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From Parents to Offspring: Influence of Approaching/Avoidance parental phenotypes on Progeny

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MAUDE: […] What flower would you like to be?
HAROLD: I don't know. One of these, maybe.
MAUDE: Why do you say that?
HAROLD: Because they are all alike...
MAUDE: Oooh, but they are not. Look. See – some are smaller, some are fatter, some grow to the left, some to the right, some even have lost some petals – all kinds of observable differences. You see, Harold, I feel that much of the world's sorrow comes from people who are this, and allow themselves to be treated as that…

Harold and Maude (1971)
directed by Hal Ashby

Abstract

Individuals are hardwired to approach pleasure and to avoid negative stimuli. Nonetheless, Approach/Avoidance (A/A) conflict arises when a situation elicits these two opposite drives, simultaneously.

I selected three sub-populations of male and female mice based on their withdrawing, balanced or advancing response to an A/A conflict task (*i.e.,* avoiding (AV), balancing (BA) and approaching (AP) mice). The neuronal substrates sustaining the selected phenotypes were investigated by immunofluorescence methods, focusing on the oxytocinergic modulation (OXT) of dopamine (DA) neurons via oxytocin receptors (OTR), in the ventral tegmental area (VTA).

The behavioral consequences of parental phenotypes on the progenies were evaluated.

I found that AP male mice were characterized by a greater number of VTA-DA neurons, enriched in OTR, compared to controls and the AP paternal phenotype was able to bias descendants' behaviors.

Indeed, the offspring of AP fathers were more approaching and faster at the A/A conflict task, more prone towards novel stimuli compared to the offspring of AV fathers, and less anxious compared to controls.

Conversely, AV females were characterized by lower VTA-DA cell density compared to controls, but maternal phenotype did not impact offspring's response to A/A conflict. Maternal phenotype effects on progenies were sex-specific, affecting response to novelty only in female offspring and anxiety levels only in males.

Finally, no phenotype effect on spontaneous parental care was observed. On the contrary, AV paternal phenotype influenced pup-retrieval behaviors of fathers and, indirectly, of their female mates, when separated from their pups.

Given abnormal A/A motivation has been recognized in several psychological and psychiatric diseases, investigations on the mechanisms involved in A/A phenotype transmission and the development of transgenerational animal preclinical models are coveted.

Contents

List of abbreviations

A/A: Approach/Avoidance
AP: approaching
AV: avoiding
BA: balancing
CTR: controls
DA: dopamine
EPM: elevated plus maze
OF: open field with novel object
OTR: oxytocin receptors
OXT: oxytocin
PCO: undisturbed parental care observation
PVN: paraventricular hypothalamic nucleus
pnd: post-natal day
ROI: region of interest
THY: tyrosine hydroxylase
VTA: ventral tegmental area

1 Introduction

Approach and Avoidance (A/A) are the pillars of motivated behavior (Berridge and Kringelbach, 2008; Cornwell et al., 2014; Elliot, 2006; Elliot and Thrash, 2002, 2010; Higgins, 1997). Individuals tend to approach pleasant stimuli and avoid pain and danger, nonetheless some stimuli can exhibit desirable and undesirable features, simultaneously, thus they require a contention between opposite drives (Berkman et al., 2009; Corr and Krupić, 2017; Ehrlich and Fasbender, 2017; Lewin, 1935; Miller, 1944; Wilborn et al., 2018).

Individual differences in response to A/A motivation occur spontaneously in humans and other animals (Berkman et al., 2009; Laricchiuta et al., 2012 a,b, 2015; Laricchiuta and Petrosini, 2014; Norbury et al., 2015; Petrosini et al., 2017). Some individuals are more willing to take risk for achieving gains while others are more cautious and sensitive to the undesirable aspects of A/A conflict.

Such an interindividual variability can represent a boon for the population (Wilson et al., 1994), though approaching/avoidance tendencies can go beyond the functional behavior and become maladaptive, as in many psychological and psychiatric disorders (such as substance use disorders, depression, bipolar disorder and schizophrenia) (Alcaro and Panksepp, 2011; Baskin-Sommers and Foti, 2015; Der-Avakianet et al., 2016; Whitton, Treadway and Pizzagalli, 2015; Wilborn et al., 2018).

The dopaminergic system is strictly involved in A/A responses (Baik, 2013; Ikemoto and Panksepp, 1999; Wise and Rompre, 1989) and anomalies in dopaminergic signaling have been recognized in disorders linked to aberrant motivation (Baskin-Sommers and Foti, 2015; Cardozo Pinto and Lammel, 2017; Comings and Blum, 2000;

Di Chiara et al., 2004; Nestler and Carlezon, 2006; Volkow, Wise and Baler, 2017; Whitton, Treadway and Pizzagalli, 2015).

In the recent years, striking evidence has revealed how parental life-events and characteristics may impact descendants across generations (Bohacek and Mansuy 2013, 2015; Franklin et al., 2010; He et al., 2016; Mitchell et al., 2016; Yehuda and Lehrner, 2018; Yeshurun and Hannan, 2018).

Evidence of inter- and trans-generational transmission comes from studies in humans (Mendoza Diaz et al., 2018; Pavlickova et al., 2014), non-human primates (Kinnally et al., 2018), rodents (Sauce et al., 2017; Weber-Stadlbauer et al., 2017), birds (Leroux et al., 2017) and fish (Cavalieri and Spinelli, 2017; Newman et al. 2016).

Most studies focused on transmission of trauma and stress (Franklin et al., 2010; Pang et al., 2017; Yehuda and Lehrner, 2018), drug/toxic exposure (reviewed in Bohacek and Mansuy, 2013), or on the intergenerational impact of apparent psychological or psychiatric disorders as anxiety (Gibler et al., 2018), depression (Mikkonen et al., 2016; Ronovsky et al., 2017; Sharp et al., 2014), alcohol use disorder (Long et al., 2018), gambling problem (Dowling et al., 2016).

Most of these studies emphasize the importance of parents' behavior in the transmission of vulnerability across generations, in some cases sex specific, and the increased vulnerability to disease development in children who have both parents exposed to negative psychological/environmental insults.

To date, little attention has been paid to the intergenerational consequence of spontaneous individual differences in approaching or avoidance traits.

The present work investigated the neural substrates and behavioral consequences for the progenies of maternal and paternal approaching/avoidance phenotypes, in mice, focusing on the role of dopaminergic neurons in the ventral tegmental area.

Understanding pathways of transmission of approaching/avoidance traits is a compelling challenge both to understand physiological influences from parents to offspring as well as to develop promotion/prevention/intervention strategies on vulnerable phenotypes across generations (Anttila et al., Brainstorm Consortium, 2018; Denham, 2018; Gapp et al., 2016; Jiménez et al., 2018; Maciejewski et al., 2018; Zorumski, 1988).

1.1 The Approach/Avoidance conflict

Throughout life, individuals respond to environmental stimuli crucial for wellbeing and survival (*e.g.*, food, mates, danger, competitors). Reactions to salient stimuli are biologically hardwired, species-specific and evolved to promote survival and reproduction (Berridge and Kringelbach, 2008; Higgins, 1997).

Approach and Avoidance (A/A) have been defined as basic motivational systems, virtually present in the whole animal kingdom (Alcaro and Panksepp, 2011). Approach motivation can be described as the energization of behavior by, or the direction of behavior toward, positive stimuli, whereas avoidance motivation as the energization of behavior by, or the direction of behavior away from, negative stimuli (Elliot, 2008; Lewin, 1935).

The idea that individuals seek pleasure and escape from pain, has roots that lie far behind in history (*e.g.*, the ethical hedonism by Democritus of Abdera (460–370 BC)) but still stimulates researches across fields from moral psychology (Janoff-Bulman, Sheikh and

Hepp, 2009) and neuroscience (Berridge and Kringelbach, 2008; Higgins, 1997), to behavioral economics (Simon, 1955) and animal welfare science (Fraser and Duncan, 1998).

Approaching positive stimuli (such as food) and avoiding pain and danger (such as being preyed) is so fundamental that the dedicated brain structures have very remote phylogenetic development and are maintained through many species, including invertebrate animals (Gray et al., 2005; Huber et al., 2011; Tinette et al., 2007; reviewed in Alcaro and Panksepp, 2011) up to mammals and other vertebrates (Fidler et al., 2007; Molina-Borja and Gómez-Soutullo, 1989; Panksepp, 1981).

Nonetheless, approach and avoidance are not a so simple matter. One motive can be direct toward two or more different positive stimuli simultaneously (*i.e.,* approach/approach conflict) or away from two or more negative stimuli (*i.e.,* avoidance/avoidance conflict). Moreover, the same situation can drive two different motives, unsolvable together (*i.e.,* approach/avoidance conflict) (Arkoff, 1957; Claes et al., 2016; Ehrlich and Fasbender, 2017; Lewin, 1935).

When a stimulus or a situation exhibits both desirable and aversive features simultaneously, A/A conflict arises, opposite drives collide, and the chance of eliciting of individual differences is revealed.

Individual differences in response to A/A conflict occur within species with certain animals more willing to take risk to achieve their goals and others more risk-averse in their behaviors (Wilson et al., 1994). The variability could be evolutionarily befitting, but aberrations in the elaboration of aversive or rewarding stimuli and defective responses to conflict can interfere with goal-directed behavior. This has been associated with clinical observations (*e.g.,* withdrawal from potential

rewards to avoid even potential negative outcomes, common in clinical anxiety and depressive disorders (Aupperle and Paulus, 2010; Kash et al., 2002; Muris et al., 2001) or approach of rewarding stimuli despite the negative consequences in drug abuse and gambling disorders (reviewed in Brevers et al., 2013; Cox et al., 2017)).

Sensible progress has been recently made to unravel the neuronal circuits and neurotransmitters involved in A/A behaviors.

The endocannabinoid system is implicated in individual differences in A/A behaviors through amygdaloid-hypothalamic-striatal and striatal-cerebellar networks, (Laricchiuta et al., 2012 a,b, 2014, 2016; Laricchiuta and Petrosini, 2014). Further, cerebellar levels of brain-derived neurotrophic factor (BDNF) have been linked to approaching exploratory behaviors (Laricchiuta et al., 2018).

Moreover, the mesolimbic and mesocortical dopaminergic systems, of which the ventral tegmental area (VTA) is the starting point, are closely involved in the responses to positive and negative stimuli and their conflict (Bromberg-Martin et al., 2010; Cardozo Pinto and Lammel, 2017; Cohen et al., 2012; Ikemoto, 2010; Lammel et al., 2012; Moriya et al., 2018; Nieh et al., 2015; Qi et al., 2016; Verharen et al., 2018; You, Vandegrift and Brodie, 2018).

1.2 Animal models to investigate the Approach/Avoidance conflict

In humans, A/A tendencies are inferred by several tasks, including self-report questionnaires (Aupperle et al., 2011; Kirlic et al., 2017). For instance, among the Big Five (Dyce, 1997), Extraversion trait has been linked with approach-oriented goals and Neuroticism has been linked with avoidance-oriented goals (Smits and Boeck, 2006).

Prolific efforts to identify the neural substrates of such personality dimensions have been made by using neuroimaging approaches in humans (Gonen et al., 2016; Norbury et al., 2015; Petrosini et al., 2015, 2017).

Yet, starting from the seminal work of Olds and Miller (1954) on rat brain, decisive discoveries on the circuits involved in the response to positive and aversive stimuli, have been achieved by using animal models.

Several behavioral protocols have been developed to investigate reactions to A/A conflict in animals (Kirlic et al., 2017). Most paradigms combine reward delivery and punishment, for instance by means of food and electrical shock administration, simultaneously (*e.g.,* Neil Miller conflict situation (1937, 1944), or by learned/punished responses (*i.e.,* conditioned operant conflict tests) (Vogel et al., 1971).

Many attempts have been made to characterize A/A individual differences also using selective breeding in rats (*e.g.,* Roman Low (RLA) and High (RHA) Avoidance rats selected for their extremely slow or rapid acquisition at the active-passive avoidance task; reviewed in Brush, 1991; Giorgi, Piras and Corda, 2007; Steimer and Driscoll, 2003; Steimer, la Fleur and Schulz, 1997).

Mouse models also, had demonstrated their suitability for causative study on the role of DA in motivation psychopathologies (Armario and Nadal, 2013; Bergamini et al., 2016; Young et al., 2011).

Among rodents, the use of inbred strain has grown over time (Casellas, 2011; Festing, 1979) and individual differences in response to A/A conflict have been demonstrated to occur even

within inbred strains (Laricchiuta et al., 2012 a,b, 2016, 2018; Pittaras et al., 2013, 2016).

In fact, although inbreeding guarantees a negligible genetic variability, inbred mice are far from exhibiting absolute isogeneity and behavioral uniformity (Gruneberg, 1954).

By using an A/A conflict task (the A/A Y-Maze, based on the conflict between appetitive drive toward palatable food and withdrawal drive away from an aversive environment) Laricchiuta and colleagues (Laricchiuta et al., 2012 a,b, 2016, 2018) studied spontaneous individual differences in A/A behaviors in inbred mice.

By means of this “ethological and unpunished” task the authors demonstrated that responses to A/A conflict are normally distributed within the population and detected three sub-populations of inbred C57BL/6JOlaHsd male mice. Balancing (BA) mice react to the conflict with balanced responses and represent the mean of the sample distribution; Approaching (AP) mice respond with advancing responses toward the positive stimulus despite the negative characteristics of environment; Avoiding mice (AV) react withdrawing the negative component of the conflict (Fig. 1).

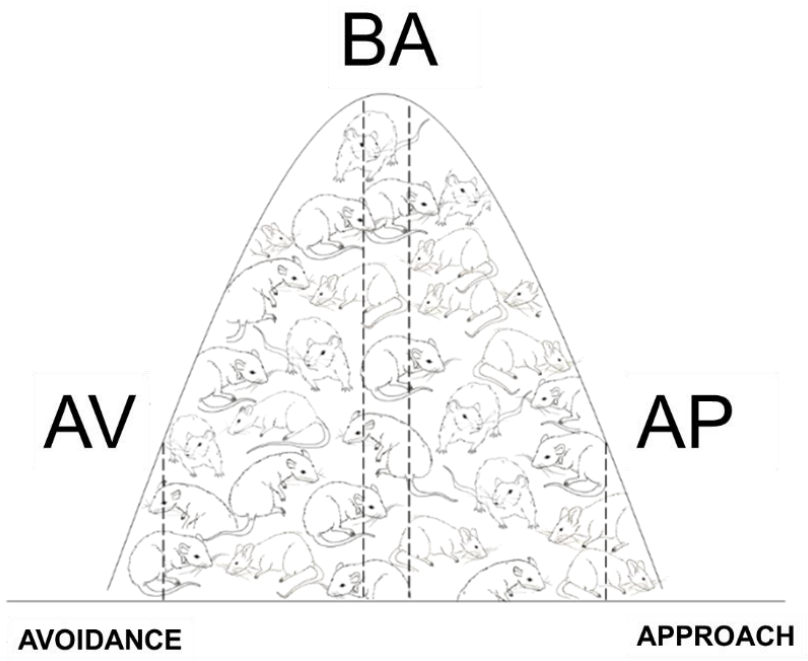

Fig. 1 Normal distribution of approaching/avoiding phenotypes in inbred mice. The image represents the distribution of avoiding (AV), balancing (BA) and approaching (AP) mice within the population. BA mice represent the mean of the distribution while AV and AP mice are positioned at -2 and +2 standard deviations from the mean, respectively.

