 doi:10.20944/preprints202105.0404.v1

SOCIAL INTERACTIONS OF DAT-HET EPI-GENOTYPES DIFFERING FOR MATERNAL ORIGINS: THE DEVELOPMENT OF A NEW PRECLINICAL MODEL OF SOCIO-SEXUAL APATHY

Anna BRANCATO † (2); Sara L.M. LO RUSSO † (1); Anna Sara LIBERATI (3); Cristiana CARBONE (1); Silvia ZELLI (1) Giovanni LAVIOLA (1); Carla CANNIZZARO (2); Walter ADRIANI (1, 3)

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

2 - Dept Sciences for Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties "G. D'Alessandro", University of Palermo, Palermo, Italy

3- Faculty of Psychology, Università Telematica Internazionale "Uninettuno", Rome, Italy

† = Contributed equally.

Running Title: The epigenetic subtypes of heterozigous DAT rats.

***Correspondence:**

Dr. Walter Adriani, PhD. Center for Behavioral Sciences and Mental Health, Istituto Superiore di Sanità, 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, SLL, SZ carried out the behavioral experiment; AB carried out the ex-vivo experiment; AB and SLL wrote a first draft of the paper; GL, ASL, CaC, and WA contributed to its final version. We thank Raul Gainetdinov and Damiana Leo who originally 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. Data for these experiments are stored on a computer in the office of the corresponding author; such raw data can be made available upon request. There is no conflict of interest to disclose.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

ABSTRACT

Social interaction is essential for life and is impaired in many psychiatric disorders like schizophrenia, autism, depression and major anxiety disorder. Monoamine transmission plays a key role in social behavior and both genetic and epigenetic modifications of dopamine and noradrenaline neurotransmission-related genes can affect the levels of social interaction. Since heterozygous individuals for a specific genetic trait possess only one mutant allele of that trait, in order to better evaluate the role of the interaction between genetics and epigenetics in unmasking latent genetically-determined predispositions, our interest has focused on studying the interplay between genetics and epigenetics influences on social behavior in male rats obtained by two different breeding schemes: a first group by breeding of knock-out (KO) male rats with wild-type (WT) female dams (homogeneous heterozygous offspring, termed MAT-HET), and a second group of heterozygous DAT male offspring by breeding of KO male and DAT-heterozygous female subjects (to obtain comparable control pups, termed MIX-HET). Their social behavior was then assessed by partner preference, social preference and elicited preference tests. In the first test MIX-HET and MAT-HET male mice had choice between two WT females one in estrous and the other not in estrous. In the second test they met either a MIX-HET or a WT male rodent. Also, the expression of the noradrenaline transporter (NET) was assessed in the prefrontal cortex, hippocampus and hypothalamus of MAT, MIX and WTs by immunofluorescence in order to estimate its involvement in the expression of social behavior. Our results show that MIX-HET focal rodents tend to have an asocial behavior when in contact with a female in estrous, and their behavior is similar to when the stimulus is a MIX-HET male. MAT-HET male rodents, instead, tend to be very attracted by the female in estrous, but they ignore the MIX-HET stimulus. MIX-HET progeny showed a lower expression of noradrenaline transporter in both hypothalamus and hippocampus with respect to MAT-HET rats, whereas MAT-HET rats displayed increased noradrenaline transporter immunofluorescence in the hypothalamus and in the hippocampus with respect to WT rats, while no difference was observed in the prefrontal cortex. Therefore we can hypothesize that the differences observed between the two heterozygous groups may be attributable to an epigenetic factor: the different maternal care received. These data can open new perspectives towards increased the preclinical knowledge about autism and bipolar disorder.

Keywords: dopamine transporter; socio-sexual reward; social behavior; parent-of-origin effect.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

INTRODUCTION

Social interaction is composed by complex behaviors, which are essential for life of many mammalian species. In humans, these interactions are impaired in several psychiatric disorders like autism, schizophrenia, major depression and social anxiety disorder. Pharmacological studies have demonstrated that many neurotransmitters (oxytocin, dopamine, noradrenaline and β-endorphin) are recruited in social behavior (Pearce et al., 2017). In particular, both dopaminergic and noradrenergic systems seem to be the most important systems linked to social drives. A wide literature has demonstrated that external stimuli such as stress, often result in significant changes in DA and NE concentrations in brain regions as the amygdala and the prefrontal cortex (Gutknecht et al., 2012; Strekalova et al., 2020), thus supporting the evidence for epi-genotype-governed influence in altered social and sexual behaviour.

DA neurons are located in the ventral tegmental area (VTA) and substantia nigra (SN), thereby innervating brain regions relevant to social play and affiliative behavior, such as the prefrontal cortex, the hippocampus, the striatum and hypothalamus (Lopes et al., 2020; Neumann et al., 2009; Laviola and Terranova 1998, Cirulli et al., 1996). When subjects are in contact with social stimuli, emotional processes having a positive or negative valence are triggered and the DA neurons originating in VTA are involved. This discovery has been validated via the implant of an optical fiber into the VTA. It was observed that, after the introduction of a novel stimulus mouse to the focal mouse, activity of VTA neurons was increased during same-sex social interaction (Gunaydin et al., 2014). Accordingly, optogenetic enhancement of phasic DA activity in VTA cell bodies increased social behavior (Gunaydin et al., 2014). These concordant results demonstrate that DAergic projections from VTA neurons are involved in social interaction.

In our study we exploit the dopamine transporter (DAT) knock-out KO rat (Leo et al., 2018; Cinque et al., 2018), somewhat similar to the transgenic DAT-KO mouse, developed to investigate the role of DA in the modulation of neuro-behavioral processes (Giros et al., 1996; Sora et al., 1998; Gainetdinov et al., 1999; Spielewoy et al., 2000). As far as social interaction is concerned, the heterozygous (DAT-HET) rodents could be more informative than full KO rodents (Adinolfi et al., 2019; Sanna et al., 2020): in fact DAT-HETs exhibit an evident asocial behavior in comparison with WT and even KO rats. Due to the fact that they are heterozygous, DAT-HETs possess only one functional allele for the DAT gene. Therefore, it is suspected that this specific allele may be somehow more vulnerable to epigenetic effects.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

Very recently, two possible mating combinations have been implemented to generate these rats, yet obtained via: a first group, originated by classical breeding of male and female DAT-HET subjects, led to "mixed" offspring with inactivated allele on chromosome 5 being either of paternal or of maternal origin (MIX-HET); a second group was obtained by breeding of KO male rats with WT female dams. This group was hence named "maternal" (MAT-HET; Carbone et al., 2020): the functional DAT allele is always of maternal origin in the latter group; also, the offspring likely received a full repertoire of maternal care (see Mariano et al., 2019: "HETs with WT dam" correspond to present MAT-HET). Our hypothesis was that HET offspring belonging to the two epi-genotypes would differ depending on crucial factors, among which being cared from either a WT or a HET dam, since HET mothers are less inclined to maternal care. Also, the atypical KO-WT breeding renders MAT-HET rats likely to inherit unknown patterns of DNA methylation and histone acetylation, from their KO fathers. Moreover, another source of variability is that only half of MIX-HET subjects, born from a HET/HET breeding, will have functional maternal and mutated paternal alleles, while the other half of their siblings will have functional paternal and mutated maternal alleles. These limitations can be partly circumvented: breeding KO males with HET females will simply produce a half of MIX-HET offspring which will be entirely comparable to MAT-HET subjects, apart from the mother's genotype (and cares). Such kind of "biased" breeding presently allowed to isolate the maternal epigenetic factors alone. A simpler approach would be fostering HET subjects to WT dams and WT subjects to HET dams, yet in this case maternal factors would interact with the own genotype of pups (Oggiano et al., *in preparation*).

Preliminary studies on MAT vs. MIX-HET rodents (Carbone et al., 2019; Mariano et al., 2019) have shown increased overall locomotory activity in MAT-HET rodents: they also are more active compared to MIX-HET rats during the first hour of facility lighting (resting time), suggesting sleep disorders. Using a methylphenidate challenge (methylphenidate 1 mg/kg), MAT-HET locomotory activity did not change in response to the lower dose, while the latter was already effective in MIX-HET and WT rodents. In the Porsolt forced Swim test, MAT-HET subjects showed an instable profile, in that they alternated more frequently between the passive floating and active escape behaviors. This data, if transferred from animals to man, could be translated in the alternation between states of despair and struggle due to an unstable emotional state.

