the fearful stimulus: *chamber* ($F_{1,43} = 29.322$; $p < 0.0001$) and *chamber * genotype* ($F_{3,43} = 3.113$; $p = 0.0360$).

The only genotype to show no difference at all in the number of fecal boli, found in either room was, the KO one. This means that, despite the fearful stimulus which took place in D shaped chamber,

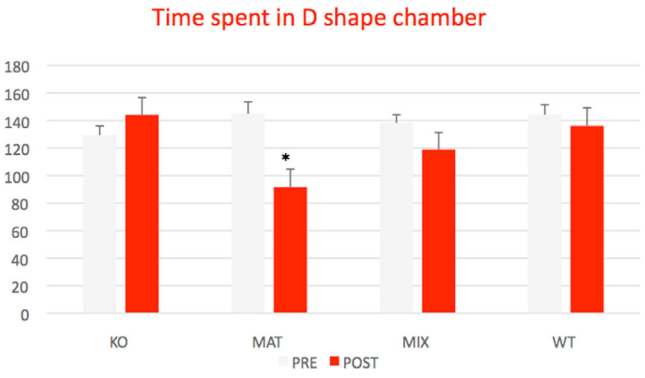

FIGURE 1 Time (mean ± SEM) spent in D shaped chamber before and after the fright. Rats were placed into the apparatus with free-choice between two differently shaped-chamber (D- and L-); the fearful experience (5-min bright light) occurred while confined in D shaped chamber; change in free-choice preference (i.e., time spent there before and after that stimulus) was measured. Only MAT-HET rats showed a change of preference: they spent much less time in D shaped chamber after the fright (*$p < 0.05$ in the D shaped chamber comparing pre- to post- fright). KO, MIX-HET, and WT rats showed no significant difference in preference for D shaped chamber

they did not change their defecation in either room. No visceral / affective consequences of the frightening experience were recorded; consequently, we propose KO rats to be totally insensitive, at least to that stimulus (see Table 1).

All the remaining genotypes showed a clear difference ($p < 0.05$), in that a defecation preference was clearly established towards the L shaped chamber after the fearful stimulus. For MIX-HET and WT rats, fecal boli were still found at small dose in D shaped chamber. Instead, no fecal boli at all were found therein for MAT-HET rats. The frightful experience had such a greater effect on their conditioned visceral / affective response, leading them not to defecate anymore inside the D shaped chamber.

3.4 | Dopaminergic singularity in MAT-HET Rats

The ex vivo markers in the prefrontal cortex considered, were consistent with an altered top-down control from the PFC onto limbic structures.

One-way ANOVA revealed a significant effect of genotype on DAT-positive puncta-density in both prelimbic and infralimbic sub-regions ($F_{2,14} = 29.47$, $p < 0.001$; $F_{2,14} = 45.32$, $p < 0.001$). In detail, Tukey's posthoc test indicated that DAT-positive puncta-density increased in MAT-HET and MIX-HET rats with respect to WT rats in both prelimbic ($q = 10.00$, $df = 14$, $p < 0.001$; $q = 8.478$, $df = 14$, $p < 0.001$) and infralimbic ($q = 12.88$, $df = 14$, $p < 0.001$; $q = 9.569$, $df = 14$, $p < 0.001$) sub-regions (Figure 3(A-E)). In addition, MAT-HET group showed a higher DAT- positive puncta-density than MIX-HET ones ($q = 3.764$, $df = 14$, $p < 0.05$) in the infralimbic sub-region of the prefrontal cortex (Figure 3(E)).

When data from HDAC2-positive immuno-fluorescence were analyzed, no significant effect of the genotype was observed in the prelimbic cortex ($F_{2,15} = 1.611$, $p = 0.2325$) (Figure 4(A,B)), whereas a significant effect of genotype was present in the infralimbic cortex ($F_{2,15} = 7.475$, $p = 0.0056$). Tukey's posthoc test indicated that HDAC2 immunopositivity decreased in MAT-HET rats with respect to WT ($q = 5.059$, $df = 15$, $p < 0.01$) and MIX-HET rats ($q = 4.327$, $df = 15$, $p < 0.05$) (Figure 4(C,D)).