1.3 Oxytocin-dopamine interplay in motivated behaviors

The dopaminergic (DA) system has a special place in motivation (Goto and Grace, 2005; Baik, 2013; Di Chiara et al., 2004; Nestler and Carlezon, 2006; Volkow, Wise and Baler, 2017¸ Wise and Rompre, 1989). In vertebrates, dopamine-producing nerve cells are located in discrete brain areas, mainly in the VTA and substantia nigra pars compacta (SNc) but affect extended brain circuits via widespread efferents. The VTA neurons receive input from the hypothalamus, raphe, ventral pallidum, striatal regions, globus pallidus, laterodorsal tegmentum and lateral habenula (Lammel et

al., 2012) and project to the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) (*i.e.,* the mesocorticolimbic pathway), mediating motivated behaviors (Cohen et al., 2012; Lammel et al. 2012; Moriya et al., 2018), as well to other areas such the amygdala and hippocampus, facilitating emotional memory (Alcaro and Panksepp, 2011) (Fig. 2). Furthermore, SNc DA neurons project to the dorsal striatum (*i.e.,* nigrostriatal pathway), mediating the control of motor function.

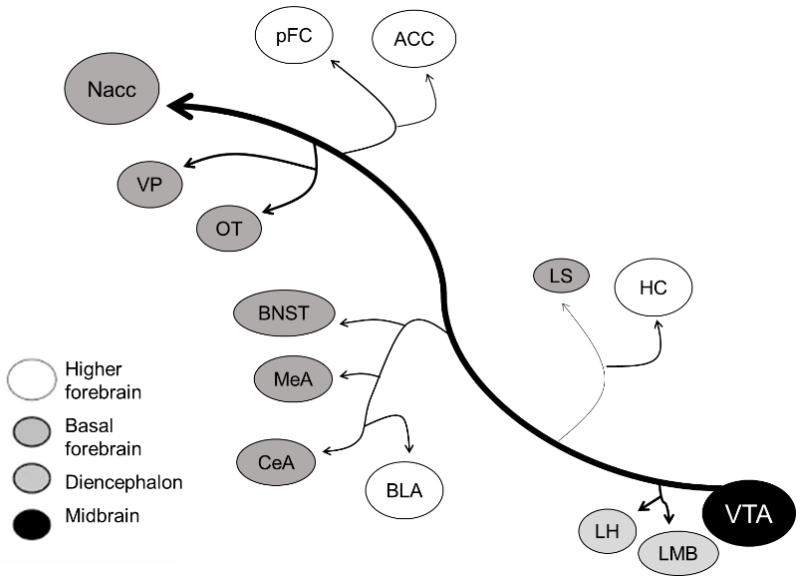

Fig. 2 The VTA dopaminergic system. Figure shows the main brain areas involved in dopaminergic transmission and motivated behaviors. (From bottom to top) ventral tegmental area (VTA); lateral mammillary body (LMB); lateral hypothalamus (LH); basolateral amygdala (BLA); hippocampal complex (HC); lateral septum (LS); central nucleus of amygdala (CeA); medial nucleus of the amygdala (MeA); bed nucleus of stria terminalis (BNST); olfactory tubercle (OT); ventral pallidum (VP); nucleus accumbens (Nacc); anterior cingulated cortex (ACC); prefrontal cortex (pFC). (Image adapted from Alcaro, Huber and Panksepp, 2007)

In turn, the DA transmission is influenced by neurotransmitters (*e.g*., endocannabinoid and endogenous opioid system), hormones and microglia (reviewed in Fuxe et al. 2015; Laricchiuta et al., 2014; Nash, 2017; Wenzel and Cheer, 2018; Yoest et al., 2018).

Recently, the oxytocin (OXT), a neuropeptide classically linked with pregnancy, lactation, maternal care and pair bonding (Lee et al., 2009; Mitre et al., 2016; Numan and Young, 2016), has been recognized to play a role in mediating VTA dopaminergic transmission via oxytocin receptors (OTR) (Love, 2014; Peris et al., 2017; Xiao et al., 2017).

OXT is mainly synthesized within the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus and, from PVN, OXT-neurons send projections to extrahypothalamic regions including the VTA and the nucleus accumbens. In fact, the activation of OXT stimulates the mesocorticolimbic pathway and enhanced DA levels in the nucleus accumbens have been detected after OXT infusion in the rats' VTA (Melis et al., 2007; Shahrokh et al., 2010).

Electrophysiological studies in mice confirmed that both the application of oxytocin and optogenetic stimulation of OXT terminals lead to increased DA neuron activity in the VTA (Xiao et al., 2017).

Peris and colleagues by using OTR-Cre mice Tg(Oxtr-cre)ON66Gsat/Mmucd expressing Cre-recombinase under the control of the promoter for the OTR gene, identified OTR-expressing VTA neurons projecting to nucleus accumbens, prefrontal cortex and extended amygdala (Peris et al., 2017).

Together these findings provide evidence for a role of the oxytocinergic regulation in midbrain DA systems. Further studies will be necessary to understand the mechanistic interplay and function

of this modulation inspired by the intriguing hypothesis that OXT and DA can cooperate to refine the response to salient environmental stimuli (Xiao et al., 2017).

2 Rationale

Individuals are not all equal in facing A/A conflict (Gonen et al., 2016; Laricchiuta et al., 2012 a,b, 2016, 2018; Laricchiuta and Petrosini, 2014; Norbury et al., 2015; Pittaras et al., 2013, 2016).

The sensitiveness to positive and negative salient stimuli varies within the population and the functionality of the DA system has proved to have a special role in sustaining individual differences in motivated behaviors both in physiological and in pathological conditions (Baik, 2013; Baskin-Sommers and Foti, 2015; Cardozo Pinto and Lammel, 2017; Comings and Blum, 2000; Di Chiara et al., 2004; Ikemoto and Panksepp, 1999; Nestler and Carlezon, 2006; Volkow, Wise and Baler, 2017; Whitton, Treadway and Pizzagalli, 2015; Wise and Rompre, 1989).

Recently, the intergenerational impact of apparent psychological or psychiatric disorders linked to aberrant DA signaling as depression (Mikkonen et al., 2016; Ronovsky et al., 2017; Sharp et al., 2014), alcohol use disorder (Long et al., 2018) and gambling problem (Dowling et al., 2016) has been strictly demonstrated. These studies evidenced that the children of parents with a psychopathology are more likely to develop the same disorder themselves or otherwise are vulnerable to the development of problems related to it (Maciejewski et al., 2018).

Several lines of evidence suggest that neuronal and psychological anomalies, featuring such disorders, can be present before the patent expression of the disease (Alcaro and Panksepp, 2011; Bardo et al., 1996; Khantzian, 2003; Panksepp, 2010). For instance, some individuals characterized by extreme exploratory/foraging/seeking

dispositions are more susceptible to the development of substance use disorders (Alcaro and Panksepp, 2011; Panksepp, 2010).

Despite the importance of investigating these vulnerability factors in depth for intervening in advance, to date only few studies have focused on the variations of A/A behaviors and of the respective neuronal substrates in the healthy population (Bradberry et al, 1991). Even less attention has been paid to the intergenerational consequence of spontaneous individual differences in A/A traits.

The present Thesis work aimed to investigate in a mouse model the transmissibility and possible VTA-DA association of maternal and paternal A/A behavioral phenotypes in mice, by using an individual difference-based approach and by segregating individuals based on the response to an "ethological and unpunished" conflict task (Campos, 2013), namely selecting AV, BA and AP individuals (Laricchiuta et al., 2012 a,b, 2016, 2018; Laricchiuta and Petrosini, 2014).

Given that males and females neurobiologically and behaviorally differ (Palanza and Parmigiani, 2017; Wald and Wu, 2010), as demonstrated also in mice with attention to avoidance, approach-related exploratory behaviors, and attribution of incentive salience to reward cues (Carreira, 2017; Dickson et al., 2015; Yokota et al., 2017), I firstly investigated sex-difference in AV, BA and AP male and female mice.

Secondly, I formed breeding couples with AP/BA/AV mothers mated and reared with BA males (Maternal effect) and AP/BA/AV fathers mated and reared with BA females (Paternal effect) and evaluated phenotype effects on biparental care and pup-retrieval behaviors. As OXT has been linked both to maternal care and retrieval and to the

regulation of midbrain DA systems, the number of OXT neurons in the PVN and the OXT modulation of DA neurons via OTR, in the VTA of AV, BA and AP male and female mice were evaluated by immunofluorescence methods.

Finally, I assessed intergenerational outcome of the parental phenotype on the offspring's response to A/A conflict, exploratory behaviors, response to novelty and anxiety, all behaviors previously linked to A/A tendencies.

3 Materials and methods

3.1 Experimental design

The study evaluated two generations of mice: parents (F0) and offspring (F1) (Fig.3).

To arrange the F0, male and freely cycling female C57BL/6JOlaHsd mice were tested at post-natal day (pnd) 35-40 at the A/A Y-Maze to select AV, BA and AP mice (Laricchiuta et al., 2012 a,b, 2016, 2018). Responses to novelty, exploratory and anxiety-like behaviors were investigated in adult AV, BA and AP mice (pnd 55-65) by the Open Field with novel object (OF) and Elevated Plus Maze (EPM).

After the behavioral testing, mice were coupled based on their phenotypes and used for breeding the F1 generation (see paragraph 3.2 for details on phenotypic assortments of couples and sample size).

At offspring's pnd 3, 30-min undisturbed parental care (PCO) observation was performed to evaluate time spent contacting the litter by dams and sires.

At weaning (pnd 21) the offspring was separated from parents and arranged in same-sex cages. Dams and sires remained caged together for a second and a third mating.

The response of mothers and fathers to pups' separation was evaluated by the pup-retrieval test (RTV) at pnd 6 of the second litter. Finally, at pnd 6 of the third litter, brains of mothers and fathers were collected and processed for immunofluorescence staining for OXT in the PVN and OTR/DA colocalization in the VTA.

The entire first litter born to each different couple was used for the behavioral characterization of the F1 by the A/A Y-Maze task at pnd

35-45 and the OF and EPM in adulthood. After testing animals were sacrificed and brain collected and stored for biochemical analysis.

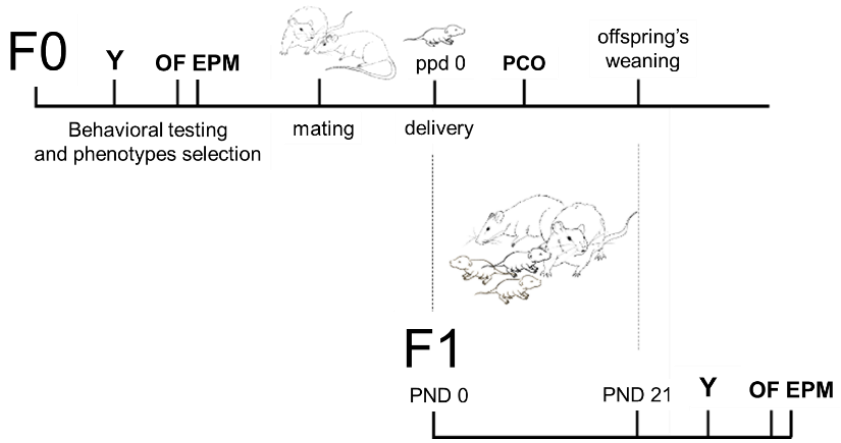

Fig. 3 Timeline of the experimental design. Schematic representation of the main experimental steps. Generation of parents (F0); Offpsring's generation (F1); Approach/Avoidance Y-Maze (Y); Open Field with novel object (OF); Elevated Plus Maze (EPM); *post-partum* day (ppd)*;* post-natal day (pnd); undisturbed parental care observation (PCO).

3.2 Animals and rearing conditions

219 C57BL/6JOlaHsd 21-day old mice (100 females) were purchased by ENVIGO (Netherland). Animals were arranged 4/5 per cage (standard cages with bedding sawdust and house supplementation: nesting material and a "Mouse House™" as refuge (Sherwin, 2007; Wirz et al., 2015) and reared according to the European Directive 2010/63/EU and Italian D.L. 26/2014 and to the Federation of European Laboratory Animal Science Associations' guidelines and recommendation for rodents.

Mice were kept under a 12-h light/dark cycle (lights on at 07:00 am), controlled temperature (22-23 °C), and constant humidity (60 ± 5%),

with water and food (Mucedola 4RF21, Italy) *ad libitum*. Cages were cleaned twice a week.
Mice were identified using the non-invasive temporary identification method of tail-coloring (Dahlborn et al., 2013). Given the well-known stress effect of bedding change (Gray and Hurst, 1995), no cage-cleaning during behavioral tests was made.

Starting from the initial sample of 219 animals, 46 subjects (50% females) were selected according to their AV, BA and AP phenotypes. Namely, 4 AV, 13 BA and 6 AP males; 3 AV, 16 BA and 4 AP females. Selected animals were tested at the OF and EPM in adulthood and then used as F0 for breeding the F1.
Precisely, 23 couples have been set up to obtain different phenotypic assortment:

- BA♀♂: both BA mother and father; N=6
  (used as controls, properly the contrast group CTR)
- AV♂BA♀: AV father paired with BA mother; N=4
- AP♂BA♀: AP father paired with BA mother; N=6
- AV♀BA♂: AV mother paired with BA father; N=3
- AP♀BA♂**:** AP mother paired with BA father; N=4

Each couple (one male and one female) was placed in a standard cage and reared as previously described, and remained together for mating, during gestation and delivery and until offspring's weaning (pnd 21).

To note, those monogamous and biparental rearing conditions were similar to the breeding condition provided by the company where the animals were purchased (ENVIGO).

The entire first litter born to each different couple was used for the behavioral characterization of the F1.

Litters with less than 2 animals *per* sex were excluded from the experiment. Finally, I included 128 F1 animals (68 females) born to 3-6 different couples for each phenotypic assortment (Fig. 4).