Our present study is focused on the sociality of MIX-HET and MAT-HET rodents, to determinate if MIX-HET and MAT-HETs could become a new preclinical model of behavioral symptoms of mental

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

disorders based on their epigenetic modification. Since the genotype is heterozygous in both groups and changes are likely due to epigenetic impact of their dam, the two groups are hence-forward termed “epi-genotypes” and underwent three tasks: the partner preference (PPT), the social preference (SPT) and the elicited preference (EPT) tasks. The PPT was originally developed in the laboratory of Dr. Sue Carter (Williams et al., 1992) and assesses the extent of social contact and time in proximity to a partner relative to a stranger. The PPT has been used extensively to assess how different manipulations alter formation and maintenance of *preferences for a mate* in monogamous prairie voles and to a lesser degree in other monogamous species (Ahern et al., 2009; Kingsbury and Goodson, 2014). The PPT is also used to assess factors affecting social preferences for *same-sex peers* in meadow and prairie voles (Beery and Zucker, 2010; Anacker et al., 2016a,b), and occasionally other rodents (e.g., Triana-Del Rio et al., 2011). We adapted this kind of simple-choice tests to laboratory rodents. The previous paper (Adinolfi et al., 2019) was based on encounters among all possible genotypes (WT, HET, KO, in a “3x3” design); presently, we tested offspring from HET mothers and KO fathers (MIX epi-genotype) *versus* WT mothers and KO fathers (MAT epi-genotype). In our-own ethogram for scoring of the preference protocol, we had a total of 8 behavioral items: one item serving position, 3 for social behaviors and 4 for non-social behaviors. In addition, since social alterations in heterozygous DAT rats were associated with neurochemical alterations in norepinephrine neurotransmission, with increased levels of norepinephrine in the hippocampus and hypothalamus with respect to WT counterparts (Adinolfi et al., 2019), we investigated the levels of noradrenaline transporter in the prefrontal cortex, hippocampus and hypothalamus, brain regions relevant to social behavior (Lopes et al., 2020) by employing immunofluorescence.

2. METHODS

All experimental procedures have been approved by the ISS 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 on March 2020; plus formal license 1008\2020-PR for project D9997.110; both delivered to W. Adriani). Procedures were 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 made to minimize the suffering of animals and to use as few animals as possible, according to the 3Rs principle.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

2.1 SUBJECTS

The generation of Wistar-Han DAT knockout rats was previously described elsewhere (Leo et al., 2018). The original colony was maintained in a heterozygous-heterozygous breeding fashion; the 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 new G0 of 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. We tested a total of 24 male rats belonging to two different epigenotypes (MIX-HET vs MAT-HET). The first was offspring of HET father X HET mother breeding while the second was offspring of KO father x WT mother breeding; two siblings per dam out of six dams were used. All 24 rats were used for the partner and social preference tests, and as stimulus for the elicited preference test. Additionally, we also used: another 7 of the adult heterozygous rats, 18 WT males and 6 females, hosted in the colony. The 7 HET rats and 7 WT rats were used as stimuli for the social preference test (termed “HZ”); the remaining 11 WT rats were used as focal for the elicited preference task. The 24 main subjects, all heterozygous but belonging to two epigenotypes (i.e MIX offspring from HET mothers and KO fathers *versus* MAT offspring from WT mothers and KO fathers), underwent counter-balanced encounters with either free choice for various kind of stimulus rats or acting themselves as stimulus. For the experiment 1 (partner preference), a female in estrous and a female not in estrous were used as stimuli; for experiment 2 (social preference), 7 WT and 7 HET males (thereafter termed “HZ”) were used as stimuli; for experiment 3 (elicited preference), the previously focal MAT and MIX-HETs were used as stimuli whereby the non-previously stimuli WT rats acted as focal.

2.2 APPARATUS

The experimental apparatus was a plexiglass box with smooth walls and floor (70x30x35 cm). It was composed of two equal environments separated by a central wall with a door and distinguished by the end walls’ colour: the walls on the long sides and in the centre were grey whereas

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

those on the short end sides of the maze were either black or white. The central door was always open.

Inside the two end-chambers, aluminium cages (15x15x40 cm) were positioned against the corner with short walls and raised 3 cm above floor; we gently positioned the stimulus rodent inside the aluminium cage. As for the “black wall” chamber we placed: for experiment 1 the female not in estrous; for experiment 2 it was left empty; for the experiment 3 either a MAT or a MIX-HET. As for the “white wall” chamber we placed: the female in estrous for experiment 1; either WT or HET stimulus (“HZ”) for experiment 2; either MIX- or MAT-HET (to provide choice) for experiment 3. All the procedure (experimental encounters) has been videotaped for experiments 1 and 2 while for experiment 3 we exploited automatic data.

2.3 PROTOCOL

Partner, social and elicited preference tasks, spaced apart of at least one month, were each effectuated in a total of 4 consecutive days. First two days were used for habituation and other two days were needed for test. Habituation was divided for focal and stimulus rats; during the first habituation day, to get stimulus rats used to be confined within the aluminium cage, we put them individually inside it for 15 minutes; on second of habituation days, all focal rats were placed for 30 minutes in the apparatus in which there where the two empty aluminium cages. From third to fourth day we run the encounters, to measure preference for stimuli shown by focal rats.

For the partner and social preference tasks, both epi-genotype groups of heterozygous animals (MIX- and MAT-HET) acted as focal subjects while they acted as stimulus for the elicited preference task. At the beginning of all encounters, we gently put stimulus rats inside the aluminium’s cages; immediately then (second step), we gently placed the focal rats in the start room. For the experiment 1, rats started from the “black wall” room (in whose cages there was the female non in estrous); for experiment 2, rats also started from “black wall” room (where the cage was empty). For experiment 3, MAT- and MIX-HET rats which acted previously as focal were placed as stimuli in either chamber’s aluminium cage in a counterbalanced order. The focal WT rats (tested twice on days 3 and 4) started once in the MAT-HET stimulus chamber and once in the MIX-HET stimulus chamber. Both stimulus and focal rats were left there for a total of 15 minutes: focal rats had the possibility to freely cross the door and enter in either chambers.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

During the second test day (day 4), we used again the same focal subjects who met different stimulus rats: this was to prevent final preference to be biased by one given stimulus in particular. Thus, male rats of different epi-genotypes met as stimulus other female rats with estrous and non estrous conditions (exp 1) as well as different genotypes (exp 2: WT or HET male "HZ") in counterbalanced order. For experiment 3, the same MAT- and MIX-HET subjects, this time acting as a stimulus, inverted the chamber (inside aluminium cage) for days 3 and 4 in counterbalanced order. The floors of each chamber and cage were cleaned between each animal with water and ethanol (2:1) and test was carried out under red and dim white illumination.

For experiment 1 and 2, The Observer XT 10 (NOLDUS, NL) was used to analyze videotapes. We created a behavioral ethogram in order to describe the interactions between focal and stimulus rats: we had a total of 8 behavioral items.

We divided the ethogram according firstly to two main positions of rats (namely "inside" and "outside" the white and black wall chamber) and secondly to two main groups of behaviors:

- non social behaviors: wall exploration, wall rearing, self-grooming, inactivity;

- social behaviors: cage exploration, cage rearing, social sniffing.

We analyzed for each behavior the total duration, total number (frequency) and the latency.

For experiment 3, we used Cage controller 1.27 for Dark light for Rat and Mouse (PRS, Rome, Italy), that allows to score: 1) each subject's motor activity (beam interruption per second) in either compartment; 2) time spent in each compartment (both forepaws and hind paws in a same compartment); 3) transitions (number of times a subject crosses the door between the two compartments). The compartment was divided into two sectors, one with two photocells close to the aluminium cage and the other one with two photocells close to the central door. This allowed to get the above parameters separately for sectors "near to" and "far from" the cage.

Data were divided into 300-second intervals (bins).

2.4 EX-VIVO NET IMMUNOFLUORESCENCE

The expression levels of noradrenaline transporter (NET) in WT, MIX-HET and MAT-HET rats were investigated by immunofluorescence in the prefrontal cortex (prelimbic and infralimbic sub-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

regions), hippocampus (dentate gyrus, CA1, CA2 and CA3 subregions) and hypothalamus (ventromedial and arcuate nuclei), by employing n = 6 rats per group.

2.4.1 PERFUSION AND TISSUE PROCESSING

All rats 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 in PBS. 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 immunofluorescence processing.

2.4.2 IMMUNOFLUORESCENCE STAINING

Immunofluorescence 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 2 h at room temperature under gentle shaking. Sections were then incubated in primary antibody solution for 24 h at 4°C under gentle shaking (3% NGS, 0.3% Tween-20 in PBS, with anti-NET antibody, 1:200, Invitrogen). Sections were washed in PBS for 1 h, incubated in secondary antibody for 2 h under gentle shaking (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.

NET immunofluorescence was assessed in the prefrontal cortex (prelimbic and infralimbic subregions, from 3.2 to 2.7 mm from bregma), hippocampus (dentate gyrus, CA1, CA2 and CA3 subregions, from -3.14 to -3.6 mm from bregma) and dorsomedial hypothalamus (from -3.14 to -3.6 mm from bregma), according to Paxinos and Watson; 2013, and the previously described regional distribution of NET in the rat brain (Schroeter et al., 2000). Images (one per section) were acquired on a Meiji Techno fluorescence microscope at 40x magnification, by employing Deltapix Insight imaging software. NET-p positive immunofluorescence was quantified by using ImageJ (NIH) and expressed as arbitrary units of integrated density values for each individual subject.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

2.5 DATA ANALYSIS

Results of behavioral scoring were analyzed in the experiments 1 and 2 using repeated measures analysis of variance (ANOVA). Our model was a 2 x 2 x 3 design: one between-subjects factor was focal genotype (MIX-HET and MAT-HET) while the other factors were within-subject. One factor was constituted by stimulus features (phenotype for Exp 1: WT female in estrous vs. WT female not in estrous; genotype for exp 2: WT male vs HET male "HZ"); the last factor was time constituted by 3 partial bins of five minutes each.