On the other hand, when data from HDAC4 immunofluorescence were analyzed, one-way ANOVA revealed a significant effect of genotype in both prelimbic and infralimbic sub-regions ($F_{2,14} = 60.57$, $p < 0.001$; $F_{2,14} = 24.97$, $p < 0.001$). In details, Tukey's posthoc test indicated that HDAC-4 increased in MAT-HET and MIX-HET with respect to WT rats in both prelimbic ($q = 10.60$, $df = 14$, $p < 0.001$; $q = 15.07$, $df = 14$, $p < 0.001$; Figure 5(A,B)) and infralimbic ($q = 7.663$, $df = 14$, $p < 0.001$; $q = 9.294$, $df = 14$, $p < 0.001$) sub-regions (Figure 5 (C,D)). In addition, MAT-HET rats displayed significantly lower HDAC4-positive immuno-fluorescence than MIX-HET ones ($q = 3.769$, $df = 14$, $p < 0.05$) in the prelimbic cortex (Figure 5(A,B)).

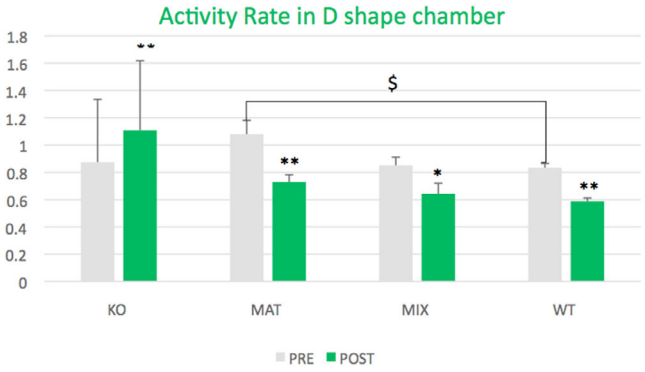

FIGURE 2 Activity rate in D shaped chamber before and after the fright, measured as number of beam interruptions per second (mean ± SEM); rats were the same as in Figure 1. As regards the pre-fear phase, MAT-HET rats showed a significant difference, with higher locomotor activity ($ $p < 0.01$) compared to WT rats. During the post-fear phase, all subjects but KO ones displayed a significantly reduced locomotor activity (**$p < 0.01$), compared to before such frightening experience. Within KO group, locomotor activity was slightly but significantly higher (*$p < 0.05$) during the post-fear phase compared to the pre-fear one

4 | DISCUSSION

In order to better understand anxiety symptoms in numerous psychiatric disorders, behavioral paradigms on rodent models[18] are widely used. The classic anxious behavior is evaluated, preclinically, by tests that take advantage of stimuli that are perceived by the animal as threatening. Overall, fear produces behavioral responses that stimulate defensive behavior in rodents. For example, in the presence of a predator or when exposed to stimuli associated with it, such as the predator's smell,[32] freezing and avoiding behaviors are displayed. These negative (aversive) emotional experiences depend on the intervention of three main factors (and transmitters\brain areas): one (dopamine\striata) determines the motivation toward or away from the stimulus, the second (noradrenaline\hypothalamus) controls the state of excitation, the last one (serotonin\prefrontal cortex) mediates the resulting activation with approach or avoidance.[33]

Time spent with new objects suddenly introduced in an arena (neophobia) is measured and used as a putative indicator of anxiety.

TABLE 1 Fecal boli in L- and D-shaped rooms after the sudden fright

	KO	MAT-HET	MIX-HET	WT
L shaped chamber	2.25 ± 0.70	4.33 ± 1.64	3.20 ± 0.87	4.056 ± 0.60
D shaped chamber	2.25 ± 1.61	0.00 ± 0.00*	0.33 ± 0.18*	0.333 ± 0.28*

*$p < 0.05$ in the D shaped chamber compared to the L shaped one.