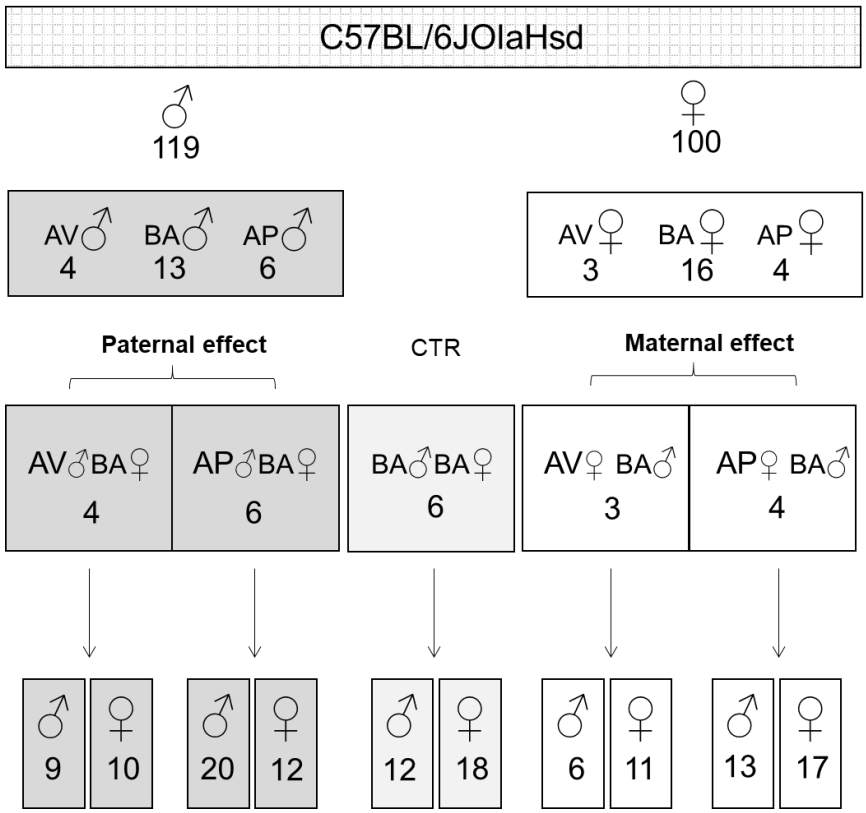

Fig 4. Experimental groups. Schematic representation of the groups used in the experiment. C57BL/6JOlaHsd mice were used. Males (♂); Females (♀); avoiding (AV), balancing (BA); approaching (AP) mice; control group (CTR) (properly, the F1 contrast group); AV father paired with BA mother (AV♂BA♀); AP father paired with BA mother (AP♂BA♀); both BA mother and father (BA♂BA♀); AV mother paired with BA father (AV♀BA♂); AP mother paired with BA father (AP♀ BA♂). Numbers indicate relative sample size.

3.3 Behavioral testing

All behavioral tests took place during the light phase (10:30 am - 04:00 pm) in a slightly lit and silent behavioral room. Each apparatus used was cleaned thoroughly with 30% ethanol and dried before and after each trial to remove scent cues.

3.3.1 Approach/Avoidance Y-Maze

The A/A Y-Maze task allows to detect individual differences in response to an A/A conflict. Namely, animals can advance or withdraw the conflicting salient stimulus or react with balanced response (Laricchiuta et al., 2012 a,b, 2014, 2018; Laricchiuta and Petrosini, 2014).

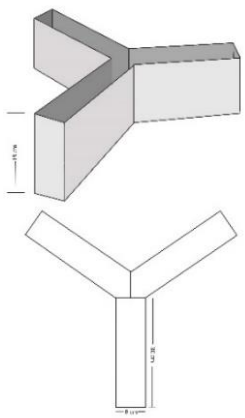

Fig 5. Approach/Avoidance Y-Maze. Figure shows the Y-shaped apparatus used for the A/A Y-Maze task. The maze is made of three arms (each 8x30x15 cm) and is provided with black and white removable walls and floors that allow to set up one black arm and one white arm, alternately.

Apparatus: The A/A Y-Maze consists of a Y-shaped apparatus made of three plexiglass arms (each 8x30x15 cm). The starting arm is gray-colored and is divided from other two arms by a T-guillotine gray door (Fig. 5). One of the two remaining arms (arranged at an angle of 90° to each other) has black and opaque walls and floor, the other one has white walls and floor and is lighted by a 16-W neon lamp (the aversive arm). At the end of each arm there is a food tray (3 cm in diameter, 1 cm deep). Apparatus' walls, floors and the lamp are exchangeable to alternate the spatial position of the white and black arms among trials.

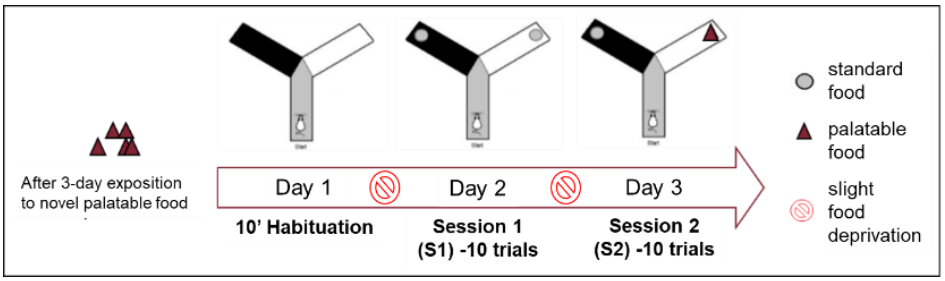

Fig 6. Approach/Avoidance Y-Maze procedure. Figure shows the A/A Y-Maze procedure. One week after 3-days exposition to the novel palatable food, 3-day of testing interspersed with slight food deprivation.

Procedure: A week before the behavioral testing the animals were exposed to the palatable food (Fonzies, KP Snack Foods, Munchen, Germany) in their home cages for three consecutive days, to get them used with the novel food later used for the test. The A/A Y-Maze procedure lasted three days and consisted of a habituation phase (day 1) and two 10-trial-sessions: Session 1 (S1, day 2) and Session 2 (S2, day 3).

The habituation phase comprised 10-min-free exploration of the maze. All arms of the apparatus were opened, and no food was present in the food trays. After the habituation and 12 hours before the beginning of the S1, the animals were slightly food deprived by limiting food access.

The S1 consisted of 10 trials with 1 min-inter-trial interval. The animal was placed in the starting gray arm and chose to enter one of the two arms, both containing the same standard food. At the end of each trial food was always replaced.

During the S2, starting 24 h after S1, the A/A conflict was generated rewarding the white arm with the palatable food (Fonzies), while the black arm was still rewarded with the standard food. Again, the animal chose to enter one of the two arms, for 10 trials (Fig. 6).

Notably, the A/A Y-Maze allowed to exhibit two different behaviors: reaching the palatable reward despite the aversive environment or avoid the conflict reaching standard food placed in a reassuring environment.

Behavioral parameters: Number of white arm choices in S1 and S2 (an arm entry was defined as four paws entering one of the arms); latency to enter in the white arm (seconds); A/A conflict index (Δ of white arm choices): the number of S2 white arm choices *minus* the S1 white arm choices.

As in females it has been shown that some approaching behaviors (*e.g.* drug- or palatable food-related approach) may be influenced by the estrous cycle (Egan et al., 2018; Kerstetter et al., 2013), vaginal smears were collected to evaluate influences of estrous on behavioral performances (see Supplementary materials).

3.3.2 Open Field with novel object

The Open Field (OF) is a widely used test for assessing exploratory behavior, general activity and anxiety levels in rodents (Campos et al., 2013; Gould, Dao and Kovacsic, 2009; Prut and Belzung, 2003). The OF procedure used allowed also to assess reaction to novelty, placing a novel object in the center of the arena during an additional second session (S2) (Kazvauckas, 2005; Laricchiuta et al., 2012a).

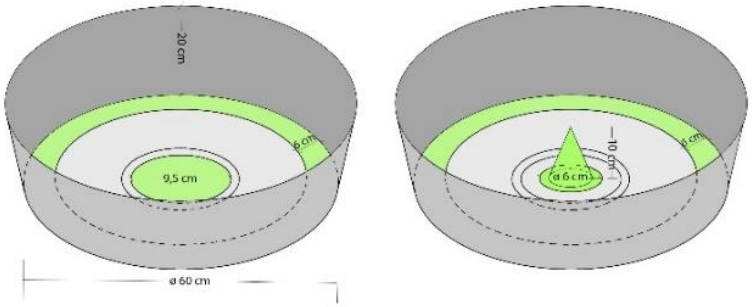

Fig 7. Open Field with novel object. Figure shows the Open Field apparatus. A circular arena (60 cm) is surrounded by 20-cm high walls. A 6-cm peripherical anulus and an arena center (10.5 cm) are virtually defined on the arena surface (highlighted in green). In the second session a novel object (the cone highlighted in green) is place in the arena center.

Apparatus: The Open Field apparatus consists of a circular arena (diameter 60 cm) delimited by a pale gray 20 cm-high wall. It is possible to define upon the apparatus different zones of interest: a 6cm peripheral anulus and a 10.5-cm arena center.

A novel object (a gray plastic cone: 10x6 cm; base diameter =9.5 cm) was used in the S2 and placed at the center of the arena (Fig. 7).

Procedure: In S1, a single animal was allowed to explore the empty open field. In S2, the novel object was positioned in the arena center and the animal was placed again in the open field. Sessions lasted 10 minutes with a 5-min inter-session interval (Laricchiuta et al., 2012a). The whole testing was recorded by a video camera and processed by a behavioral analysis software (EthoVision, Noldus, Wageningen, The Netherlands). The contact with the novel object in S2 was also recorded with a second camera and manually scored by using Noldus EthoVision XT 7.1.

Behavioral parameters: S1 percentage of total distance traveled in the peripheral annulus and arena center, total distance travelled in

the arena (cm), mean velocity (cm/sec); S2 duration (sec), frequency (number) and latency (sec) of contact with the novel object. The contact with object was considered when the mouse snout touched the object, or when it sniffed the object for at least 1 sec.

3.3.3 Elevated Plus Maze

The EPM is used in rodents to assess anxiety levels (Hogg, 1996; Lister, 1987).

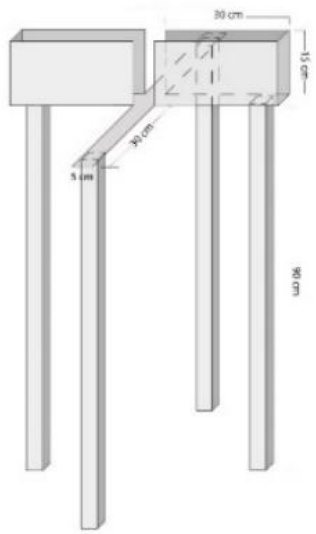

Fig. 8 Elevated Plus Maze. Figure shows the EPM apparatus. The apparatus consists of four arms (30x5 cm) starting from a central 5x5 squared region, two arms are opened, and two arms are enclosed by 15 cm high walls. The maze is 90 cm elevated from the floor.

Apparatus: EPM apparatus is a cross-shaped wooden structure with four 30x5 cm arms extending from a central (5x5 cm) region. The maze is 90 cm raised above the ground. North and south arms were open, east and west arms were enclosed by 15 cm high walls (Fig. 8).

Procedure: Single 5-minutes videotaped exposition to the elevated maze (Laricchiuta et al., 2012a).

Behavioral parameters: Time spent in the open and closed arms (sec) and arm visit frequency (number).

Duration and frequency were manually scored by using Noldus EthoVision XT 7.1.

3.3.4 Undisturbed Parental care Observation

The protocol for the undisturbed parental care observation was adapted from Orefice and Heinrichs (2007).

Procedure: On pnd 3/4, between the 02:00-03:00 pm, biparental care behaviors of mice were videotaped in their home cage for 30 consecutive minutes. The father was colored on the back and on the tail for identification during the scoring phase. The cage with animals was placed on a cart inside the animal facility 3 hours before the recording to allow acclimatization.

Behavioral parameters: Time spent in pup-directed behaviors (sec) were scored for mothers and fathers, separately by using the Noldus EthoVision XT 7.1 software.

3.3.5 Retrieval test

Retrieval is a specific behavior consists in returning separated pups to the nest by carrying them in the mouth, burrowing under sawdust, to arrange the nest.

At pnd 6 a retrieval test was done for mother and father separately (Liu et al., 2013).

Procedure: The house supplementation was removed from the home cage to leave inside only parents with pups and the bedding nest. After 3 hours room- acclimatization parents were separated from pups for 10 min, placed in a cage with clean sawdust, located near the home cage. Three pups were selected (mix-sex and with milk spots, when possible) and gently placed at the opposite corner of the nest, immediately before the 10-min separation ended. Then the mother was placed in the home cage on nest-side and the latency to reach separated pups at the opposite corner and to retrieve all separated pups to the nest were measured (Fig. 9). The protocol

lasted 10 min, videotaped, and maternal pup-directed behaviors, digging, self-grooming and rearing were manually scored by Noldus EthoVision XT 7.1.

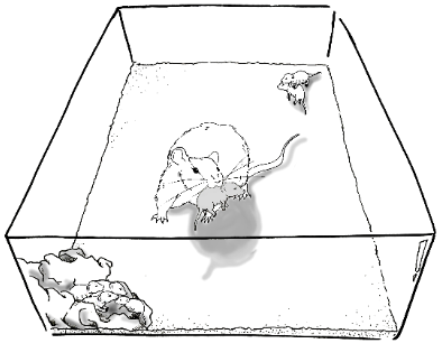

Fig. 9. Retrieval test. Figure shows pup-retrieval behavior at the RTV test. Three pups were separated from the nest and located at the opposite corner of the cage.

At the end of the test the mother was placed again in the clean cage and the entire procedure was repeated with the father.

3.4 Biochemical analysis

3.4.1 Brain collection

Animals were sacrificed by decapitation. Brains were rapidly collected and post-fixed overnight by 4% paraformaldehyde fixative in 0.1M phosphate buffer (pH 7.4). Afterward, brains were equilibrated in a 30% sucrose phosphate buffer, frozen with dry-ice and stored at -80°C until staining. Brains were cut in 40 µm thick coronal sections with a freezing microtome. Regions of interest (ROI) were identified by Franklin and Paxinos brain mouse atlas (Franklin and Paxinos, 1997).

The ROI considered were:

- paraventricular nucleus of the hypothalamus (PVN) from -0.82 to -0.06 in relation to bregma
- ventral tegmental area (VTA): from -2.92 to -3.16 in relation to bregma

3.4.2 Immunofluorescence

PVN coronal sections were stained for OXT and VTA coronal sections were double-stained for OTR and DA (properly for tyrosine hydroxylase) to measure OTR-DA colocalization (details of the antibodies used for immunofluorescence staining are reported in Table 1S).

3.4.2.1 Oxytocin in the PVN

Brain slices were permeabilized with 0.3% Triton X-100 in PBS for 10 min. To saturate the non-specific sites where then incubated in a blocking solution containing 3% normal donkey serum (NDS) in 0.3% Triton X-100 in PBS for an hour. Afterwards, they were incubated in a blocking solution containing a mouse monoclonal anti-oxytocin

antibody (1:1000, clone OT-NP, PS38, a generous gift from Dr. Hal Gainer, NIH, Bethesda MD USA) for 16-18 h at room temperature. To visualize the primary antibody, donkey secondary antibodies conjugated to Cy2 against mouse were used (Jackson Immunoresearch, West Grove, USA; 1:200 in PBS). Nuclei were observed incubating sections with Hoechst (1:500).

3.4.2.2 Dopamine-oxytocin receptors colocalization in the VTA

Brain slices were pretreated with 0.1% Triton X-100 in PBS for 10 min, then blocked in 1% bovine serum albumin (BSA) in 0.1% Triton X-100 in PBS for an hour. Afterwards, they were incubated in a blocking solution containing mouse anti-tyrosine hydroxylase (1:700, MAB318, Millipore) and rabbit anti-oxytocin receptors (1:1000, AVR-013, Alomone lab) antibodies for 16-18 h at room temperature. To visualize the primary antibodies, donkey secondary antibody conjugated to Cy2 against mouse and goat secondary antibody conjugated to Cy5 against rabbit were used (Jackson Immunoresearch, West Grove, USA; 1:200 in PBS). Nuclei were observed incubating sections with Hoechst (1:500).