In experiment 3 we evaluated automatically-collected data by ANOVA with a 2x2x3 design. All FACTORS were within-subject: stimulus genotype (two levels: MIX-HET vs MAT-HET), position of the focal rat in the environment (two levels: "near to" or "far from" the aluminum cage) and time (3 levels: three partial bins of 5 minutes each). In a second step of analysis we run two separated ANOVAs, one focalized on data linked to the position "near" and the other one focalized on data linked to the position "far" (both ANOVAs thus had a 3x2 design). Multiple post hoc comparisons were performed with the Tukey's HSD test. Immunofluorescence data from each brain region were tested for normality and equal variances, and analyzed by using one-way ANOVA, considering epi-genotype as statistical factor, followed by Tukey's post hoc test when necessary.

Statistical analysis was performed using StatView II (Abacus Concepts, California, USA) and Prism 8.2 (Graphpad Software Inc). Data are expressed as mean ± SEM. Significance level was set at $P \leq 0.05$, NS = not significant.

3 RESULTS

3.1 PARTNER PREFERENCE

3.1.1 INSIDE CHAMBER WITH FEMALE IN ESTROUS

Total duration: analyzing the total duration of time spent inside the room with either target female rats, we observe MAT-HET male rats spending more time in the chamber of the female stimulus rat that was in estrous compared to the other that it was not in estrous [stimulus phenotypes * time, $F(2,44)=4.626$; $p=0.0150$]. This was not true for the epi-genotype MIX-HET (indicated in figure 1 as MIX-HZ).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

Latency: The POST HOC analysis with Tukey resulted in HSD (44; K=2)=14.56). The average latency is lower in the epi-genotype MAT-HET when target female is in estrous compared to latency observed for the other not in estrous (see figure 1). In the epi-genotype MIX-HET this effect was not observed. This piece of data can suggest the MAT-HET epi-genotype has a clear preference and motivation to reach the female in estrous compared to the epi-genotype MIX-HET ($p<0.05$).

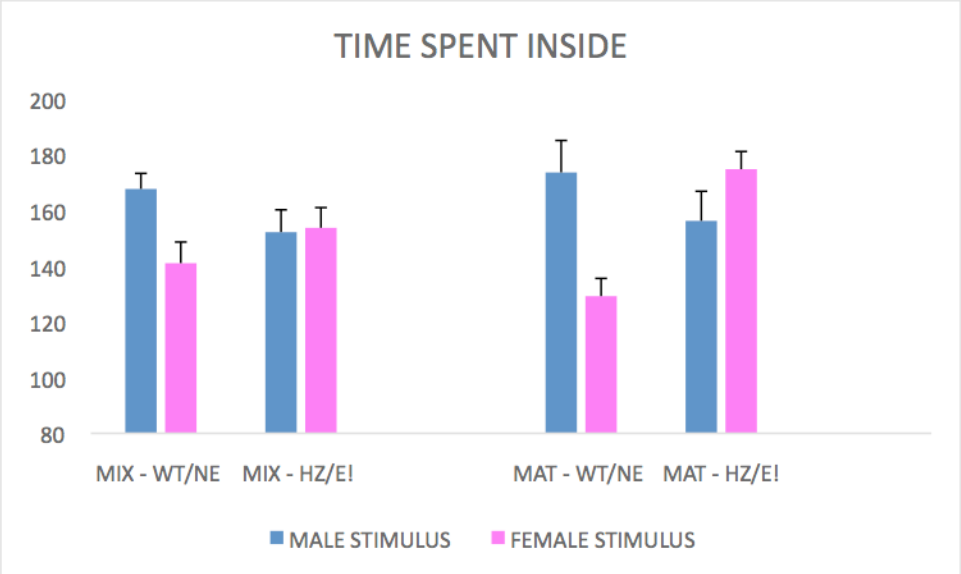

Figure 1) Total duration of time spent inside the chamber with a stimulus-conspecific subject (Bars represent performance of focal rats towards male stimuli, in Blue/ exp. 1, or towards female stimuli, in Pink/ exp. 2). MIX-HET (left bars) focal male rats show a profile in which, by having as stimulus a female in estrus (E!) or a HET male ("HZ"), the total preference doesn't drastically change compared to having a female not in estrus (NE) or a WT male, respectively. MAT- HET focal male rats, instead, show a clear preference for the female in estrus (E!) but decreased preference for HET stimulus (HZ) rodents compared to male WT controls.

3.1.2 SELF-GROOMING

Total duration: we observed (Figure 2) that the epi-genotype MAT-HET was more involved in self grooming behavior when in the chamber with the female in estrous, compared to that with the female non in estrous. This different profile was not exhibited by epi-genotype MIX-HET [stimulus phenotypes * focal genotypes, F(1,22)=5.237; p=0,0321].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

Total number: we note a greater frequency for self-grooming in the epi-genotype MAT-HET when they are in the chamber with the female in estrous. This phenomenon was absent for the epi-genotype MIX-HET.

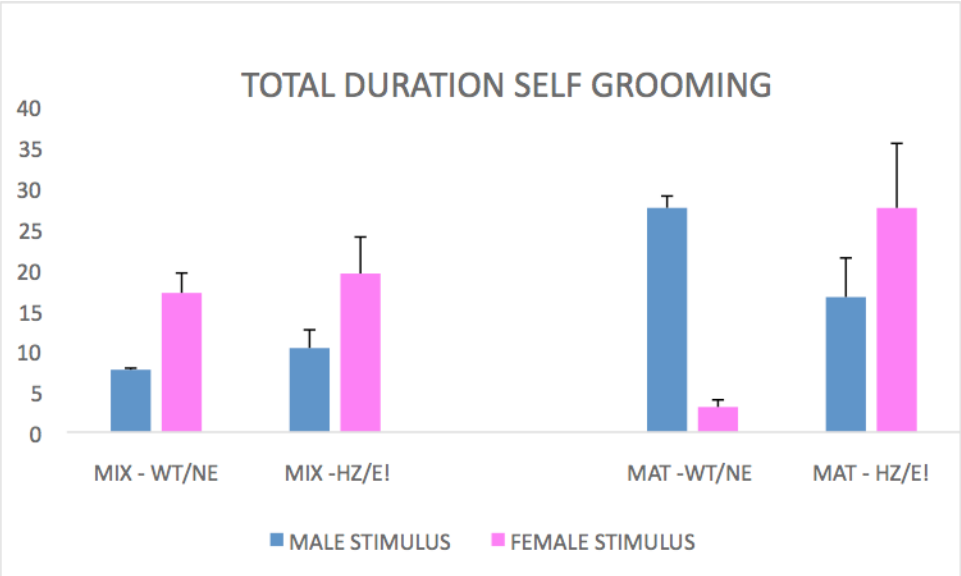

Figure 2) Total duration of self grooming, whose profile is quite different for the two focal epi-genotypes (Rats are the same as in figure 1). In the focal rats of epi-genotype MIX-HET (left bars), the total duration of self grooming is enhanced when the stimulus is a female. On the contrary, in the focal rats of epi-genotype MAT-HET (right bars), the total duration of self grooming is enhanced when the stimulus is a female in estrus (E!) while it decreases with a HET stimulus (HZ).

3.1.3 WALL EXPLORATION

Total number: POST HOC ANALISYS with Tukey resulted in HDS (44;K=2)=1,12. The average frequency for ”wall exploration” is statistically reduced the subjects are in the room with a female in estrus but this is found mostly in the MAT-HET male epi-genotype, which seems to be more concerned with the female itself than with the environment [stimulus phenotypes F(1,22)=5.737; p=0.0256; stimulus phenotypes * time F(2,44)=3.308; p=0.459]. Such profile is not true for the male epi-genotype MIX-HET which therefore is not distracted by the receptive female.

3.1.4 WALL REARING

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

Total duration: POST HOC ANALISYS with Tukey resulted in HSD (44;K=2)=3,46. We noted that the epi-genotype MIX-HET spent more time in rearing the wall despite the female in estrous while this effect was absent for epi-genotype MAT-HET.

Total number: the average number of times that rodents start rearing the wall is greater in the chamber with the female in estrous [stimulus phenotypes, F(1,22)=6.768; p=0.0163]. This is true for the MAT-HET while this phenomenon is absent in the epi-genotype MIX-HET

Latency: we note that the average latency for rearing the wall is greater in the chamber with the female in estrous. This appeared only for the epi-genotype MAT-HET while this was not true for the epi-genotype MIX-HET.