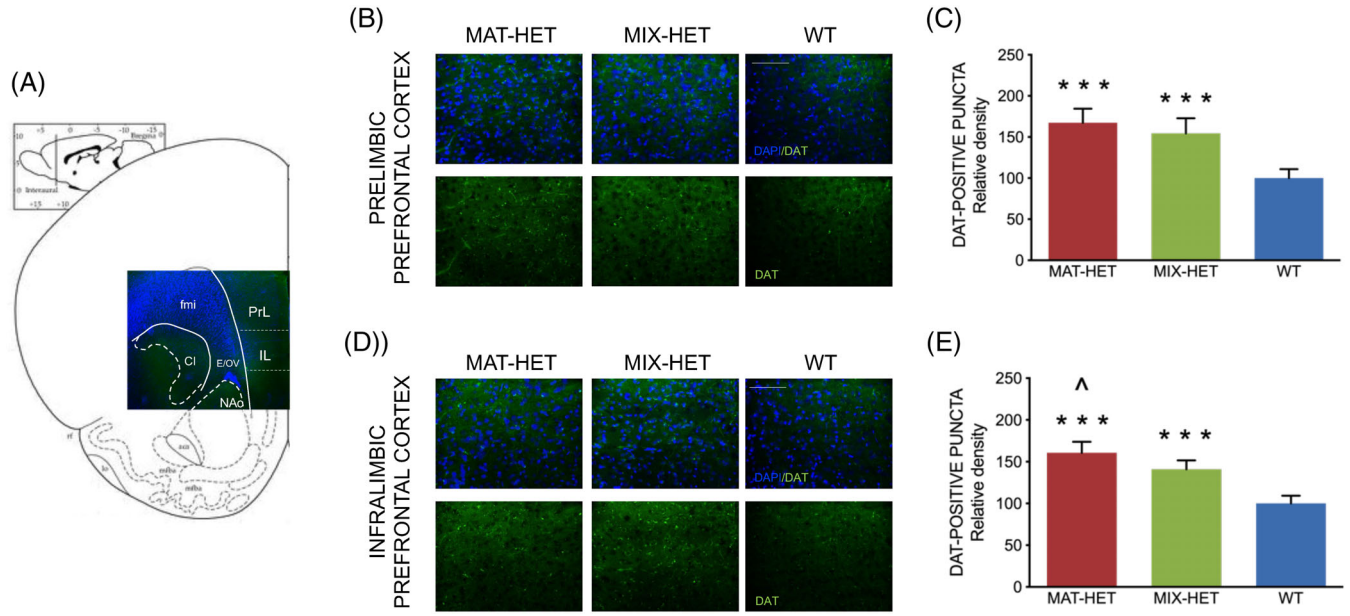

FIGURE 3 DAT immunofluorescence in the prefrontal cortex. DAT-HDAC2 and HDAC4 relative immunofluorescence was evaluated in the prelimbic (PrL) and infralimbic (IL) subregions of the prefrontal cortex (A). MAT-HET and MIX-HET rats displayed increased DAT-positive puncta density in the prelimbic (B, C) and infralimbic (D, E) prefrontal cortex with respect to WT rats. Moreover, increased DAT-positive puncta density was observed in the infralimbic cortex of MAT-HET rats with respect to MIX-HET rats (E). Each bar represents the mean ± SEM. of n = 5–6 rats. ***$p < 0.001$ versus WT. ^$p < 0.05$ versus MIX-HET, scale bar 100 μm

Another approach is to evaluate the behavior of one animal in the presence of another conspecific ("partner"): loss of social interaction can reflect therefore the relative state of anxiety.[34] In the same way, the plus maze is another classical test that combines natural preference for safe spaces and aversion to open or high areas. Time spent in open arms of the maze is evaluated in order to identify the anxiety level.[35-37] A widely used test is also the light–dark emersion test, consisting of a box divided into two sections: a dark and roof-protected side and a brightly illuminated one. Time spent inside the lit side is intended as an index of a less anxious behavior.[38,39]

The procedure presently developed is somewhat reminiscent of a light–dark box, with the difference that light is initially absent and just suddenly turned on for 5 min, while the subject is confined and cannot escape. The classical DAT genotypes, offspring of a classical HET by HET breeding, did not differ so much in the light–dark test.[2] This new paradigm shows no significant difference between MIX-HET and WT ones, accordingly. Notably, a different behavior emerged between MAT-HET rats, specifically, and MIX-HET as well as WT rats. Their genotype is always HET, yet maternal care was different[21]: genotype of the dam, and consequent caring style, can influence offspring behavior more easily than own genotype. These notions tap onto epigenetics as a major determinant in the development of depression and lack of stress resilience.[7] In this line, we recently found that MAT-HET rats display, in the Porsolt test, enhanced diving with more frequent transitions toward behavioral despair (floating), compared to MIX-HET group.[4] Furthermore, a lack of social and exaggerate sexual motivation in MAT-HET rats has been seen, compared to the MIX-HET ones.[40] Specifically, MIX-HET rats show no approach in presence of a female in estrous; however, although MAT-HET males show a very high attraction toward a female in estrous, they tend to ignore a male HET stimulus.