3.4.3 Confocal microscopy acquisition and cell counting

Digital pictures of brain slices were captured with a confocal microscope (20x magnification for OXT and THY; 40x for OTR).

Cell counting was performed using the open access ImageJ 1.41n (Wayne Rasband, National Institute of Health, USA). Four bilateral slices per subject were counted for OTX and THY, using the ImageJ plug in for Particle Analysis. Results express cell density (cell/mm^2). Three cells/slice were counted for OTR. Results express receptorial density (receptor/mm2).

3.5 Statistics

The R free software environment for statistical computing and graphics was used. (R Core Team; 2017). Graphs were generated by using R basic tools and ggplot2 package[1] (Wickham, 2016).

Prior to statistical analysis a data screening was led using graphical inspections, looking for outliers, points of high influence and missing data that could bring to misinterpret results.

To compare the A/A conflict index sample distributions in F0 males and females and in F1 offspring, Kolmogorov-Smirnov tests were performed (Conover, 1971; Marsaglia et al., 2003). Additionally, the Bhattacharyya coefficient (Bhattacharyya, 1943) was used to calculate the probability of overlap between males and females' distributions.

To evaluate influence of the estrous cycle on behavioral performances at the A/A Y-Maze, an analysis of variance considering the cycle phase as independent variable was run on 83 females (17 missing data from the initial sample).

Parametric t-tests were run on the initial sample (N=219) to compare males and females' latency at the A/A Y-maze. After phenotypes selection (N=46) Kruskal Wallis and Mann-Whitney-Wilcoxon non-parametric analyses were used for comparing selected groups

[1] F0 results in which male and female mice are compared are shown in bordered graphs with grid background; Results on dams and maternal effects are shown in graphs with white background; Results on sires and paternal effects are shown in graphs with gray background; Offspring's sex is highlighted by respective symbols on the top of graphs' legends (male offspring: ♂; female offspring: ♀).

(Sinclair, 1988). Sex and phenotype effects were investigated for each behavioral and neurobiological parameter.

Before analyzing the results on the F1 I checked by Kruskal Wallis tests that the litters born to the couples selected, belonging to the same phenotypic assortment, were not significantly different from each other, thus ensuring that there was not a significant effect of a single breeding pair in pulling the results.

4 F0 Results

Graphs with grid-background indicate data of male and female mice; graphs with white background indicate data of female mice; graphs with gray background indicate data of male mice. Asterisks on graphs indicate significant p-values (* $p \leq .05$; ** $p \leq .01$; *** $p \leq .005$; **** $p \leq .001$).

4.1 Approach/Avoidance Y-Maze

219 mice (100 females) were evaluated at the A/A Y-Maze for phenotype categorization. Mean values and standard deviations of the parameters considered are reported in Table 2S.

WHITE ARM CHOICES

Kolmogorov-Smirnov tests showed no sex effect on the number of white arm choices made by males and females in both S1 ($D = 0.077395$, $p = .900$) and S2 ($D = 0.035882$, $p = 1$).

During the S1, when both black and white arms were reinforced with standard food, mice preferred the black arm, as demonstrated by the higher number of visits in the black arm compared to the visit in the white arm (7 in the black arm *vs.* 3 in the white arm on average).

In the S2, when the white arm was reinforced with palatable food, the number of white arm choices significantly increased in respect to the S1 ($D = 0.14612$, $p = .018$).

WHITE ARM LATENCY

The latency to enter the white arm significantly increased from S1 to S2 in both male ($t = -2.5913$, $df = 194.9$, $p = .010$) and female ($t = -3.6958$, $df = 147.17$, $p = .0003$) mice. No sex effect on latency was found during the S1 ($t = -0.94625$, $df = 195.37$, $p = .345$).

Notably, females were significantly slower than males during the S2 (t = -2.3278, df = 174.7, p = .021) (Fig. 10).

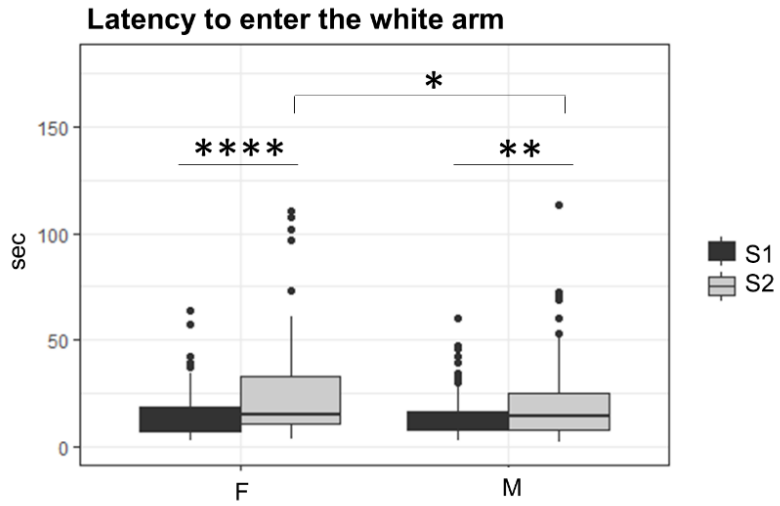

Fig 10. Latency to enter the white arm. Boxplots indicate females (F) and males' (M) latency to enter the white arm (seconds) during the Session 1 (S1) and Session 2 (S2) of the A/A Y-Maze.

A/A CONFLICT INDEX

The male and female distributions of the A/A conflict index 98% overlapped (Bhattacharrya coefficient = .985; Fig. 11) and Kolmogorov-Smirnov test confirmed that males and females were not statistically different (D = 0.047227, p = .999).

Both male and female samples approximated the normal distribution and reproduced values previously described in males by Laricchiuta and colleagues (Laricchiuta et al., 2012 a) (Table 1).

Table 1. Frequencies of AV, BA and AP phenotypes in male and female mice. Table shows the relative frequencies of avoiding (AV), balancing (BA) and approaching (AP) phenotypes in males (♂) and females (♀). Values are expressed in percentage (%). Remaining animals (to reach the 100%) were at ± 1 standard deviation from BA mice and were not included in this experiment.

	AV	BA	AP
♂	9 %	49 %	7 %
♀	5 %	48 %	8 %

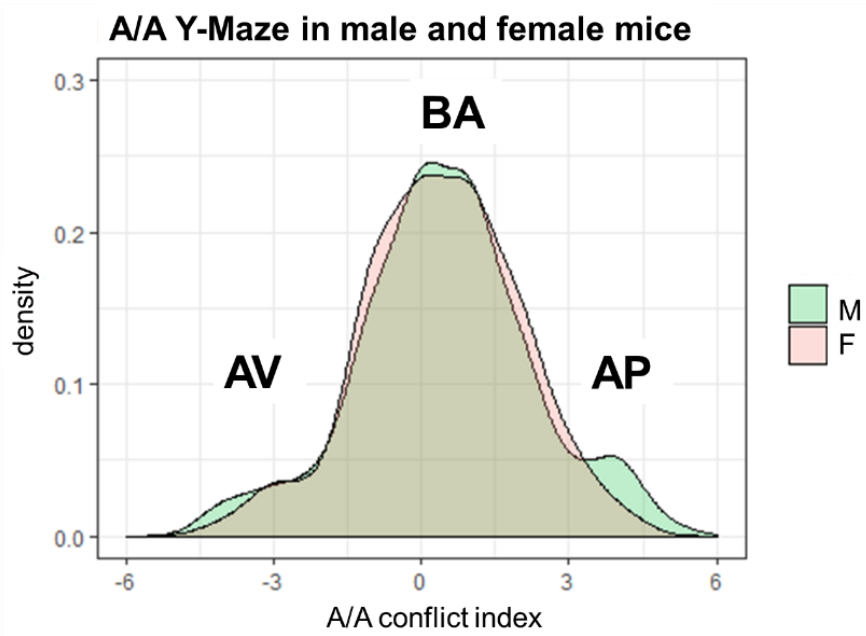

Fig. 11: Approach/Avoidance conflict index. Density plots indicate the distributions of the Approach/Avoidance (A/A) conflict index in males (M, green) and females (F, pink). Abbreviations indicate the location on the curve of avoiding (AV), balancing (BA) and approaching (AP) mice.

According to these distributions the phenotypes of interest were selected within males and females.

Namely, I selected

- BA mice whose behavioral performances represented the mean of the distribution (controls, CTR);
- AV mice whose behavioral performances fell almost 2 standard deviation under the mean of the distribution;
- AP mice whose behavioral performances fell almost 2 standard deviations upon the mean (Fig. 11). The descriptive analysis of the A/A conflict index is reported in Table 2.

Table 2: Approach/Avoidance conflict index. Table shows the descriptive analysis of the Approach/Avoidance (A/A) conflict index in male (♂) and female (♀) mice[2].

A/A conflict index	♂	♀
Mean	0.47	0.40
st dev	1.77	1.56
se	0.16	0.16
skew	-0.05	-0.19
kurtosis	0.27	0.02

No significant effect of cycle phase on female A/A conflict index was found (see S2, Supplementary materials).

[2] From top to bottom (rows): Mean value (Mean); standard deviation (st dev); standard error (se); skewness index (skew) (index of symmetry, = 0 for normal distribution); kurtosis (0 for mesokurtic distribution).

PHENOTYPE EFFECT

After the selection of phenotypes of interest (*i.e*., AV, BA, AP mice), data were analyzed considering phenotype as grouping factor within each sex-group.

The animals used for coupling and breeding the F1 generation were included in the analyses.

To balance the groups' sample size, only the BA animals that formed the BA ♀♂ couples (CTR) were included, while those used for the couplings with AV and AP mates were excluded. Thus, I analyzed 16 males (4 AV, 6 BA, 6 AP) and 13 females (3 AV, 6 BA, 4 AP).

WHITE ARM LATENCY

Kruskal-Wallis analyses showed no significant phenotype effects on S1 latency both in male ($H = 5.4345$, $df = 2$, $p = .06$) and female ($H = 1.6099$, $df = 2$, $p = .447$) mice.

Nevertheless, Mann-Whitney-Wilcoxon (W) planned comparisons showed that AP males were faster in entering the white arm during the S1 compared to AV ($W = 2$, $p = .038$) but not BA ($W = 7$, $p = .092$) males (Fig. 12). No significant differences were found between AV and BA ($W = 10$, p-value = .748) male mice.

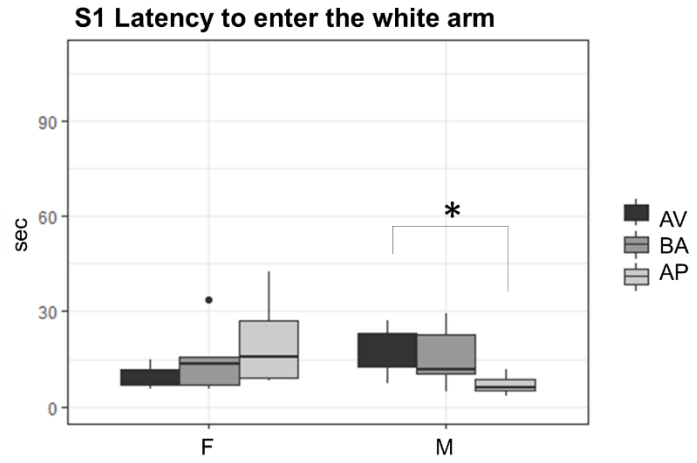

Fig 12. S1 Latency to enter the white arm. Boxplots indicate latency (seconds) to enter the white arm in avoiding (AV), balancing (BA) and approaching (AP) female (F) and male (M) mice during the Session 1 (S1).

No phenotype effect on S2 latency was found in males (H = 3.3088, df = 2, p = .191) and females (H = 1, df = 2, p = .606) (Fig. 13). Mean values and standard deviations are reported in Table 3S.

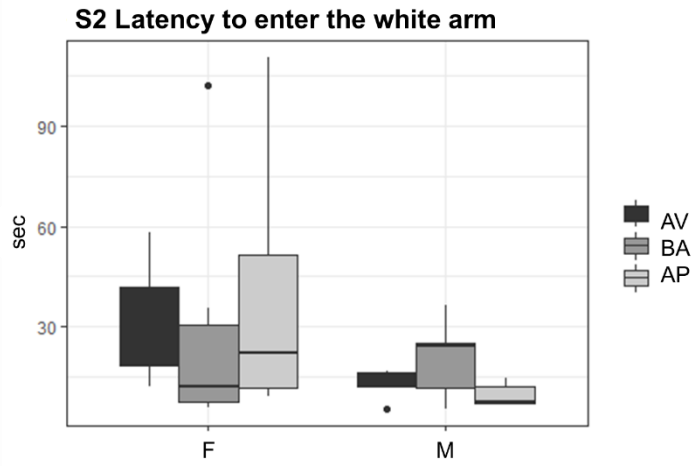

Fig 13. S2 Latency to enter the white arm. Boxplots indicate latency (seconds) to enter the white arm in avoiding (AV), balancing (BA) and approaching (AP) female (F) and male (M) mice during the Session 2 (S2).

No sex effects were found comparing AV, BA and AP males and females by Mann-Whitney-Wilcoxon tests. Mean values, standard deviations and non-significant p-values are reported in Table 3S and Table 4S.

4.2 Open Field with novel object

Results on the OF behavioral parameters related to anxiety levels showed no sex effects. Male and female mice did not differ for the percentage of time spent in the periphery (W = 106, p = .948) and center (W = 116, p = .619) of arena during the S1.

Nevertheless, in S1 males and females were different in parameters linked to general locomotor and exploratory activity as distance traveled (W = 54, p = .028) and mean velocity (W = 44, p = .007). Namely, males travelled more distance and were faster than females (Fig. 14 and Fig. 15). Mean values and standard deviations are reported in Table 2S.

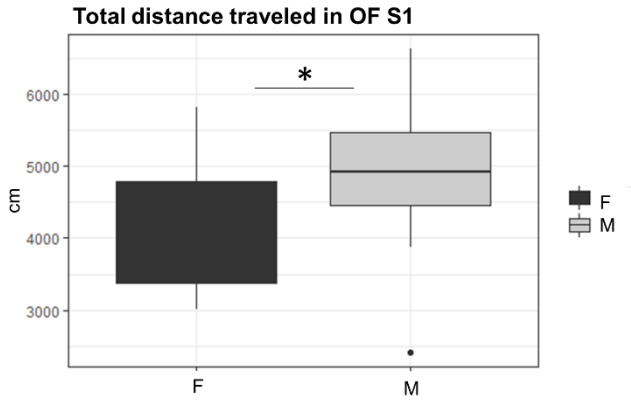

Fig. 14 Distance traveled. Boxplots indicate females (F) and males' (M) total distance traveled in centimeters (cm) during the Session 1 (S1) of the Open Field (OF).