3.1.5 CAGE EXPLORATION

Latency: we observe (Figure 3) that the latency for exploration of the cage is decreased in the epi-genotype MAT-HET when they are in chamber with the female in estrous [stimulus phenotypes F(1,22)=4.862; p=0.0382; stimulus phenotypes * time F(2,44)=2.741; p=0.0755]. This is interesting compared to the epi-genotype MIX-HET where this phenomenon is less pronounced.

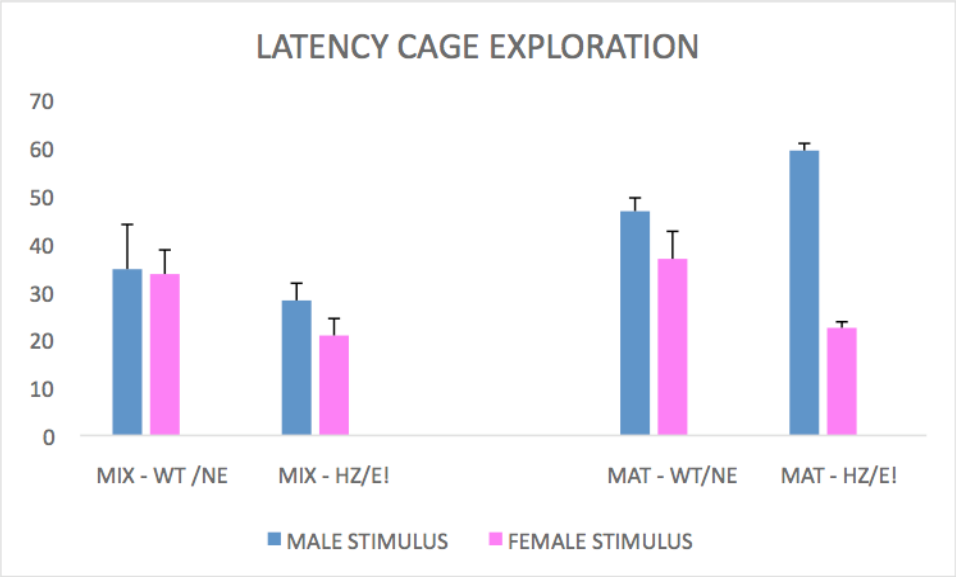

Figure 3) Latency to the exploration of the cage with stimuli (Rats are the same as in figure 1). Within focal rats of epi-genotype MIX-HET (left bars), latency to exploration of the cage is quite similar across all stimuli and if it is slightly reduced when the stimulus is a female in estrus. For the

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

focal rats of epi-genotype MAT-HET (right bars), latency changes drastically depending on the stimulus: it is lower with the female in estrus (E!) and higher with a HET stimulus (HZ).

3.1.6 CAGE REARING

Total duration: we observe that both MAT-HET and MIX-HET epi-genotypes spent more time in "CAGE REARING" when in the chamber with the female in estrous (as expected) [stimulus phenotypes $F(1,22)=7.980$; $p=0.0099$].

Latency: we observe that the average latency for rearing of the cage (when in the chamber with the female in estrous) is decreased for the epi-genotype MAT-HET and conversely increased for the epi-genotype MIX-HET [stimulus phenotypes * focal genotypes $F(1,22)=5.058$; $p=0.0349$; stimulus phenotypes * focal genotypes * time, $F(2,44)=4.045$; $p=0.0244$].

3.1.7 SOCIAL SNIFFING

Total duration: POST HOC analysis with Tukey resulted in HSD $(44;k=2)=4.24$ when there is a female in estrous, total duration is reduced in the epi-genotype MIX-HET [stimulus phenotypes $F(1,22)=7.581$; $p=0.0116$; stimulus phenotypes * time, $F(2,44)=3.308$; $p=0.459$]. Although this profile was expected to be found, it nonetheless emphasizes no sex-related interest, compared to the epi-genotype MAT-HET.

3.1.8 INACTIVITY

Total number: we note that, similarly for the MAT-HET and MIX-HET epi-genotypes, rats started more often being "inactive" in the chamber with the female in estrous (i.e. fragmented inactivity).

3.2 SOCIAL PREFERENCE TEST

3.2.1 INSIDE THE CHAMBER WITH A MALE STIMULUS

Total duration: as expected focal rats of epi-genotype MIX-HET tend to spend less time in the chamber with a same-genotype stimulus compared to stimulus WT rats ($p<0.05$). This result (see

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

Figure 1) is even more pronounced in the epi-genotype MAT-HET [stimulus genotypes * focal genotypes, F(1.15)=3453; p=0.0829; stimulus genotypes * focal genotypes * time, F(2,30)=860; p=0.09422]. In fact, focal MAT-HET rodents spent much less time in the chamber with a MIX-HET stimulus ("HZ") rodent.

Total number: we can note that the number of times in which the focal rat enters in the chamber with a "HZ" (MIX-HET STIMULUS) is statistically lower for a MAT-HET compared to a MIX-HET focal rat. MIX-HET subjects still prefer to enter a considerable number of times in the room with a MIX-HET stimulus, while a MAT-HET focal enters in the chamber much less (nearly a -25%) [stimulus genotypes, F(1,13)=3.539; p=0.0825].

Latency: we observe (Figure 2) that the average latency to enter in a chamber with a MIX-HET stimulus is a bit lower for the epi-genotype MIX-HET compared to WT control stimuli, instead it is much greater ($p<0.05$) for the epi-genotype MAT-HET [stimulus genotypes * focal genotypes F(1,15)=3.875; p=0.0678; stimulus genotypes * time F(2,30)=3.370; p=0.0478; stimulus genotypes * focal genotypes * time, F(2,30)=3.928; p=0.305].

3.2.2 SELF GROOMING

Total duration: Tukey post hocs resulted in HDS (15; K=2)=3.375. We note (Figure 2) opposed results for the two epi-genotypes: when they are in the chamber with a "HZ" MIX-HET stimulus, the average total duration of self grooming in focal rats is greater for the epi-genotype MIX-HET and lower for the epi-genotype MAT-HET when compared to control WT stimuli in the same conditions ($p<0.05$).

Latency: when they are in the same chamber with a "HZ" MIX-HET stimulus, the average latency is lower for the focal MIX-HET and higher for the focal MAT-HET ($p<0.05$) when compared to control WT stimuli in the same condition.

3.2.3 WALL EXPLORATION

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

Total duration: data have shown that total duration of wall exploration is different for focal MIX-HET and MAT-HET rodents when they have a MIX-HET rodent ("HZ") as stimulus. The total duration of this behavior increases in focal MAT-HET rodents compared to WT control stimuli. Interestingly we don't see this trend in MIX HET rodents [stimulus genotypes * focal genotypes, $F(1.15)=5.011$; $p=0.0408$]. MAT-HET appear asocial.

Latency: The average latency for focal MAT-HET rats is greater when they have as stimulus a MIX-HET rodent compared to a WT stimulus control [stimulus genotypes * focal genotypes $F(1,15)=3453$; $p=0.0829$]. The same trend is not shown at all in the focal epi-genotype MIX-HET; the two epi-genotypes, therefore, differ considerably due to having been cared by a DAT-HET dam versus a WT one.

3.2.4 WALL REARING

Latency: the average latency for rearing a wall is lower for both MIX-HET and MAT-HET epi-genotypes when they are in contact with a MIX-HET stimulus ("HZ") rat rather than a WT one ($p<0.05$) [stimulus genotypes, $F(1,15)=4.562$; $p=0.0496$].

3.2.5 CAGE EXPLORATION

Total number: data have shown that average frequency is decreased for the focal rats of MAT-HET epi-genotype when they are in the same chamber with a stimulus, particularly if it was a "HZ" of MIX-HET epi-genotype ($p<0.05$) [focal genotypes, $F(1,15)=5.001$; $p=0.0410$]. MAT-HET appear therefore asocial.

Latency: data have shown that the average latency is greater for focal MIX-HET and lower for the focal MAT-HET rodents ($p<0.05$) when they are in contact with a MIX-HET ("HZ"), rather than a WT control stimulus [stimulus genotypes * focal genotypes * time, $F(2,30)=3.593$; $p=0.0399$].

3.2.6 CAGE REARING

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

Total number: POST HOC analysis with Tukey yielded HSD=(30;K=2)=1.50. We note that the frequency for the rearing over a cage is decreased for focal rodents of the MAT-HET epi-genotype when they are in the same chamber with the "HZ" MIX-HET stimulus rat, confirming that they are actually asocial.

3.2.7 SOCIAL SNIFFING

Latency: Data analysis has shown an emerging trend in the last bins [Stimulus genotypes * focal genotypes * time, (2,30)=3198; p=0.0551]. MAT-HET focal rodents, when in contact with the stimulus "HZ" (MIX-HET rat), show a more pronounced latency for this behavior compared to the focal MIX-HET rodent.