4.1 | Neuro-biological comments on dopaminergic singularity

In our previous studies about HET rats for DAT, we have shown lower levels of DAT in ventral (nucleus accumbens) and dorsal striatum, both in MAT- and MIX-HET rats compared to WTs.[4] Surprisingly, in the present study, HET rats showed higher levels of DAT in the prefrontal cortex, despite just one functional copy of the gene, compared to WTs. As for prefrontal cortex, while slow DA uptake is due to the noradrenaline transporter,[41-43] the COMT is the major regulator of DA clearance.[44] Yet, alterations in DAT positive terminals may deeply affect general activity, motivation, and survival-directed actions.[4]

This is highly relevant, as DA afferents to various subregions of the PFC are implicated in seeking behavior, which is inhibited by prefrontal dopaminergic self-control functions[45] and promoted by the ventral striatum.[46-48] Enhanced prefrontal DAT may therefore promote seeking behavior in DAT-HET rats.[49] Increased DAT expression in MAT- and MIX-HET rats is indeed associated with higher seeking of an escape in the Porsolt test, yet in different ways, as shown by (respectively) increased diving and climbing behaviors,[4] with respect to WT rats. Furthermore, the higher DAT levels should indicate

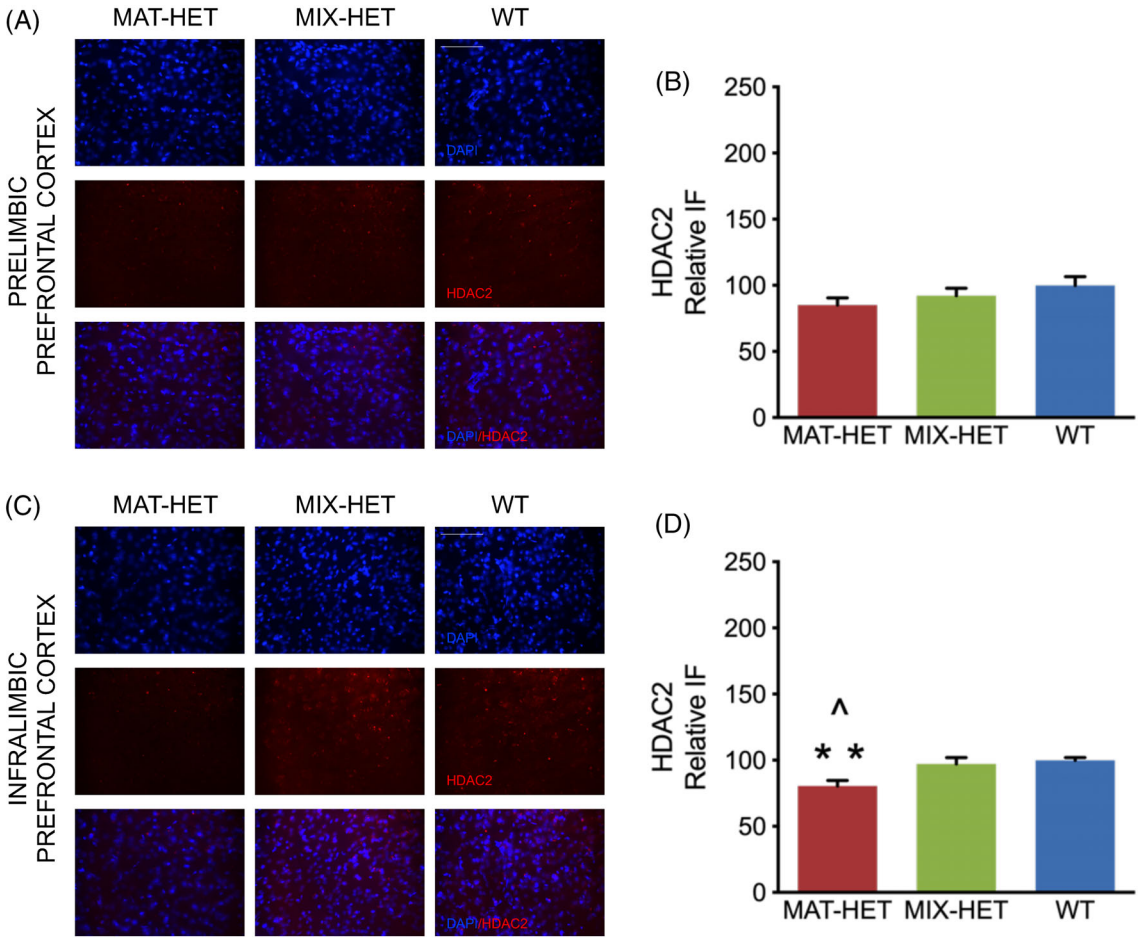

FIGURE 4 HDAC2 immunofluorescence in the prefrontal cortex. As regards HDAC2 immunofluorescence, no significant differences were observed in the prelimbic cortex (A,B), whereas MAT-HET rats displayed decreased HDAC2 immunofluorescence in infralimbic cortex with respect to WT and MIX-HET rats (C,D). Each bar represents the mean ± SEM of n = 6 rats. $^{**}p < 0.01$ versus WT. $^{\wedge}p < 0.05$ versus MIX-HET, scale bar 100 μm

elevated DA, which may cause prefrontal cortex over-activation abolishing fear extinction.[50] In this way, we could explain the significant difference about time spending in D shaped room before and after the stimulus in MAT-HET rats. Instead WT and MIX-HET rats showed no significant difference. Interestingly, this evidence is suggestive of a relevant role of nurturing—thus epigenetics—in shaping the functioning of the prefrontal cortex.