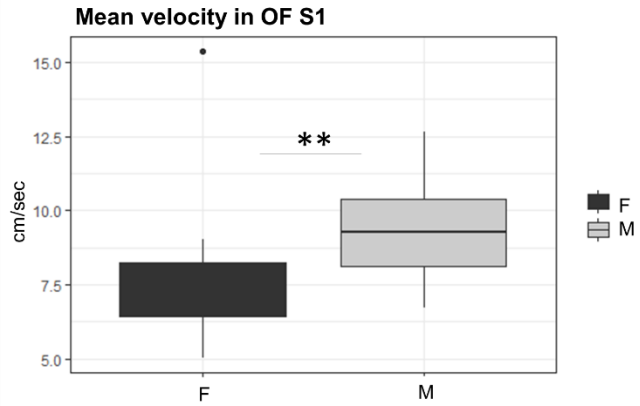

Fig. 15 Mean velocity. Boxplots indicate females (F) and males' (M) mean velocity in centimeters *per* seconds (cm/sec) during the Session 1 (S1) of the Open Field (OF).

Results from the S2 of OF test, showed no gender-dependent effects in novelty response. Male and female mice did not differ in time spent contacting the novel object (W = 88, p = .502) and in frequency (W = 97.5, p = .792) and latency (W = 122, p = .439) of novel object's contact. Mean values and standard deviations are reported in Table 2S.

No phenotype effects were found in the percentage of time spent in the periphery and center of the arena, total distance traveled and mean velocity, time spent contacting the novel object and in frequency and latency of novel object's contact neither within male or female mice (mean values, standard deviations and non-significant p-values are reported in Table 5S and Table 6S).

No sex effects were found in each parameter comparing AV, BA and AP males and females (Table 7S).

4.3 Elevated Plus Maze

Results on EPM parameters showed no sex effects. Male and female mice did not statistically differ in time spent in the open ($W = 77$, $p = .245$) and closed arms ($W = 117$, $p = .589$) and in open ($W = 98$, $p = .807$) and closed ($W = 192.5$, $p = .098$) arms' frequency. Mean values and standard deviations are reported in Table 2S.

No phenotype effect was evident for all parameters considered in both males and females (non-significant p-values are reported in Table 8S). Notably, comparing male and female mice for each AV, BA and AP phenotype no sex differences were found in time spent in the open and closed arms (Fig. 16 and Fig. 17) and in open arms' frequency (Fig. 18) but a sex effect emerged regarding closed arm entries' frequency: AV, BA and AP females more frequently entered the closed arm compared to AV ($W = 12$, $p = .047$), BA ($W = 31.5$, $p = .033$) and AP ($W = 24$, $p = .013$) males (Fig. 19). Mean values, standard deviations and p-values are reported in Table 9S and Table 10S.

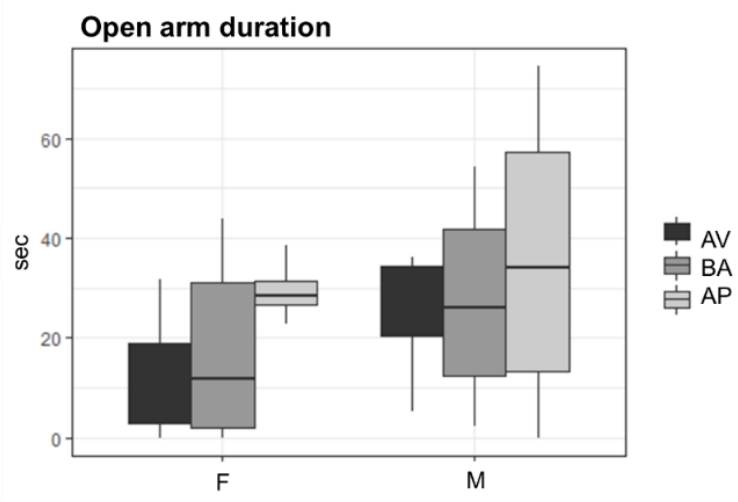

Fig. 16 Time spent in the open arm of the Elevated Plus Maze. Boxplots indicate time spent in the open arm of the EPM, in seconds (sec), in avoiding (AV), balancing (BA) and approaching (AP) female (F) and male (M) mice.

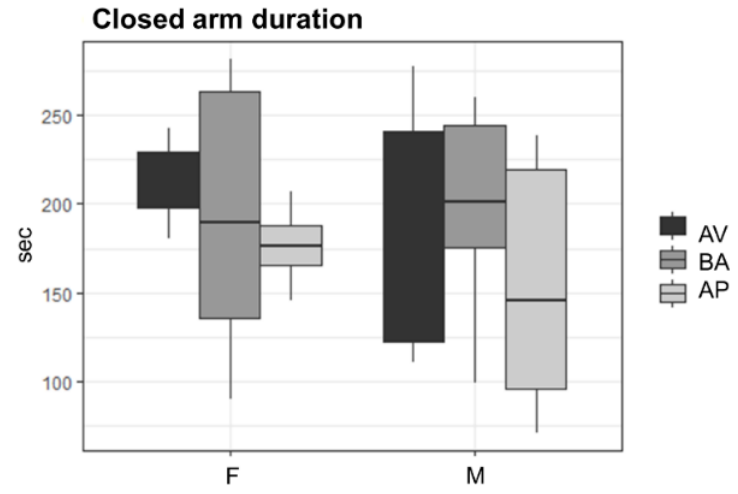

Fig. 17 Time spent in the closed arm of the Elevated Plus Maze. Boxplots indicate time spent in the closed arm of the EPM, in seconds (sec), in avoiding (AV), balancing (BA) and approaching (AP) female (F) and male (M) mice.

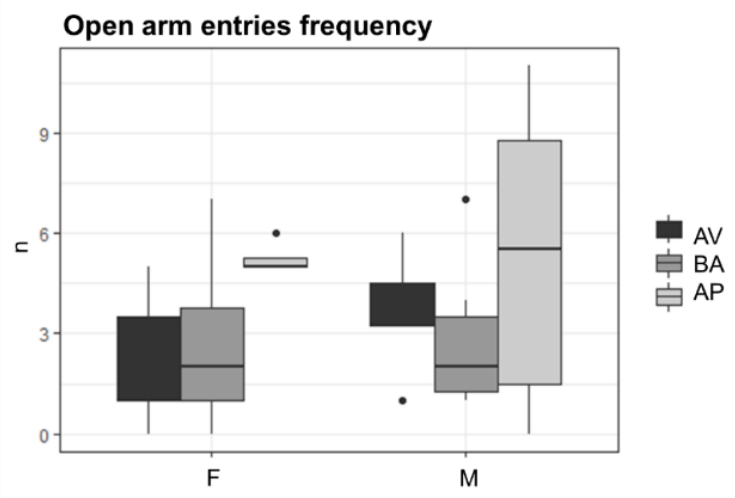

Fig. 18. Open arm entries at the Elevated Plus Maze. Boxplots indicate number (n) of entries in the open arm of the EPM in avoiding (AV), balancing (BA) and approaching (AP) female (F) and male (M) mice.

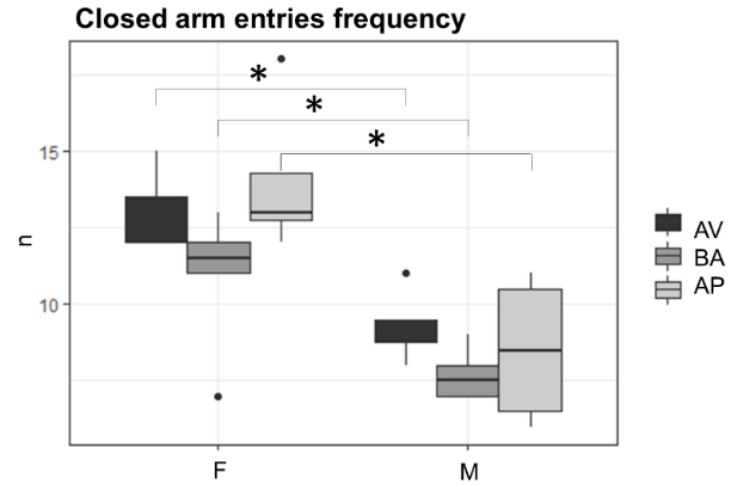

Fig. 19 Closed arm entries at the Elevated Plus Maze. Boxplots indicate number (n) of entries in the closed arm of the EPM in avoiding (AV), balancing (BA) and approaching (AP) female (F) and male (M) mice.

Summary of F0 results:

- **No significant difference was found between male and female A/A conflict index distributions;**
- **Females were slower in entering the white arm during the conflicting S2 of the A/A Y-maze and exhibited reduced general locomotor and exploratory activity in the OF than males, regardless their phenotype;**
- **In the EPM AV, BA and AP females entered more often in the closed arm compared to the respective AV, BA and AP males;**
- **A specific phenotype effect was found for AP male mice that showed reduced latency to enter the white arm of the A/A Y-Maze compared to AV male mice.**

4.4 Undisturbed Parental care Observation

Mann-Whitney-Wilcoxon tests on PCO revealed no significant sex differences in time spent in pup-directed behaviors ($W = 220$, $p = .613$) and in pup-contact frequency ($W = 316$, $p = .079$). Mean values and standard deviations are reported in Table11S.

MATERNAL CARE

AV, BA and AP mothers (coupled with BA fathers) did not differ in time spent in pup-directed behaviors ($H= 1.5604$, $df = 2$, $p = .458$) and pup-contact frequency ($H = 2.1368$, $df = 2$, $p = .343$) (Table 12S; Fig 1S).

No paternally-induced effect on maternal care was found. In fact, BA mothers coupled with AV, BA and AP fathers did not differ in time spent in pup-directed behaviors ($H = 0.525$, $df = 2$, $p = .769$) and pup-contact frequency ($H = 1.765$, $df = 2$, $p = .413$) (Table 12S and Fig 2S).

PATERNAL CARE

AV, BA and AP fathers (coupled with BA mothers) did not differ in time spent in pup-directed behaviors ($H = 0.78891$, $df = 2$, $p = .674$) and pups-contact frequency ($H = 0.34615$, $df = 2$, $p = .841$) (Table 12S and Fig 3S).

No maternally-induced effect on paternal care was found. In fact, BA fathers coupled with AV, BA and AP mothers did not differ in time spent in pup-directed behaviors ($H = 3.4308$, $df = 2$, $p = .179$) or in pup-contact frequency ($H = 0.38769$, $df = 2$, $p = .823$) (Table 12S and Fig 4S).

No sex effects in parental care were found comparing AV, BA and AP mothers and fathers (Fig. 20). Non-significant p-values are showed in Table 13S.

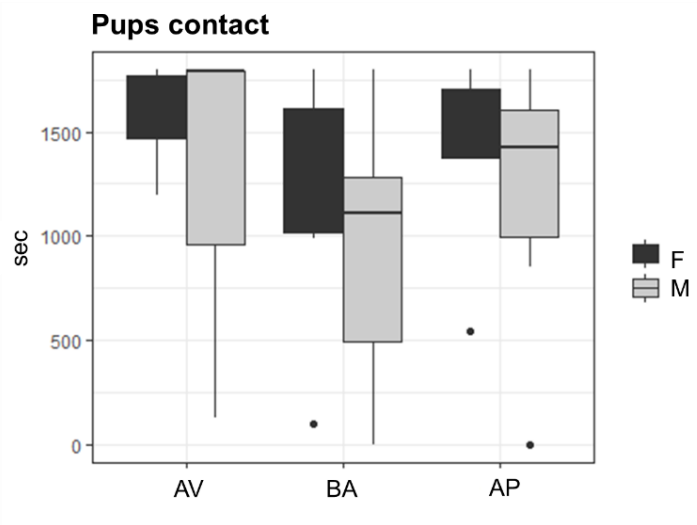

Fig. 20: Undisturbed parental care observation. Boxplots indicate time spent in pup-directed behaviors (seconds) in avoiding (AV), balancing (BA) and approaching (AP) female (F) and male (M) mice.

4.5 Retrieval test

No sex effects were found in latency to reach separated pups (W = 152, p = .763), pup-directed behaviors (W = 202, p = .214) and digging (W = 219, p = .073).

As expected, mothers exhibited pup-retrieval behavior while almost all fathers did not (W = 34, p = <.0001; Fig. 21).

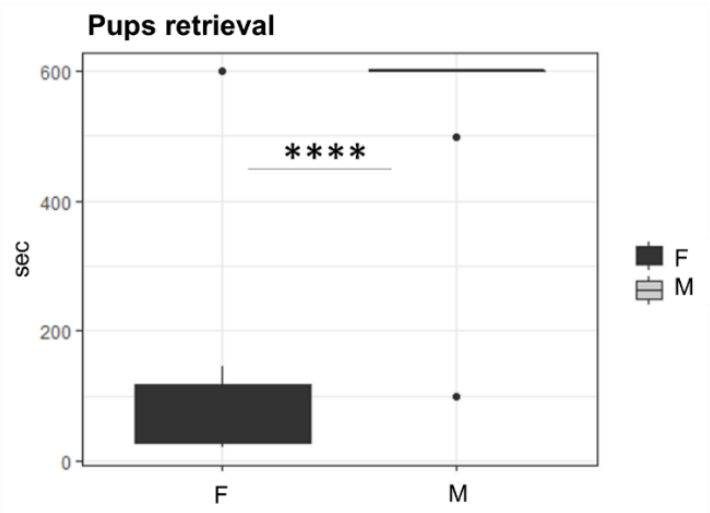

Fig. 21: Retrieval test. Boxplots indicate latency to retrieve all separated pups to the nest in female (F) and male (M) mice. To note, 600 sec is the cut-off.

In addition, females showed less self-grooming (W = 58, p = .0008; Fig. 22) and rearing's frequency (W = 55, p = .0007; Fig. 23) compared to males, regardless their phenotype. Mean values and standard deviations are reported in Table 11S.

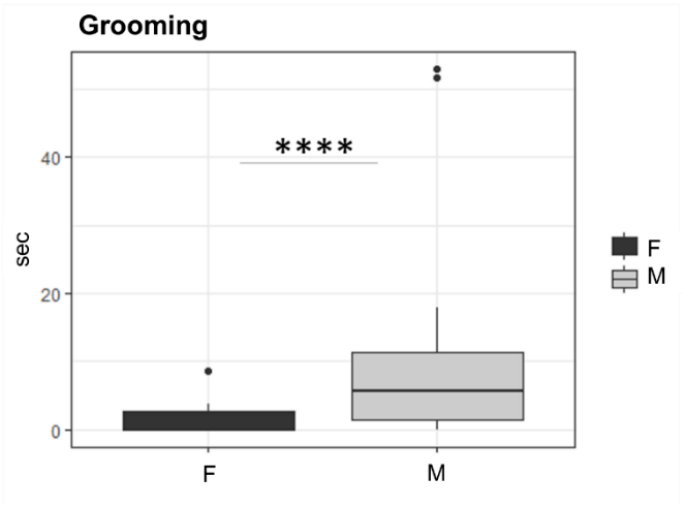

Fig. 22: Retrieval test, self-grooming. Boxplots indicate time spent in self-grooming (seconds -sec) in female (F) and male (M) mice.