3.2.8 INACTIVITY

Total duration and frequency: data have shown that MAT-HET focal rodents start to be inactive more often [stimulus genotypes * focal genotypes, F(1,15)=9.185; p=0.084] and remain inactive for much more time facing MIX-HET stimulus [stimulus genotypes * focal genotypes, F(1,15)= 3.563; p=0.0786]. In other words, when focal MIX-HET meet the MIX-HET stimulus, the frequency and total duration of "inactivity" are lower ($p<0.05$) compared to results with a WT control stimulus; contrarily, the frequency and total duration of "inactivity" raise for focal MAT-HET rodents when put with MIX- HET stimuli. Once again the latter epi-genotype appears asocial.

3.3 ELICITED PREFERENCE TEST

By the analysis of automatically-collected data we evaluated if there was a social preference for one of the two epi-genotypes (MAT-HET vs MIX-HET of the same sex).

Activity rate and transitions: ANOVA didn't show significance for interaction of environment with epi-genotype of stimulus rats (stimulus genotype * environment-position * time, F [2,70]=0.48; p=0.9532) while interestingly a significant profile appeared for a number of transitions (F[2,70] = 5.989; p=0.0040). Data indicate that there was a relatively lower frequency of cage approaches compared to room entries towards the MAT-HET cage. Comparable entries and approaches

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

emerged with a MIX-HET stimulus especially during the first partial bin. A reduction in the approach rate to the cage, when the time that the subjects spend in the sector containing that cage increases (see below), implies a longer average duration for each single approach: MAT-HETs seem to be the most attracted to it.

Time spent with stimulus rats: ANOVA for time spent in "sectors" within the chambers containing the stimulus rats didn't show any significance (stimulus genotype * environment-position * time, $F[2,68] = 2.535$; $p = 0.867$). In the presence of a strong effect of environment position ($F[1,34]=96.784$; $p<0001$) separate ANOVA for the position "far" was carried out (stimulus genotype*time, $F[2,70]=1.59$; $p=0.2096$) Another ANOVA for the position "near" showed significance ($F[2,70]=4.96$; $p=0.0231$). Post-hoc analyses and the threshold obtained with Tukey was 10.41 ($df=70$; $K=3$). Specifically, focal WT rats spent significant ($p<0.05$) more time in the last partial bin (10-15 min) with the subtype MAT-HET stimulus partner than with the MIX-HET one (Data not shown).

3.4 EX-VIVO NET IMMUNOFLUORESCENCE

NET-positive immunofluorescence was quantified in brain regions relevant to social behavior. The results of one-way ANOVA showed no significant effect of the epi-genotype on NET immunofluorescence in both prelimbic ($F (2, 15) = 0.07783$, $p = 0.9255$) and infralimbic ($F (2, 15) = 0.3277$, $p = 0.7256$) prefrontal cortex. On the other hand, data from NET immunofluorescence in the dorsomedial hypothalamus showed a significant effect of epi-genotype ($F (2, 15) = 7.806$, $p = 0.0047$), with MAT-HET rats displaying a significant increase with respect to WT ($q = 4.485$, $df = 15$, $p= 0.0164$), whereas MIX-HET showed a significant decrease when compared to MAT-HET rats ($q = 5.129$, $df = 15$, $p= 0.0066$) (fig. 4 B, C). No significant difference was observed in the arcuate nucleus ($F (2, 15) =$ respectively $0.2.357$, $p = 0.1288$) (figure 4 D, E). When NET immunofluorescence in the hippocampus was analyzed, one-way ANOVA revealed a significant effect of the epi-genotype in the dentate gyrus ($F (2, 15) = 8.439$, $p = 0.0035$), with a significant increase in MAT-HET rats with respect to WT ($q = 4.634$, $df = 15$, $p= 0.0133$), and a significant decrease in MIX-HET rats with respect to MAT-HET group ($q = 5.352$, $df = 15$, $p = 0.0048$) (figure 4 F, G). Moreover, the effect of epi-genotype was statistically significant in the CA1 region ($F (2, 15) = 7.619$, $p = 0.0052$), where MIX-HET rats displayed a significant decrease with respect to MAT-HET group ($q = 5.417$, $df = 15$, $p= 0.0044$) (figure 4 H, I); in addition, we observed significant differences in the CA2 region ($F (2, 15) = 6.858$,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

p = 0.0077), with a significant increase in MAT-HET epi-genotype with respect to WT rats (q = 3.837, df = 15, p= 0.0401) and a significant decrease in MIX-HET rats with respect to MAT-HET group (q = 5.006, df = 15, p= 0.0079) (figure 4 L, M); furthermore, one-way ANOVA revealed a significant effect of the epi-genotype in the CA3 region (F (2, 15) = 10.58, p = 0.0014), and Tukey's post hoc test highlighted a significant increase in MAT-HET rats with respect to WT rats (q = 4.209, df = 15, p= 0.0241) and a significant decrease in MIX-HET group when compared to MAT-HET rats (q = 6.401, df = 15, p= 0.0011) (figure 4 N, O).

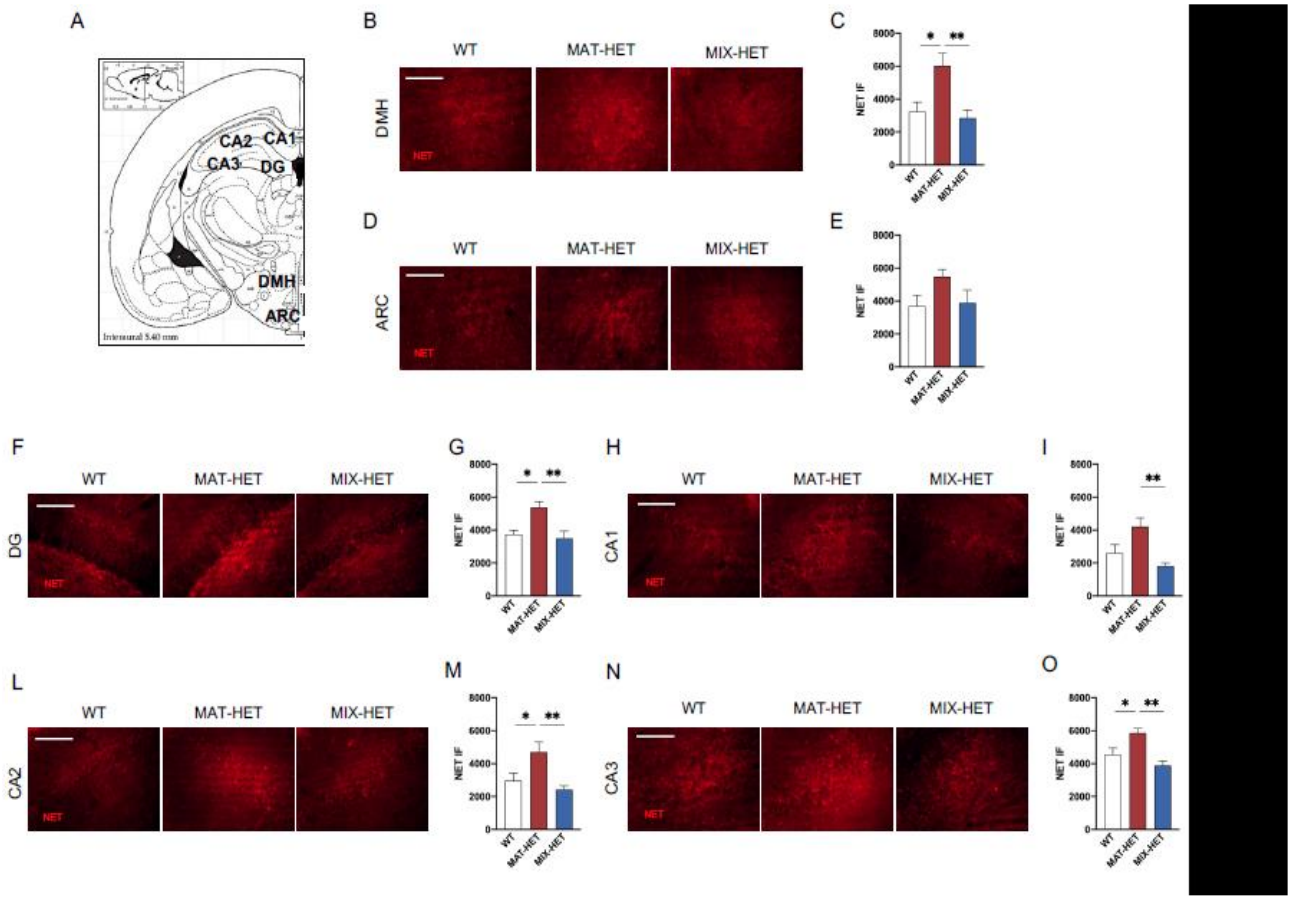

Figure 4) Comparison between MIX-HET and MAT-HET focal subjects with NET immunofluorescence in the hypothalamus and hippocampus. Images from NET-positive immunostaining were acquired at 40x magnification in the dorsomedial (DMH) and arcuate (ARC) hypothalamus, dentate gyrus (DG), CA1, CA2 and CA3 subregions of the hippocampus (A). MAT-HET rats displayed increased NET immunofluorescence with respect to WT and MIX-HETs in the dorsomedial hypothalamus (B, C), while no differences were observed in the arcuate nucleus (D, E). Moreover MAT-HET rats showed higher NET immunofluorescence than WT and/or MIX-HET rats in the DG (F, G), CA1 (H, I), CA2 (L, M) and CA3 (N, O). Each bar represents the mean ± S.E.M. of n = 6 rats. * $p < 0.05$; ** $p<0.01$. scale bar 100 μm