So far, little is known about epigenetic regulation of DAT. This high concentration of DAT in MAT-HET prefrontal cortex could be related to epigenetic regulation of transcriptional factors as Nurr1 in the ventral tegmental area, a key regulator of DAT expression. Indeed, Nurr1 KO rats show no expression of DAT and other dopaminergic genes.[51] One explanation to this profile could be linked to different PFC HDAC enzymes concentration, however. Alterations of maternal care have been reported to affect the relationship between Nurr1 and DAT expression.[52] In our experimental conditions, the early interactions between WT dams and MAT-HET offspring may prompt a compensatory Nurr1 mechanism, able to increase DAT-positive terminals in the infralimbic cortex and counteract the reduction in DA levels.

In turn, the altered DA transmission, asides from affecting the functioning of the prefrontal cortex, likely induces a dysregulation of HDAC, which contributes to aberrant transcriptional profiles. Our ex-vivo immunofluorescence investigation reveals a reduction of HDAC2 in the prefrontal cortex of MAT-HET compared to WT rats. Intriguingly, decreased levels of HDAC2 were highlighted in the prefrontal cortex of SZ patients[53] and may be responsible for reduced histone deacetylation and lack of gene-transcription repression. On the other hand, both HET groups showed higher HDAC4 immunofluorescence than WT rats. As a member of class IIa deacetylases, HDAC4 is controlled by neuronal activity and provides input-specific gene expression, shuttling between the nucleus and cytoplasm following phosphorylation. Interestingly, repeated administration of methamphetamine, which reverses the reuptake of DA and increase DA release, has been shown to decrease the expression of HDAC2 and increase the mRNA of