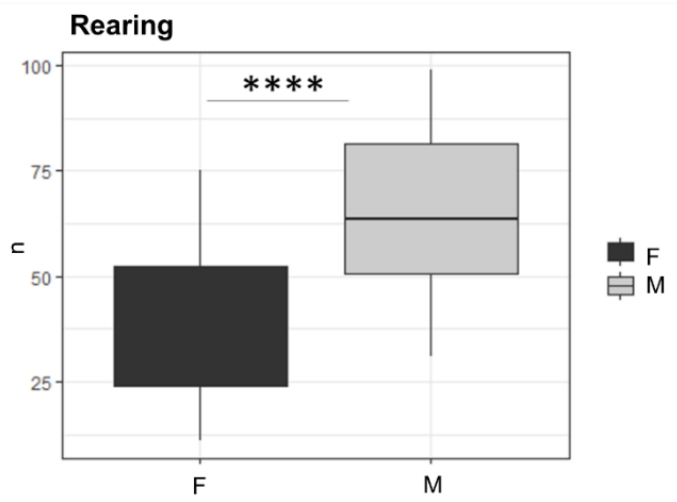

Fig. 23 Retrieval test, rearing. Boxplots indicate the frequency of rearing (number -n) in female (F) and male (M) mice.

MATERNAL RETRIEVAL

AV, BA and AP mothers (coupled with BA fathers) did not differ in latency to reach the separated pups ($H = 1.2606$, $df = 2$, $p = .532$; Fig. 24), time to retrieve all pups to the nest ($H = 0.97412$, $df = 2$, $p = .614$), time spent in pup-directed behaviors ($H = 0.13333$, $df = 2$, $p = .935$), time spent in digging ($H = 0.53333$, $df = 2$, $p = .765$), self-grooming ($H = 0.77117$, $df = 2$, $p = .680$) and frequencies of rearing ($H = 2.7275$, $df = 2$, $p = .255$). Mean values and standard deviations are reported in Table 14S A.

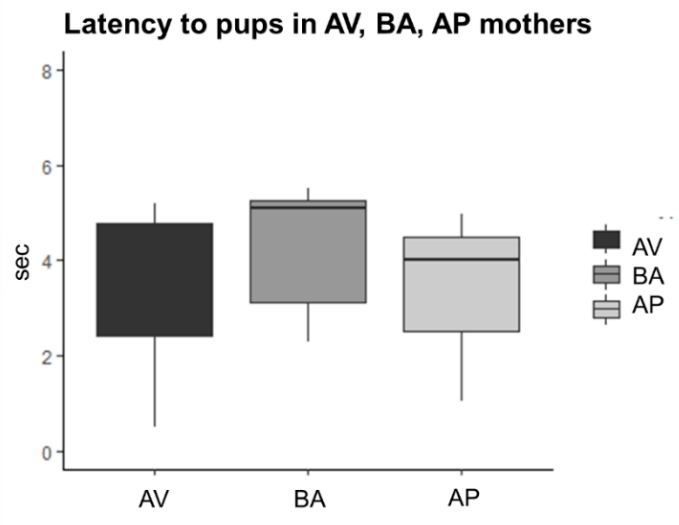

Fig. 24: Retrieval test, latency to reach the separated pups. Boxplots indicate latency (seconds -sec) to reach the separated pups in the retrieval test in avoiding (AV), balancing (BA) and approaching (AP) mothers coupled with BA males.

Notably, a paternally-induced effect on maternal latency to reach the separated pups was observed (Fig. 25). Despite the Kruskal-Wallis analysis did not evidence significant effects ($H = 3.3115$, $df = 2$, $p = .190$), planned Mann-Whitney-Wilcoxon comparisons showed that BA mothers paired with AV mates were faster in reaching separated pups compared to the BA mothers paired with BA ($H = 0$, $p = .035$) but not with AP ($W = 6$, $p = 1$) fathers. No significant differences were found in latency to reach the separated pups between mothers paired with AP or BA fathers ($W = 7$, $p = .555$).

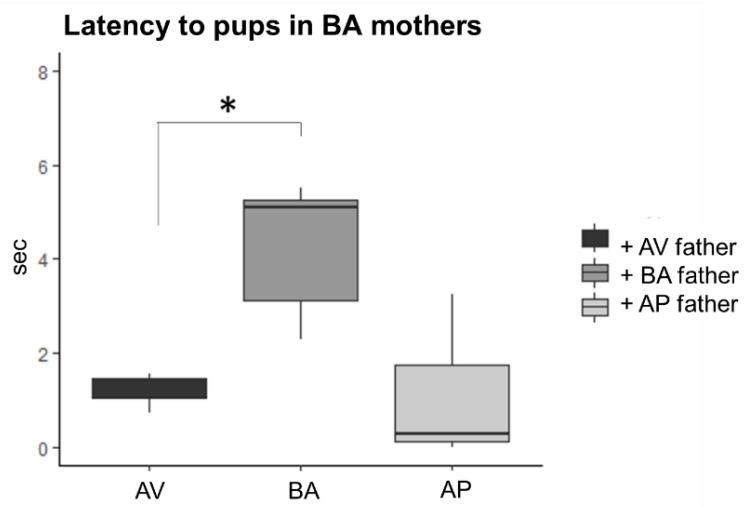

Fig. 25: Retrieval test, latency to reach the separated pups. Boxplots indicate latency in seconds (sec) to reach the separated pups in the retrieval test in balancing (BA) mothers coupled with avoiding (AV), BA and approaching (AP) males.

No other paternally-induced maternal differences were found neither in time to retrieve all pups (H = 1.0558, df = 2, p = .589) or in pup-directed (H = 1.4872, df = 2, p = .475), digging (H = 1.2603, df = 2, p = .532), self-grooming (H = 0.29971, df = 2, p = .860) and rearing (H = 3.4141, df = 2, p = .181) behaviors. Mean values and standard deviations are reported in Table 14S C.

PATERNAL RETRIEVAL

No significant phenotype effect was found in latency to reach the separated pups (H = 3.3232, df = 2, p = .189; Fig. 26), time spent in pup-directed (H = 0.83077, df = 2, p = .660), digging (H = 0.42949, df = 2, p = .806), self-grooming (H = 1.0038, df = 2, p = .605) and frequencies of rearing (H = 1.4625, df = 2, p = .481) behaviors.

Notably, paternal phenotype significantly affected the latency of fathers to retrieve all separated pups to the nest (H = 6.5455, df = 2,

p = .037). AV fathers were the only male group exhibiting a maternal-like retrieval behaviors, while BA and AP fathers reached the test cut-off without ever retrieve pups (see Table 14S B and Table 14S D).

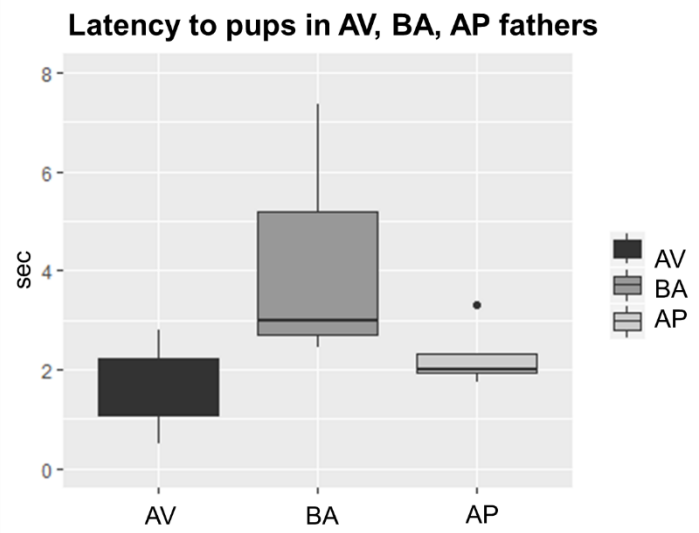

Fig. 26: Retrieval test, latency to reach the separated pups. Boxplots indicate latency in seconds (sec) to reach the separated pups in the retrieval test in avoiding (AV), balancing (BA) and approaching (AP) fathers coupled with BA females.

No significant maternally-induced effects on paternal latency to reach the separated pups (H = 4.4323, df = 2, p = .109, Fig. 27) and to retrieve separated pups (see Table 14S), time spent in pup-directed behaviors (H = 1.7455, df = 2, p = .417), digging (H = 0.1697, df = 2, p-value = 0.9187) and self-grooming (H = 1.6485, df = 2, p-value = 0.4386) and frequency of rearing (H = 1.3425, df = 2, p = .511) were found.

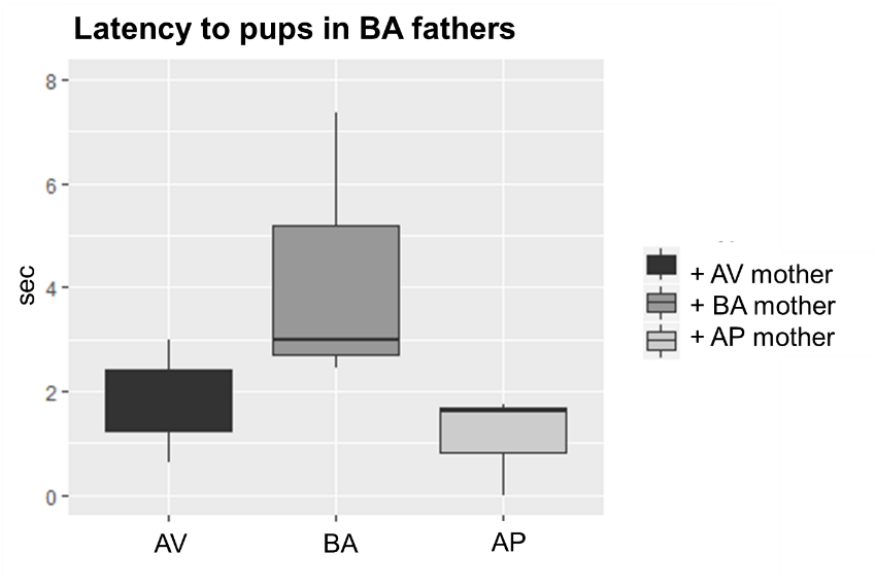

Fig. 27: Retrieval test, latency to reach the separated pups. Boxplots indicate latency in seconds (sec) to reach the separated pups in the retrieval test in balancing (BA) fathers coupled with avoiding (AV), BA and approaching (AP) females.

Sex effects for each phenotype was calculated by Mann-Whitney-Wilcoxon tests on the RTV parameters (p-values are reported in Table 15S). AV, BA and AP males and females were different only for time to retrieve all pups to the nest and time spent in self-grooming. Namely, among AP animals only females retrieved their pups ($W = 12$, $p = .031$) and AP females showed less grooming duration than AP males ($W = 12$, $p = .049$).

Summary of F0 biparental care behaviors:

- **No sex and phenotype effects were found on PCO;**
- **On the RTV test mothers showed more pup-retrieval, less self-grooming and less rearing behaviors compared to fathers, regardless their phenotype;**
- **A/A phenotype did not affect maternal retrieval, but the paternal phenotype significantly influenced maternal latency to reach the separated pups;**
- **Paternal phenotype influenced retrieval of fathers: AV fathers showed maternal-like pup-retrieval;**
- **The AP phenotype produced different effects in males and females with AP females exhibiting more retrieval and less self-grooming behaviors than males.**

4.6 Oxytocin in the PVN

Wilcoxon-Mann-Whitney test revealed no significant sex effect on OXT cell density in the PVN (W = 61, p = .625; Table 16S).

No significant phenotype effects on OXT cell density were found in the PVN of female (H = 1.8636, df = 2, p = .393; Fig. 28; Table 17S) and male (H = 0.75429, df = 2, p = .685; Fig. 29; Table 17S) mice.

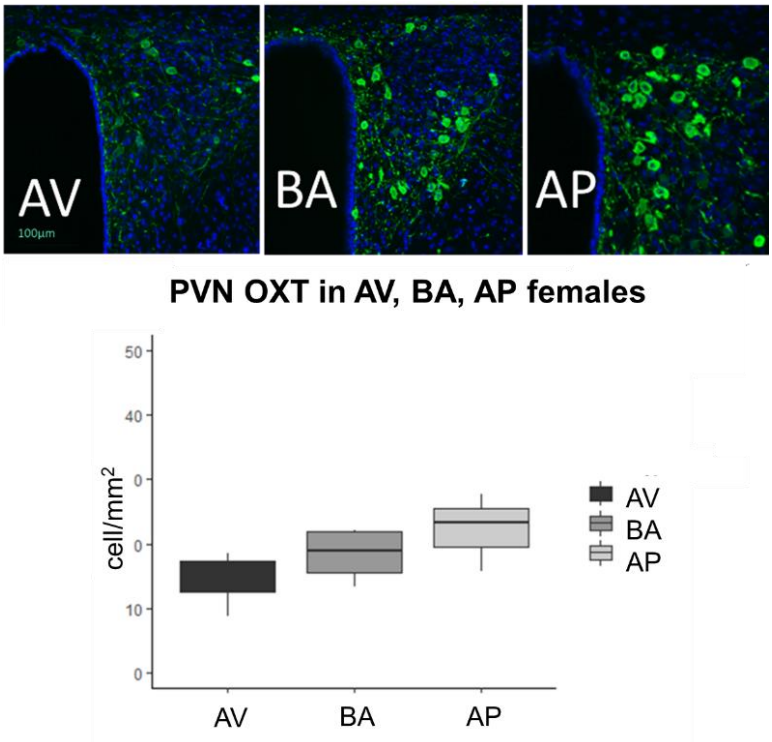

Fig. 28: OXT cell density in the female PVN. Boxplots indicate cell density (cell/mm2) of oxytocinergic (OXT) neurons in the paraventricular hypothalamic nucleus (PVN) of avoiding (AV), balancing (BA) and approaching (AP) female mice. Figures on the top of the graph represent OXT immunofluorescence, OXT in green; Hoechst (nuclei) in blue. Scalebar: 100 µm.

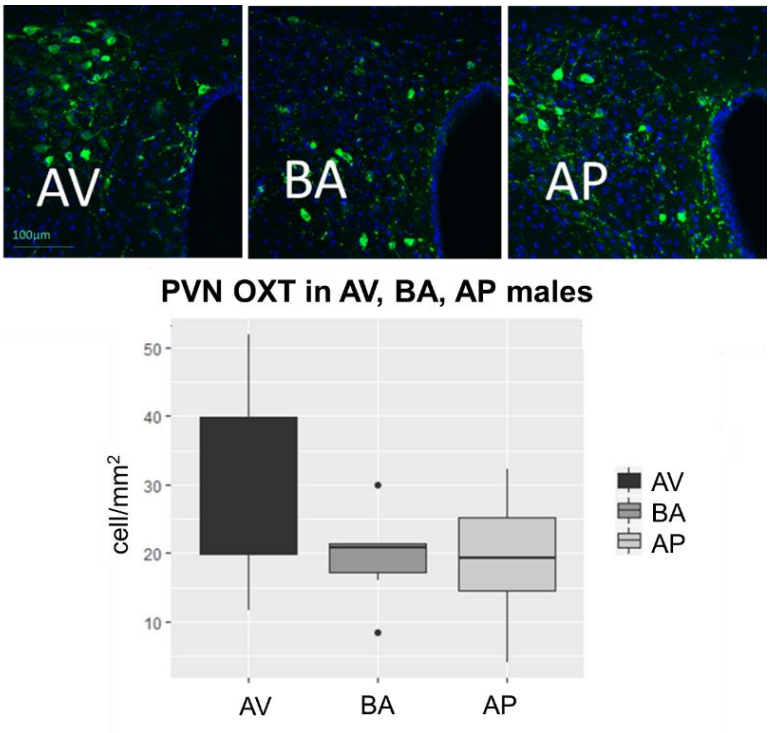

Fig. 29: OXT cell density in the male PVN. Boxplots indicate cell density (cell/mm2) of oxytocinergic (OXT) neurons in the paraventricular hypothalamic nucleus (PVN) of avoiding (AV), balancing (BA) and approaching (AP) male mice. Figures on the top of the graph represent OXT immunofluorescence, OXT in green; Hoechst (nuclei) in blue. Scalebar: 100 µm.