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

4 DISCUSSION

The epi-genotype MAT-HET exhibited a masked social profile only when found in proximity to a WT rat stimulus. On the other hand, these focal rats appear similar to a wild-type rat when in contact with a female in estrous. Specifically, MAT-HETs exhibited a higher "total duration" and frequency and a reduced "latency" for the exhibition of the social behaviors (cage exploration, cage rearing, social sniffing) toward the female in estrous while the contrary was displayed toward the "HZ" (namely, a stimulus of the epi-genotype MIX-HET). This behavioral phenotype was associated with increased NET immunofluorescence in hippocampus and hypothalamus, with respect to WT and MIX-HET progeny. When acting as focal, MIX-HET rodents' social profile was a bit different from a WT rat because it was not changing at all depending on available stimuli, just being lower when in contact with another HET male. These rats also exhibited a higher quantity of two non social behaviours, such as "wall exploration" and "wall rearing". Hence, a major interest for the environment was always shown, also when they were in the same chamber with a female in estrous. Although MIX-HET rats did not differ significantly in behaviour from WT rats, they displayed decreased NET immunofluorescence in the hippocampus and hypothalamus with respect to MAT-HET rats, while the latter are greatly asocial only towards a same-sex HZ. Thus, because MIX-HETs display a behavioural profile which resembles that of non-mutant rats, the combination of external stimuli and epi-genotype can present itself as a promising basis to speculate that these rodents could possibly become a behavioural model for socio-sexual apathy.

4.1 DETAILED SUMMARY OF DATA

After the analysis of data, some key differences were evidenced in the behavior of focal MAT- vs. MIX-HET rats. Indeed, when they had a choice between various social stimuli, different results came out from the partner compared to the social preference test (SPP). While considering total duration spent inside the chamber allowing contact with a stimulus, focal male MIX-HET rodents appeared less interested toward a female in estrous compared to a female not in estrous. Thus, MIX-HET males are showing somewhat an altered sex-driven behavior. We noted that focal male MAT-HET rats tended to show a normal socio-sexual behavior, in that they were clearly more attracted by the female in estrous than by a female not in estrous. The opposite was true for what concerns time spent with a same-sex conspecific. Indeed, when compared to focal MIX-HET male rodents, MAT-HET male rodents tended to spend slightly less time in the chamber with a MIX-HET

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

stimulus (HZ) compared to control WT stimulus rodents. Therefore rats from those epi-genotypes exhibited a marked reduction in general social interest.

For what concerns the latency to enter a room, the MAT-HET epi-genotype quickly reached a female in estrous. On the other hand, when faced with a MIX-HET stimulus (HZ) of the same sex, MAT-HET male rats seem to lose any interest. In fact, focal rats of the MIX-HET epi-genotype show similar latency to social interact regardless of type of genotype or sex of the stimulus. Indeed they showed comparable levels with a MIX-HET male and a WT male, or with a female in estrous or one not in estrous. This confirms the extremely different socio-sexual approach behavior shown by either MIX- or MAT-HET epi-genotypes.

For what concerns self-grooming, we can note opposite results in the performance by two epi-genotypes. Total duration of self-grooming by MAT-HET epi-genotype focal rats was greater when in the chamber with a female in estrous than with a female not in estrous. On the contrary, self grooming activities were lower when they could come in contact with the MIX-HET male stimulus, compared to WT male controls. Opposite results appeared for focal MIX-HET males that seemed to be more stressed (than focal MAT-HET ones) when in contact with a female (regardless of her hormonal condition), compared when in proximity to the male stimuli.

For what concerns the dynamics of chamber-wall or cage exploration, it is noteworthy that both duration and frequency was reduced for the former, and increased for the latter, respectively, when the focal rats showed interest for the stimulus (namely, by sniffing the stimulus rat itself or its aluminum cage). The finding of a significant reduction in the interest for the chamber-wall was offered when a male MAT-HET focal rat was in the chamber with the female in estrous. On the other hand, this profile was absent for the epi-genotype MIX-HET denoting an abnormal behavior: in fact these males ignored sex triggers and displayed a strange “wall preference”. Instead, when the stimulus was represented by a MIX-HET rodent (“HZ”) compared to a WT control, the dynamics of the behavior was different. In fact, the interest shown by this subject for the MIX-HET focal male was greater, while it was much lower for the MAT-HET focal male. For what concerns the cage rearing, we noted that the average total number was decreased for the focal MAT-HET rodents (both if they have a female in estrous compared to control one, or a MIX-HET stimulus “HZ” compared to WT controls).

The MIX rodents (characterized by having been reared by a heterozygous dam) exhibited as adults strong tendencies to not discriminate among diverse stimuli. On the contrary the MAT rats (all

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

heterozygous subjects reared by a WT dam) were showing overall overt climbing toward the receptive partner but never tried reaching heterozygous males. Namely specifically exclusive interest for mating and no interest in same-sex social behavior was shown at all. In the elicited preference test, a slight but significant preference was shown by focal WT male rats , for the MAT-HET male stimulus. This behavior indirectly provide indications that the MIX-HET subjects are somewhat generically avoided, above all in the last partial bins of a session.

When NET immunofluorescence was analysed in brain regions relevant to social behavior, our data showed no significant difference in the prefrontal cortex amongst the three epi-genotypes. On the other hand, MAT-HET rats showed increased NET levels in discrete hippocampal subregions with respect to WT rats. In addition, MAT-HET rats showed significant higher level in NET-positive immunofluorescence with respect to MIX-HET rats in the four subregions considered.

A similar picture emerged from the analysis of data from NET immunofluorescence in the hypothalamus, where MAT-HET rats showed increased NET levels, with respect to both WT and MIX-HET rats.

DAT KO rats have been reported to exhibit some positive symptoms of schizophrenia such as psychomotor agitation, stereotypy, deficits of prepulse inhibition (PPI) and cognitive impairment. (Wong et al., 2015). Furthermore Rodriguiz et al., (2004) reported that DAT-KO mice exhibit a marked aggressive phenotype. DAT-KO rats often engage in stereotyped and perseverative social behaviors which become more robust over time. These findings suggest that stereotypy and perseveration could distort the social interactions of KO rats. As far as their behavioral repertoires become restricted and inflexible, KO rats show dominant traits. So far, nothing is known about the sociality of heterozygous subjects. Yet, social behavior is highly rewarding, thus insights can be drawn from literature. Previous research has shown that both DA and NA are involved in social behavior. In detail, while DA modulates the rewarding and motivational aspect of social interaction, noradrenergic systems is involved in the attentional processes relevant to social behavior (Vanderschuren et al.; 1997). Interestingly, recent evidence shows that DA is strongly associated with motivation for social play whilst NA enhancement negatively modulates both motivation and expression of social play (Achterberg et al., 2016). Sociality could be influenced as well by quality of received maternal care during development . In a study, rats that received high levels of licking and grooming by their mothers spent significantly more time in social contact as adults with unfamiliar individuals, compared to subjects that received fewer levels of maternal care. In adult ro-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

dents which received more maternal care, a reduced level of anxiety is linked with their greater social interaction (Starr-Philips et al., 2014). In contrast, one study which has focused on the C57BL/6J strain, affirmed that rats receiving lower doses of licking and grooming from their mothers play more frequently and exhibit a more pronounced social behavior, compared to those who receive higher doses of licking and grooming (Franks et al., 2015).

Based on these observations, we aimed to compare brain levels of norepinephrine in MAT, MIX and WT specimens in order to evaluate their possible involvement in the behavioral abnormalities found in MATs.

Multiple brain regions including prefrontal cortex, hippocampus, and hypothalamus display active neurons during social interactions (Zhang, 2020). The *ex vivo* analysis performed on these subjects, in particular the dentate gyrus (DG) and its subregions (CA1, CA2 and CA3), have clearly shown that concentration of NA in MATs is equal to almost the double than levels found, in the same regions, in WT and MIX specimens.