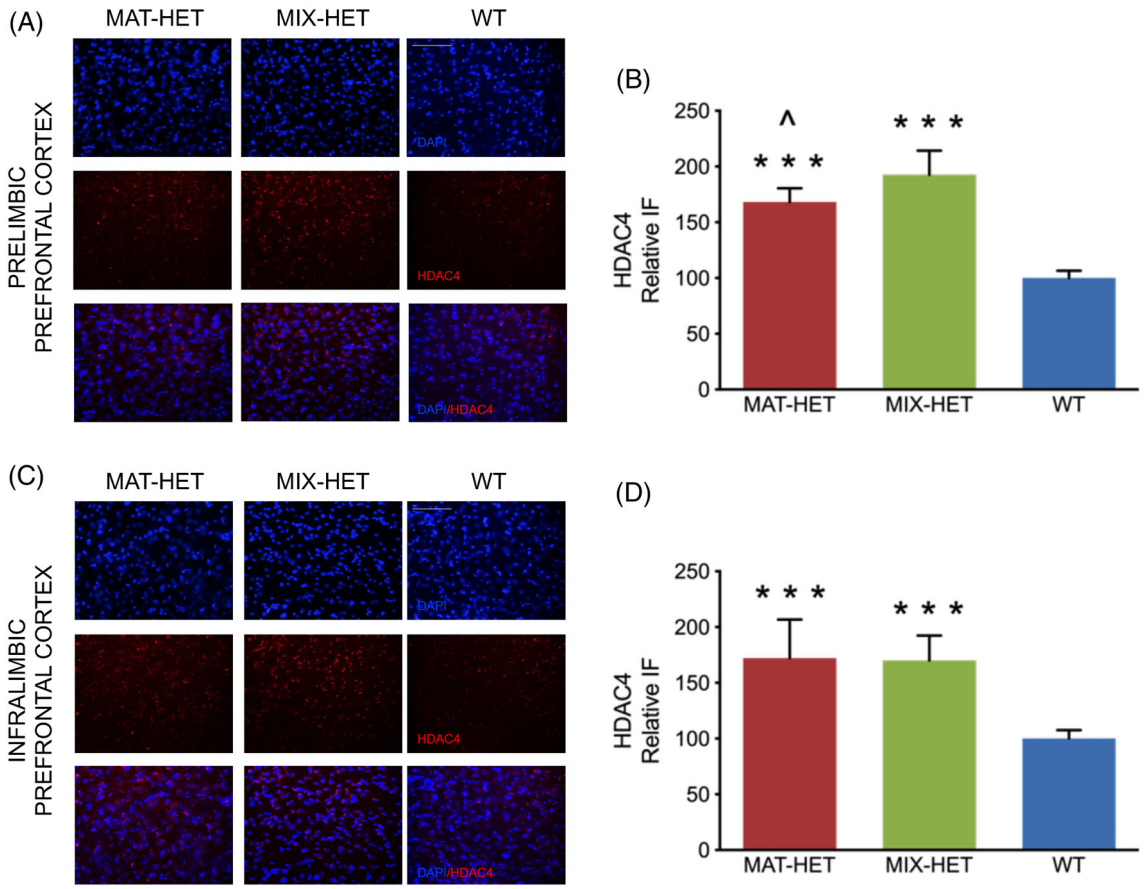

FIGURE 5 HDAC4 immunofluorescence in the prefrontal cortex. MAT-HET and MIX-HET rats displayed increased HDAC4 immunofluorescence in the prelimbic (A,B) and infralimbic (C,D) cortex, with respect to WT rats. Moreover, MAT-HET rats showed decreased HDAC4 immuno-positivity with respect to MIX-HET rats in the prelimbic cortex (A,B). Each bar represents the mean ± SEM of n = 5–6 rats. ***$p < 0.001$ versus WT; ^$p < 0.05$ versus MIX-HET, scale bar 100 μm

HDAC4 in the prefrontal cortex in mice.[22] Thus, altered DA availability in the prefrontal cortex of HET rats may be responsible for an aberrant pattern of input-specific gene expression via increased HDAC4 levels.

As far as we know, HDAC can deacetylate the N-terminal tails of the core H3 and H4 histones enhancing their interaction with DNA causing a more compacted chromatin. This compressed chromatin may prevent access to transcriptional machinery, causing a transcriptionally repressed state.[54] Decreased HDAC may similarly disinhibit Nurr1 action within the ventral tegmental area; this hypothesis could be a start point for future studies. Overall, the current set of finding is in agreement with an altered DA neurotransmission in HET rats: this can explain cognitive distortions that would potentiate depression-associated maladaptive behavior.[55-58]

There is strong evidence of prefrontal cortex projections to striatum. One of the first studies suggests that there is a topographic organization; therefore, the rostral areas of the whole cerebral cortex project to rostral striatum, while caudal areas project to caudal striatum.[59] Based on connections, the whole dorsal striatum has been divided into associative and sensorimotor functional zones, based on receiving projections from corresponding cortical areas, just above. The projections have been described as forming spatially and functionally segregated corticostriatal-thalamic feedback sub-loops.[60]

Alexander and colleagues proposed a model composed of five segregated functional loops, which receive input from a particular cortical area and send efferent to specific basal ganglia nuclei.[61] Recently, using MACM (Meta-Analytic Connectivity Modeling), an unbiased approach to generate a precise functional connectivity map,[62] Tziortzi and colleagues demonstrated that limbic subregions show a significant functional connectivity with medial PFC: those co-activations were related mainly to the emotion and cognition domains for the smooth performance of reward processes.