Comparison between AP, BA and AP males and females on the OXT cell density was calculated for each phenotype by Mann-Whitney-Wilcoxon tests. No significant differences were found (p-values are reported in Table 18S).

4.7 Dopamine in the VTA

Wilcoxon-Mann-Whitney test revealed no significant sex effect on THY cell density in the VTA (W = 43, p = .282; Table 16S).

A significant phenotype effect on THY cell density in the VTA of female mice was found (H = 6.3, df = 2, p-value = .042; Fig. 30; Table 17S). AV females showed lower DA cell density compared to BA females (W = 0, p = .057), while no differences between AV and AP (W = 0, p = .1) and between AP and BA (W = 3, p = 0.4) females were found.

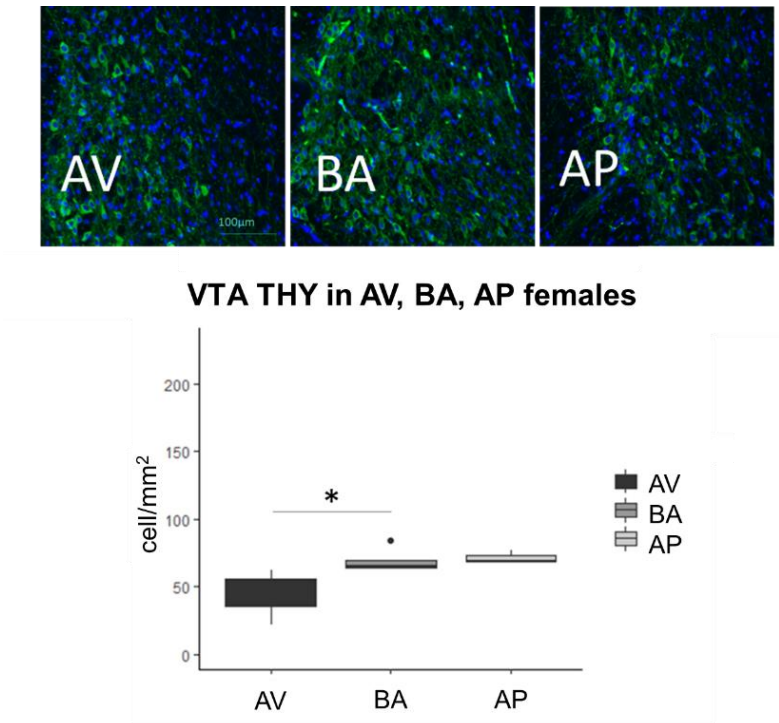

Fig. 30: THY cell density in the female VTA. Boxplots indicate cell density (cell/mm2) of dopaminergic (THY) neurons in the ventral tegmental area (VTA) of avoiding (AV), balancing (BA) and approaching (AP) female mice. Figures on the top of the graph represent THY immunofluorescence, THY in green; Hoechst (nuclei) in blue. Scalebar: 100 µm.

Results on THY immunofluorescence staining in the VTA revealed also a significant difference among AV, BA and AP male mice (H = 5.8615, df = 2, p = .053; Fig. 31). Namely, AP males showed a significantly higher DA cell density compared to BA males (W = 19, p = .031), while no differences were found between AP and AV (W = 11, p = .114) and between AV and BA (W = 5, p = .571) males.

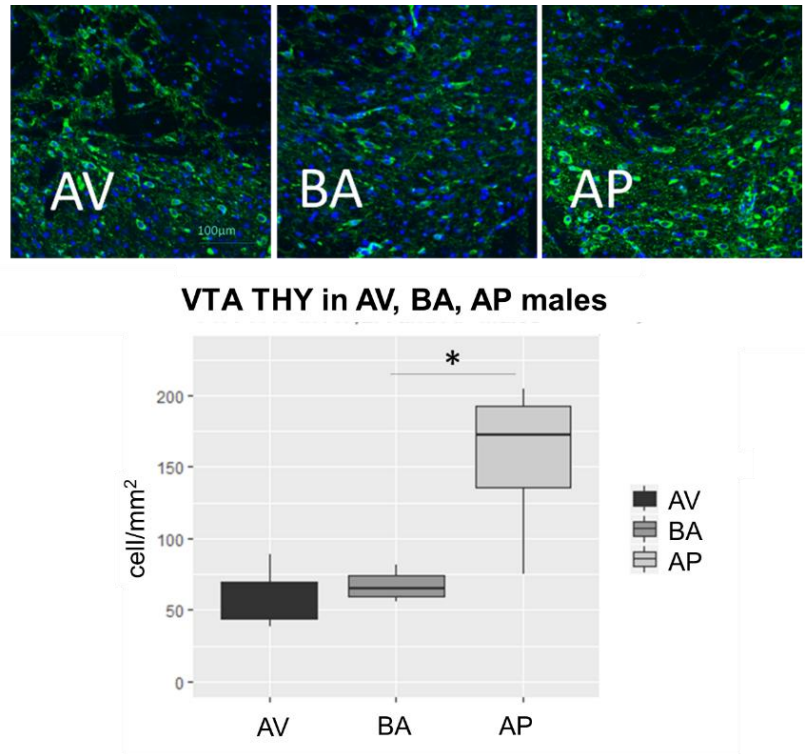

Fig. 31: THY cell density in the male VTA. Boxplots indicate cell density (cell/mm2) of dopaminergic (THY) neurons in the ventral tegmental area (VTA) of avoiding (AV), balancing (BA) and approaching (AP) male mice. Figures on the top of the graph represent THY immunofluorescence, THY in green; Hoechst (nuclei) in blue. Scalebar: 100 µm.

Sex effects on the THY cell density was calculated for each phenotype by Mann-Whitney-Wilcoxon tests. No significant differences were found (p-values are reported in Table 18S; Fig. 32).

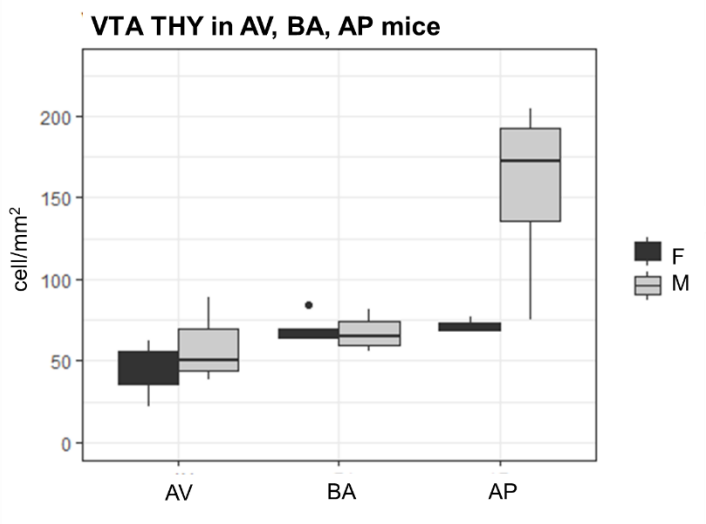

Fig. 32: THY cell density in the VTA. Boxplots indicate cell density (cell/mm2) of dopaminergic (THY) neurons in the ventral tegmental area (VTA) of avoiding (AV), balancing (BA) and approaching (AP) female (F) and male (M) mice.

4.8 Dopamine-oxytocin receptors colocalization in the VTA

No sex effect was found OTR density on VTA-DA neurons (W = 25, p = .190). Mean values and standard deviations are reported in Table 16S.

No phenotype effect was found in the OTR density on VTA-DA neurons in females (H = 1.8667, df = 2, p = .393; Fig. 33).

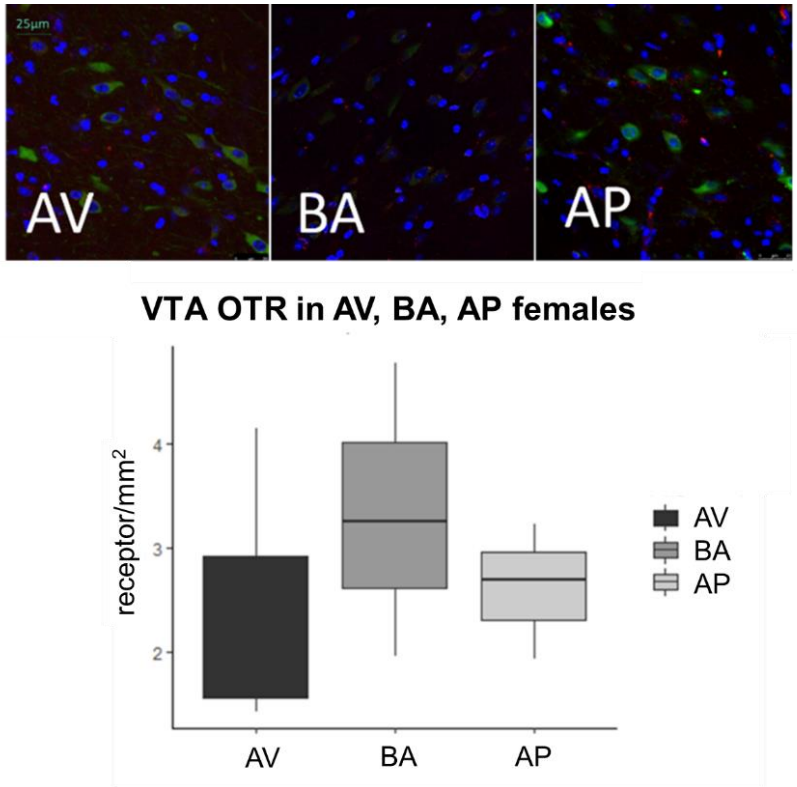

Fig. 33 OTR density in the female VTA. Boxplots indicate receptor density (receptor/mm2) of OTR on DA neurons in the ventral tegmental area (VTA) of avoiding (AV), balancing (BA) and approaching (AP) female mice. Figures on the top of the graph represent OTR/THY double immunofluorescence, OTR in red; THY in green; Hoechst (nuclei) in blue. Scalebar: 25 µm.

Kruskal-Wallis analysis revealed no significant effect of phenotype on OTR/DA colocalization in the VTA of male mice (H = 0.62222, df = 2, p = .732; Fig. 34).

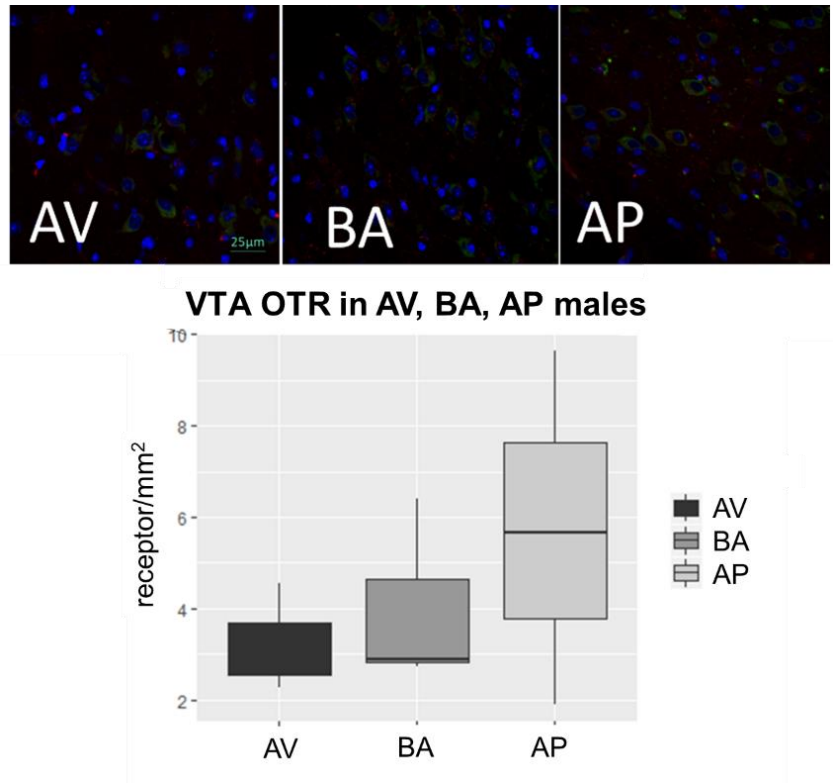

Fig. 34 OTR density in the male VTA. Boxplots indicate receptor density (receptor/mm2) of OTR on DA neurons in the ventral tegmental area (VTA) of avoiding (AV), balancing (BA) and approaching (AP) male mice. Figures on the top of the graph represent OTR/THY double immunofluorescence, OTR in red; THY in green; Hoechst (nuclei) in blue. Scalebar: 25 µm.

Sex effects on OTR density was calculated for each phenotype by Mann-Whitney-Wilcoxon tests. No significant differences were found (p-values are reported in Table 18S).

Summary of F0 brain correlates:

- **No significant relationship was observed between phenotypes and the OTX cell density in the PVN in male and female mice;**
- **AV females were characterized by lower level of DA in VTA compared to BA but not AP females while AP males were characterized by greater DA cell density compared to BA but not AV males;**
- **No significant effect resulted from OTR immunofluorescence, even if AP male mice showed greater OTR on VTA-THY neurons.**

5 F1 Results

Graphs with white background indicate maternal effect; graphs with gray background indicate paternal effect; symbols on legends indicate offspring's sex (male offspring: ♂; female offspring: ♀). Asterisks on graphs indicate significant p-values (* $p \leq .05$; ** $p \leq .01$; *** $p \leq .005$; **** $p \leq .001$).

Before analyzing the results on the F1 I checked by Kruskal Wallis tests that the litters born to the couples selected, belonging to the same phenotypic assortment, were not significantly different from each other, thus ensuring that there was not a significant effect of a single breeding pair in influencing the results (non-significant p-values are reported in Table 19S).

5.1 Maternal effect

5.1.1 Approach/Avoidance Y-Maze

Maternal phenotype did not influence offspring's response to A/A conflict.

FEMALE OFFSPRING, maternal effect

Kolmogorov-Smirnov analyses revealed that maternal phenotype did not influence female offspring's behaviors at the A/A Y-Maze (Fig. 35). In fact, the A/A conflict index of female mice born to BA mothers was not statistically different from the index of female born to AV ($D = 0.22727$, $p = .872$) and AP ($D = 0.065359$, $p = 1$) mothers. No significant differences were found between females born to AV and AP mothers ($D = 0.19786$, $p = .956$).