It is known that exposure to stress determines a modification in central neurotransmission (Torta R., Caldera P., 2008) in particular of noradrenaline (NA), serotonin (5-HT) and dopamine (DA): in conditions of acute stress, an increased release of these neurotransmitters represents an adaptive response to the stressful stimulus. One role of NA is to activate the paraventricular nuclei of the hypothalamus, a fundamental site for the regulation of behavioral responses that require autonomous reactivity. Anisman et al., (1987) have demonstrated the wide involvement of this neurotransmitter's increased concentration, particularly in the hypothalamus, following acute stress response. NA also plays an important role in a number of complex cognitive functions such as memory, attention, regulation of energy levels and that of emotions. Therefore, low concentrations of NA are often correlated with disorders such as depression, lack of energy, disturbances in attention and concentration, cognitive deficits. Conversely, high concentrations of these neurotransmitter appear to be correlated to stress response behaviors, including hyperactivity, anxiety, insomnia and irritability. In the hippocampus, NA has an active role in learning and consolidating memory and retrieving visual-spatial information, albeit not emotional through β_1 adrenergic receptors (Murchison C.F., et al., 2004).

The present evidence expanded our previous reports showing that HET rats displayed increased levels of norepinephrine in the hippocampus and hypothalamus, along with increased inactivity in the face of the social stimulus (Adinolfi et al., 2019). In particular, the immune-fluorescent experi-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 May 2021 doi:10.20944/preprints202105.0404.v1

ment confirmed a significant alteration in the noradrenergic transmission to the hippocampus and hypothalamus in HET rats, with genotype-specific differences of opposite type. These may underlie the different behavioral phenotypes of the two groups. Although only a tendency with respect to WT, MIX-HET male progeny showed a lower expression of NET in both hypothalamus and hippocampus with respect to MAT-HET rats. This evidence is somehow consistent with the elevated level of noradrenaline measured in the same brain regions in similarly raised MAT-HET rats, reported previously (Adinolfi et al., 2019). On the other hand, MAT-HET offspring displayed marked increment in the hypothalamus and hippocampus of NET immune-fluorescence, compared to WT and MIX-HET rats. Despite a comprehensive comparative analysis of the hippocampal circuits and mechanisms underlying the wide range of social interactions explored in the present experiments (including social recognition to compare the conspecific male/male, male/female and cross-epigenotype recognition) has not yet been reported, previous work has established that the hippocampal CA2 and CA3 regions are involved in social processing. Indeed, the genetic lesion of CA2 impaired social recognition (Hitti and Siegelbaum 2014), CA3 pyramidal cell plasticity and transmission contribute to the encoding of social stimuli (Chiang et al., 2018). Our data further suggest that altered noradrenergic transmission in DG may contribute to the wide range of social alteration observed in HET rats.

Therefore it could be hypothesized that the socially anomalous behavior of MAT individuals, which are heterozygous for DAT (KO allele inherited from the father) and had been raised from a WT mother unlike MIX subjects, which are heterozygous for DAT (from an heterozygous DAT-HET female), may be accounted by a high concentration of NA in the brain regions responsible for stress regulation. By this framework, it is possible to hypothesize the concomitant NA hyperfunction and *viceversa*, hypofunctionality of the DAT gene.

4.2 CONCLUDING HYPOTHESES ABOUT DAT-HET EPI-GENOTYPES

As a whole, MIX-HET male rats showed an altered social behavior: with the specifier of social apathy. This profile has been here associated to increased noradrenaline input to the hippocampal and hypothalamic regions. In the elicited preference test, we observed that WT rodents prefer to spend time with a MAT-HET stimulus. This can well be due to the fact that MIX-HET rats are rather apathic while WT controls are more easily compared to a MAT-HET rat. The latter, however, show an “almost bipolar-like” profile (see also Carbone et al., 2019), ranging from no interest for a “HZ”

male to a great excitation and approach toward a receptive female. Not only MAT-HETs result attractive compared to MIX-HET stimulus rodents; also, MIX-HET male rodents seem more stressed (compared to MAT-HET ones) when in contact with a female, regardless her hormonal condition.

A crucial factor seems to be represented by the fact that MIX-HET subjects had been raised by heterozygous mothers (as it is always the case in a classical colony setting). Our previous study (Mariano et al., 2019) included observations demonstrating that MIX-HET mothers are much involved in self-grooming and/or to dig away from the nest, rather than caring for the pups. Instead, WT dams provided appropriate levels of licking and grooming to the pups: we proposed that, because of the style of maternal care received , WT female pups did not differ so much from MAT-HET pups (Mariano et al., 2019), while MIX-HET females developed a somehow altered circadian cycle. In that study, due to colony HET/HET breeding, we acknowledged that both halves of MIX-HETs were comprised (including DAT functional alleles randomly of paternal origin: this factor was obviously dropped here, by using KO fathers). As mother-infant interaction is known to modulate noradrenergic neurotransmission in the pups (Kalpachidou et al., 2016), we could not exclude that, for some (up to a half) of those DAT-HET females, the inactivated DAT maternal allele played a role. However this is still under our investigation (Oggiano et al., *in preparation*).

Maternal care has been shown of particular importance for the development of mammalian infants and their social behavior. Individuals that don't receive the regular levels of licking and grooming by their mothers have been reported to exhibit higher levels of anxiety-related behavior and reduced social motivation at adulthood. This "maternal effect" could explain at least partially the difference in the social behavior of the two epi-genotypes. In a future study, we would like to evaluate the behavior of a next generation from present subjects: if female behavior is affected by her dam's behavior (Mariano et al., 2019), a carryover consequence may be hypothesized to pups which are in turn offspring from these two epi-genotypes. We plan to cross-breed the two subtypes so that all parents are of a same heterozygous genotype, yet the grandparents will differ.

REFERENCES

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. *Behavioural Brain Research 359,* 516-527. Doi: 10.1016/j.bbr.2018.11.028

Ahern, T.H., Modi, M.E., Burkett, J.P. & Young, L.J. (2009). Evaluation of two automated metrics for analyzing partner preference tests. *J Neuroscience Methods, 182*, 180-188. Doi: 10.1016/j.jneumeth.2009.06.010

Anacker, A.M.J., Christensen, J.D., LaFlamme, E.M., Grunberg, D.M. & Beery, A.K. (2016). Septal oxytocin administration impairs peer affiliation via V1a receptors in female meadow voles. *Psycho-neuro-endocrinology, 68,* 156-162. Doi: 10.1016/j.psyneuen.2016.02.025

Anisman, H., Irwin, J., Bowers, W., Ahluwalia, P. & Zacharko, R.M. (1987). Variations of norepinephrine concentrations following chronic stressor application. *Pharmacol Biochem Behav*, *26*(4), 653-9. Doi: 10.1016/0091-3057(87)90592-2

Beery, A.K., McEwen, L.M., MacIsaac, J.L., Francis, D.D. & Kob, M.S. (2016). Natural variation in maternal care and cross-tissue patterns of oxytocin receptor gene methylation in rats. *Hormones & Behavior, 77*, 42-52. Doi: 10.1016/j.yhbeh.2015.05.022

Beery, A.K., Routman, D.M. & Zucker, I. (2010). Same-sex social behavior in meadow voles: multiple and rapid formation of attachments. *Physiology & Behaviour, 97,* 52-57. Doi: 10.1016/j.physbeh.2009.01.020

Brun, V.H., Otnass, M.K., Molden, S., Steffenach, H.A., Witter, M.P., Moser, M.B. & Moser, E.I. (2002). Place cells and place recognition maintained by direct entorhinal-hippocampal circuitry. *Science*, 296, 2243–2246. Doi: 10.1126/science.1071089

Bugajski, J., Gadek-Michalska, A., Ołowska, A., Borycz, J., Głód, R. & Bugajski, A.J. (1995). Adrenergic regulation of the hypothalamic-pituitary-adrenal axis under basal and social stress conditions. *Physiol Pharmacol*, 46(3,) 297-312. PMID: 8527811

Carbone, C., Brancato, A., Adinolfi, A., Lo Russo, S.L.M., Alleva, E., Cannizzaro, C. & Adriani, W. (2020). Motor transitions' peculiarity of heterozigous DAT rats when offspring of an unconventional KO x WT mating. *Neuroscience* (in press).