As we find high concentration of DAT in prefrontal cortex of HET rats, and even higher in MAT-HET rats, we could speculate that there is a decreased top-down control of striatum, leading to an alteration of fronto-striatal circuits: as such, reward-based update of habits may become more rigid causing OCD-like symptoms. In fact, other clinical studies have demonstrated that patients who suffer from local lesions within the striatum often exhibit striking obsessive–compulsive behaviors.[63,64]

4.2 | Translational comment on dopaminergic singularity

In humans, DAT plays an important role in affective and behavioral regulation[65] together with the environment provided by parents. In literature, studies show the association of the 9- and 10-repeat allele to a greater expression of psychiatric disorders[66-68] such as Attention Deficit Hyperactivity Disorder,[69-73] Post-Traumatic Stress Disorder,[74] Oppositional Defiant Disorder,[75] Autism Spectrum Disorder,[76] Schizofrenia,[77-80] and Bipolar Disorder.[81] Already in 2002, Serretti and coworkers highlighted how the symptom analysis showed an association of the DRD2*S311C variant with "Delirium" and "Disorganization" factors[82]; this was also confirmed by other authors for the association with the schizotypic trait, seen as a set of personality dimensions that convey risk for schizophrenia (see References 83–85). We shall postulate that an altered dopamine level, related to altered DAT, should in turn lead to altered D2 levels because of its role as auto-receptor. So far, our unpublished data suggest reduced D2 and elevated dopamine in the PFC of HET rats (in preparation). Hence, we hypothesize that "paranoid delirium" may be the basis of avoidant behavior, which presupposes the attribution of an excessively threatening meaning to a neutral stimulus. Further studies are needed to confirm these putative associations.

4.3 | Limitations

There are some limitations in our study; first of all we recognize the limitations given by the use of only one behavioral test. Of note, however, this article represents the continuum of a previous article in which we studied locomotion by using methylphenidate and anxiety \depressive responses by using forced-swim test in MAT, MIX ad WT rats.[4] Moreover, in that article we underline the importance of other areas in a fear conditioning experiment, such as nucleus accumbens which shows different HDAC4 levels in relation to the different genotypes, but no effect of maternal care. Particularly, there is a strong effect of the genotype in the nucleus accumbens, as HDAC4 immunopositivity decreased in both HET rats with respect to WT rats.

Though we studied mainly PFC in this article, there are other important areas involved in fear conditioning such as the amygdala; despite the evidence for a dopamine signaling role in amygdala during aversive learning, the role of dopamine neurons in aversively motivated behavior is still controversial.[86] We cannot state that slight differences in PFC of our MAT versus MIX rats are causal, but it is well known that connections between the amygdala and medial prefrontal cortex (mPFC) are crucial for both fear conditioning and extinction.[87]

Moreover, we cannot state how much DAT-KO rats affected WT-females during mating: even if it seems likely that the hyperactivity of the male DAT-KO induces a stress response in the WT female, as they were kept in the same cage for 10 days for their mating, however the DAT-KO father was then removed and not kept in the same cage during the rearing of the MAT offspring. We only can hypothesize that this offspring's phenotype could not be exactly the same if the pups (MAT offspring) were fostered and reared by a WT female crossed to a WT male.

In conclusion, the purely genetic set-point of ventral striatum in both kinds of DAT-HET subjects (i.e., with notrace of epigenetic modulation) can open the way to reduced motivation, thus explaining internalizing and depressive-like symptoms; conversely, the epigenetic impact of maternal care, on the PFC, may well open the way to externalizing symptoms like those reminiscent of schizophrenia or maniacal phase of bipolar disorder.

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CONFLICT OF INTEREST

There is no conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

This source had in no way interference on the design and data analysis of the experiment. All original data leading to this article are stored on a computer located at ISS in the office of the corresponding author. All raw data can be made available upon request.

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