Chiang, M.C., Huang, A.J.Y., Wintzer, M.E., Ohshima, T. & McHugh, T.J. (2018). A role for CA3 in social recognition memory. *Behav Brain Res*, *354*, 22-30. Doi: 10.1016/j.neuroscience.2020.03.005

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 DAT KO rats: Compulsive traits, motor stereotypies and anhedonia. *Frontiers in Psychiatry* (in press). Doi: 10.3389/fpsyt.2018.00043

Cirulli, F., Terranova, M.L. & Laviola, G. (1996). Affiliation in peri-adolescent rats: Behavioral and corticosterone response to social reunion with familiar or unfamiliar partners. *Pharmacology Biochemistry and Behaviour*, *54* , 99-105. Doi: 10.1016/0091-3057(95)02169-8

Ecker, A., Romani, A., Sáray, S., Káli, S., Migliore, M., Mercer, A., Markram, H., Muller, E. & Ramaswamy, S. (2019). Data-driven integration of hippocampal CA1 synapse physiology in silico. *Biorixiv, 30*(11), 1129-1145. Doi: https://doi.org/10.1101/716480

Fritz, M., El Rawas, R., Klement, S., Kummer, K., Mayr, M.J., Eggart, V., Salti, A., Bardo, M.T., Saria, A. & Zernig, G. (2011). Differential effects of accumbens core vs. shell lesions in a rat concurrent conditioned place preference paradigm for cocaine vs. social interaction. *PlosOne ,6,* e26761 Doi: 10.1371/journal.pone.0026761

Gainetdinov, R.R., Bohn, L.M., Walker, J.K., Laporte, S.A., Macrae, A.D., Caron, M.G., Lefkowitz, R.J. & Premont, R. (1999). Muscarinic supersensitivity and impaired receptor desensitization in G protein-coupled receptor kinase 5-deficient mice. *Neuron*, *24*, 1029-1036 Doi: 10.1016/s0896-6273(00)81048-x

Giros, B., Jaber, M., Jones, S.R., Wightman, R.M. & Caron, M.G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature, 379,* 606-612. Doi: 10.1038/379606a0

Gunaydin, L.A., Grosenick, L., Finkelstein, J.C., Kauvar, I.V., Fenno, L.E., Adhikari, A., Lammel, S., Mirzabekov, J.J., Airan, R.D., Zalocusky, K.A., Tye, K.M., Anikeeva, P., Malenka, R.C. & Deisseroth, K. (2014). Natural neural projection dynamics underlying social behavior. *Cell ,157*, 1535-1551. Doi: 10.1016/j.cell.2014.05.017

Gutknecht, L., Popp, S., Waider, J., Sommerlandt, F.M.J., G¨oppner, C., Post, A., Reif, A., Van Den Hove, D., Strekalova, T., Schmitt, A., Colaço, M.B.N., Sommer, C., Palme, R. & Lesch, K.P. (2015). Interaction of brain 5-HT synthesis deficiency, chronic stress and sex differentially impact emotional behavior in Tph2 knockout mice. *Psychopharmacology, 232,* 2429-2441. Doi: 10.1007/s00213-015-3879-0

Ishida, Y., Hashiguchi, H., Takeda, R., Ishizuka, Y., Mitsuyama, Y. & Kannan, H., et al. (2002). Conditioned-fear stress increases Fos expression in monoaminergic and GABAergic neurons of the locus coeruleus and dorsal raphe nuclei. *Synapse*, *45,* 46-51. Doi: 10.1002/syn.10086

Kingsbury, M.A. & Goodson, J.L. (2014). Pair bond formation is impaired by VPAC receptor antagonism in the socially monogamous zebra finch. *Behavioural Brain Research*, *272,* 264-268. Doi: 10.1016/j.bbr.2014.06.042

Kummer, K.K., el Rawas, R., Kress, M., Saria, A. & Zerning, G. (2015). Social interaction and cocaine conditioning in mice increase spontaneous spike frequency in the nucleus accumbens or septal nuclei as revealed by multielectrode array recordings. *Pharmacology*, *95,* 42-49. Doi: 10.1159/000370314

Laviola, G. & Terranova, M.L. (1996). The developmental psychobiology of behavioral plasticity in mice: the role of social experiences in the family unit. *Neuroscience & Biobehavioral Review, 23,* 197-213. Doi: 10.1037/0735-7036.114.4.325

Leo, D., Sukhanov, I., Zoratto, F., Illiano, P., Caffino, L., Sanna, F., Messa, G., Emanuele, M., Esposito, A., Dorofeikova, M., Budygin, E., Mus, L., Efimova, E., Niello, M., Espinoza, S., Sotnikova, T., Hoener, M., Laviola, G., Fumagalli, F., Adriani, W. & Gainetdinov, R.R. (2018). Pronounced hyperactivity, cognitive dysfunctions and BDNF dysregulation in dopamine transporter knockout rats. *Journal of Neuro-science*, (in press). Doi: 10.1523/jneurosci.1931-17.2018

Mariano, S., Pardo, M., Buccheri, C., Illiano, P., 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). Doi: 10.1002/dev.21927

Martín-López, M., Muela, A.T., Cavas, M. & Navarro, J.F. (2018). Effects of para-methoxy-amphetamine (PMA) on agonistic encounters between male mice. *Pharmacology, Biochemistry and Behaviour, 167,* 9-16. Doi: 10.1016/j.pbb.2018.02.002

Murchison, C.F., Zhang, X., Zhang, W., Ouyang, M., Lee, A. & Thomas, S.A. (2004). A Distinct Role for Norepinephrine in Memory Retrieval. *Cell Press, 117*, 131-143. https://core.ac.uk/download/pdf/82761124.pdf

Pacak, K., Palkovits, M., Kopin, I.J. & Goldstein, D.S. (1995). Stress-induced norepinephrine release in the hypothalamic paraventricular nucleus and pituitary-adrenocortical and sympathoadrenal activity: in vivo microdialysis studies. *Front Neuroendocrinol*, *16*(2), 89-150. Doi: 10.1006/frne.1995.1004

Pearce, E., Wlodarski, R., Machin, A. & Dunbar, R.I.M. (2017). Variation in the β-endorphin, oxytocin, and dopamine receptor genes is associated with different dimensions of human sociality. *Psychological and Cognitive Sciences, 114*, 5300-5305. Doi: https://doi.org/10.1073/pnas.1700712114

Perkeybile, A.M. & Bales, K.L. (2017). Intergenerational transmission of sociality: the role of parents in shaping social behavior in monogamous and non-monogamous species. *Journal of Experimental Biology, 220,* 114-123. Doi: 10.1242/jeb.142182

Rodriguiz, R.M., Chu, R., Caron, M.G. & Wetsel, W.C. (2004). Aberrant responses in social interaction of dopamine transporter knockout mice. *Behavioural Brain Research, 148,* 185-198. Doi: 10.1016/s0166-4328(03)00187-6

Sanna, F., Bratzu, J., Serra, M.P., Leo, D., Quartu, M., Boi, M., Espinoza, S., Gainetdinov, R.R., Melis, M.R. & Argiolas, A. (2020). Altered sexual behavior in dopamine transporter (DAT) knockout male rats: a behavioral, neuro-chemical and intra-cerebral micro-dialysis study. *Frontiers in Behavioral Neuroscience,* (in press). Doi: 10.3389/fnbeh.2020.00058

Sora, I., Wichems, C., Takahashi, N., Li, X.,F., Zeng, Z., Revay, R., Lesch, K.P., Murphy, D.L. & Uhl, G.R. (1998). Cocaine reward models: Conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. *Proc Natl Acad Sci USA, 95*, 7699–7704. Doi: 10.1073/pnas.95.13.7699

Spielewoy, C., Roubert, C., Hamon, M., Nosten-Bertrand, M., Betancur, C. & Giros, B. (2000). Behavioural disturbances associated with hyperdopaminergia in dopamine-transporter knockout mice. *Behavioural Pharmacology* , *11*, 279-290. Doi: 10.1097/00008877-200006000-00011

Starr-Phillips, E.J. & Beery, A.K. (2014). Natural variation in maternal care shapes adult social behavior in rats. *Developmental Psychobiology 56,* 1017-1026. Doi: 10.1016/j.yhbeh.2015.05.022

Strekalova, T., Svirin, E., Waider, J., Gorlova, A., Cespuglio, R., Kalueff, A., Pomytkin, I., Schmitt-Boeher, A.G., Lesch, K.P. & Anthony, D.C. (2020). Altered behavior, dopamine and norepinephrine regualtion in stressed mice heterozygous in TPH2 gene. *Progress in Neuropsychopharmacology & Biological Psychiatry* (in press). Doi: 10.1016/j.pnpbp.2020.110155

Torta, R. & Caldera, P. (2008). *Che cosa è l'ansia: basi biologiche e correlazioni cliniche.* Pisa, Italy: Pacini Editore, pp. 56. https://www.pacinimedicina.it/wp-content/uploads/25594_interno-3B-1.pdf

Triana-Del Rio, R., Montero-Domínguez, F., Cibrian-Llanderal, T., Tecamachaltzi-Silvaran, M.B., Garcia, L.I., Manzo, J., Hernandez, M.E. & Coria-Avila, G.A. (2014). Same-sex cohabitation under the effects of quinpirole induces a conditioned socio-sexual partner preference in males, but not in female rats. *Pharmacology Biochemistry and Behaviour* , *99,* 604-616.

Wong, P., Sze, Y., Chang, C.C., Lee, J. & Zhang, X. (2015). Pregnenolone sulfate normalizes schizophrenia-like behaviors in dopamine transporter knock-out mice through the AKT/GSK3β pathway. *Transational Phsychiatry, 5,* e528. Doi: 10.1038/tp.2015.21

Zerning, G., Kummer, K.K. & Prast, J.M. (2013). Dyadic social interaction as an alternative reward to cocaine. *Frontiers in Psychiatry, 4,* 100. Doi: 10.3389/fpsyt.2013.00100