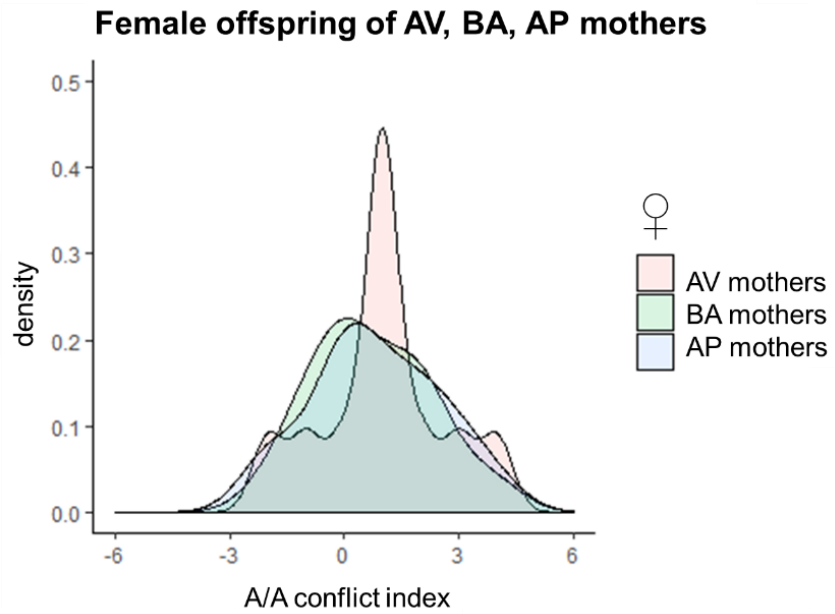

Fig. 35 Approach/Avoidance conflict index in female offspring, maternal effect. Density plots indicate the distributions of the Approach/Avoidance (A/A) conflict index in daughters of avoiding (AV, in pink), balancing (BA, in green) and approaching (AP, in blue) mothers**.**

No maternal phenotype effect was found on female offspring's latency to enter the white arm in S1 and S2 (Table 20S). Mean values and standard deviations are reported in Table 21S.

<u>MALE OFFSPRING, maternal effect</u>

Kolmogorov-Smirnov analyses revealed that maternal phenotype did not affect male offspring's behaviors at the A/A Y-Maze (Fig. 36). The A/A conflict index of sons of BA mothers was not statistically different from the index of sons of AV ($D = 0.58333$, $p = .131$) and AP ($D = 0.19872$, $p = .966$) mothers. No significant differences were found between the male offspring of AV and AP mothers ($D = 0.38462$, $p = .578$).

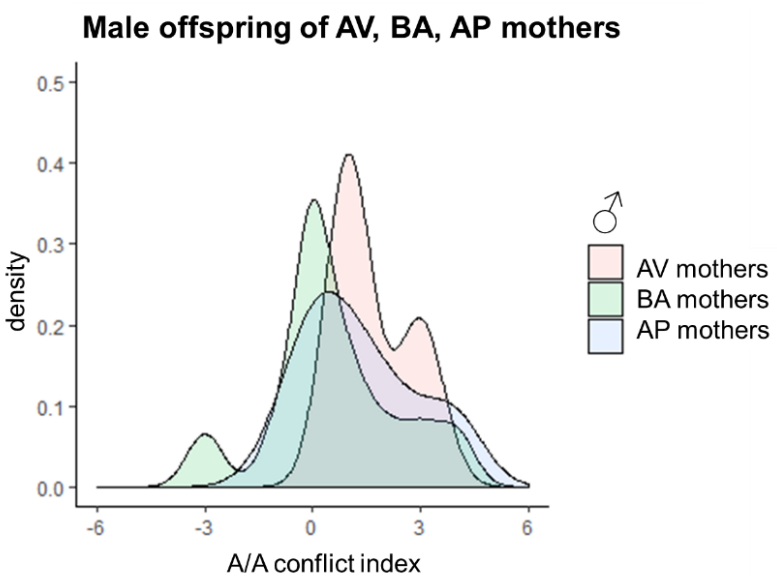

Fig. 36 Approach/Avoidance conflict index in male offspring, maternal effect. Density plots indicate the distributions of Approach/Avoidance (A/A) conflict index in sons of avoiding (AV, in pink), balancing (BA, in green) and approaching (AP, in blue) mothers**.**

Maternal phenotype influenced male offspring's latency to enter the white arm in S2 (H = 7.8561, df = 2, p = .019; Table 20S). Namely, the sons of AP mothers were faster in entering the white arm during the S2 of the A/A Y-Maze than son of BA (W = 33, p = .015) and AV (W = 14, p = .031) mothers. No significant difference was found between the sons born to AV and BA mothers (W = 38, p = .888) (Fig. 37).

Mean values and standard deviations are reported in Table 22S.

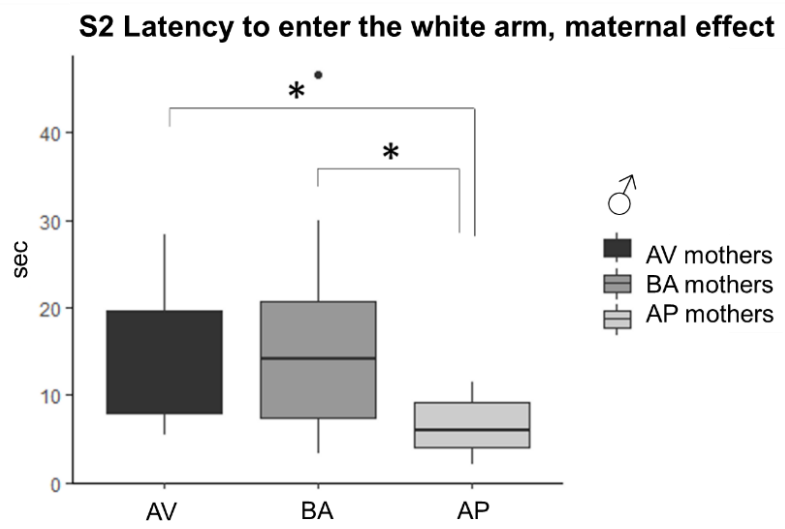

Fig. 37 S2 Latency to enter the white arm in male offspring, maternal effect. Boxplots indicate latencies (seconds -sec) to enter the white arm at the Session 2 (S2) Approach/Avoidance Y-Maze in male offspring born to avoiding (AV), balancing (BA) and approaching (AP) mothers.

Sex effects within each group born to AV, BA and AP mothers on latency to enter the white arm were calculated (p-values are reported in Table 23S).

During the first session of the A/A Y-Maze the female offspring of BA mothers exhibited shorter latency than males (W = 61, p = .048). Notably, during the S2 only the sons of AP mothers were faster than daughters to enter the white arm (W = 161, p = .036).

Mean values and standard deviations are reported in Table 21S and Table 22S.

5.1.2 Open Field with novel object

FEMALE OFFSPRING, maternal effect

Maternal phenotype significantly influenced the response to novelty of daughters (H = 8.5075, df = 2, p = .014). The female offspring born to AP mothers spent more time in contacting the novel object during the OF S2 compared to the female offspring born to AV mothers (W

= 154, p = .003). No significant differences were found among daughters of AV and BA mothers (W = 55, p = .244) and AP and BA mothers (W = 162, p = .091) (Fig. 38).

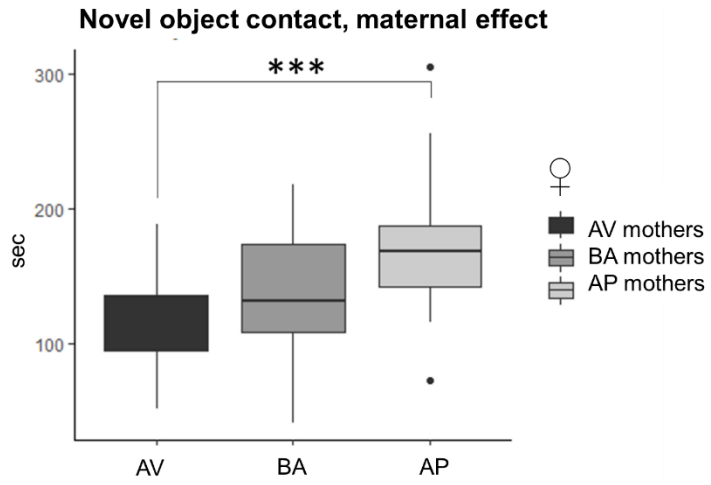

Fig. 38 Novel object contact, female offspring, maternal effect. Boxplots indicate time spent in contacting the novel object (seconds -sec) in female offspring born to avoiding (AV), balancing (BA) and approaching (AP) mothers.

No significant maternal effects were found on female offspring's behaviors on the remaining OF parameters (Table 20S). Mean values and standard deviations of all parameters are shown in Table 21S.

MALE OFFSPRING, maternal effect

No significant maternal phenotype effects on male offspring behaviors at OF were found in all parameters (see Table 20S) but in time spent in S1 arena periphery (H = 7.1939, df = 2, p = .027). Sons of AP mothers spent more time in the arena periphery compared to sons of BA (W = 122, p = .0159) but not AV mothers (W = 37, p =

.898). Sons of AV mothers also spent more time in the arena periphery compared to sons of BA (W = 58, p = .041) (Fig. 39).

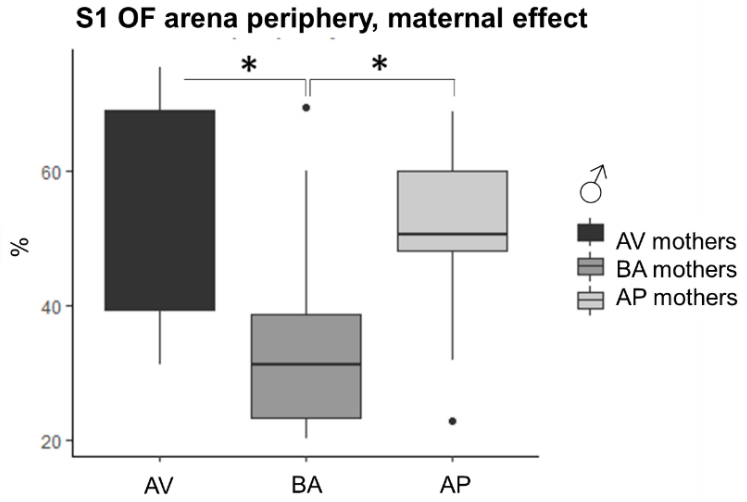

Fig. 39 Arena Periphery, male offspring, maternal effect. Boxplots indicate percentage (%) of time spent in the arena periphery during the Session 1 (S1) in male offspring born to avoiding (AV), balancing (BA) and approaching (AP) mothers.

Also, male offspring velocity was affected by maternal phenotype (H = 9.7714, df = 2, p = .007). Sons of AP mothers were slower then sons of BA (W = 25, p = .002) but not AV (W = 20, p = .106). No significant difference was found between sons of AV and BA (W = 19, p = .124).

Sex effects within each group born to AV, BA and AP mothers on OF parameters were calculated (p-values are reported in Table 23S). Mean values and standard deviations are reported in Table 21S and Table 22S. Overall, daughters of AV and BA mothers are slower than sons but traveled more distance in the arena compared to their male siblings.

5.1.3 Elevated Plus Maze

FEMALE OFFSPRING, maternal effect

No maternal phenotype effects were found on female offspring's parameters at the EPM (see Table 20S for non-significant Kruskal-Wallis comparisons and Table 21S for mean values and standard deviations).

MALE OFFSPRING, maternal effect

Maternal phenotype significantly affected male offspring time spent in the open (H = 11.388, df = 2, p = .003) and closed (H = 12.402, df = 2, p = .002) arm and open (H = 6.7413, df = 2, p = .0343) and closed (7.6061, df = 2, p = .022) arm entries' frequency at the EPM. (Table 20S).

Sons of AP mothers spent more time in the open arm of the EPM compared to sons of BA (W = 120, p < 0.001) and AV (W = 53, p = .047). No significant differences were found between sons of AV and BA mothers (W = 41, p = .673) (Fig. 40).

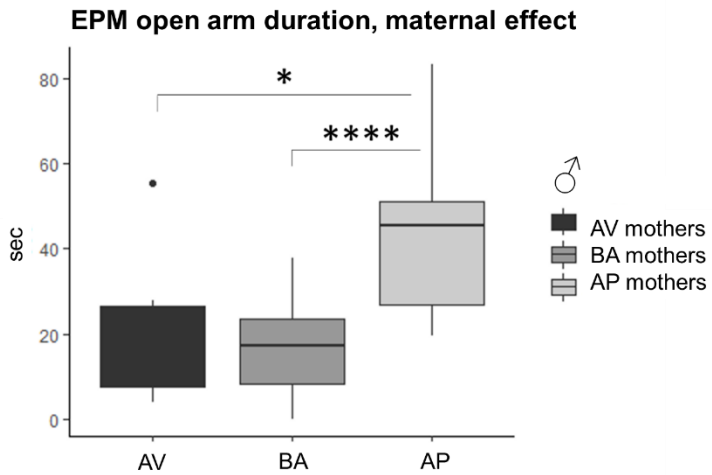

Fig. 40 Elevated plus maze, male offspring, maternal effect. Boxplots indicate time spent (seconds -sec) in the open arm of the EPM in male offspring born to avoiding (AV), balancing (BA) and approaching (AP) mothers.

Sons of AP mothers made also more open arm entries compared to sons of BA (W = 105.5, p = .015) but not AV (W = 49, p = .115) mothers. No significant differences were found between sons of AV and BA mothers (W = 44.5, p = .448).

Sons of AP mothers spent less time in the closed arm of the EPM compared to sons of BA (W = 12, p = .0004). The male offspring of AV mothers also spent less time in the closed arm of the EPM compared to sons of BA (W = 12, p = .02). No significant differences were found between sons of AV and AP mothers (W = 25, p = .462) (Fig. 41).

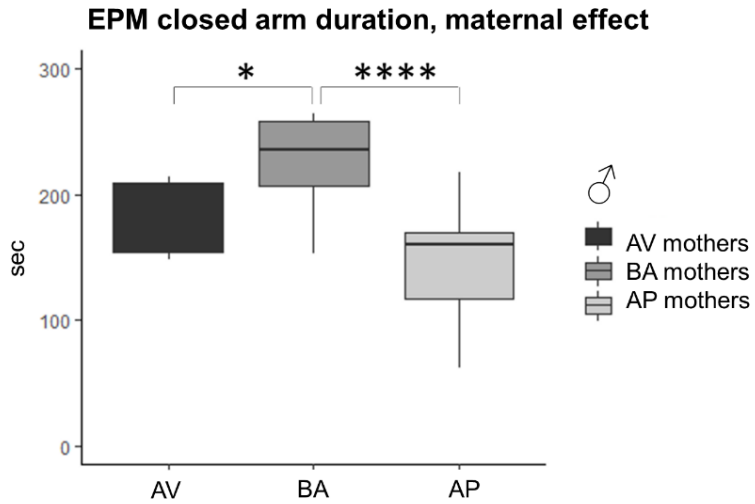

Fig. 41 Elevated plus maze, male offspring, maternal effect. Boxplots indicate time spent (seconds -sec) in the closed arm of the EPM in male offspring born to avoiding (AV), balancing (BA) and approaching (AP) mothers.

Sons of AV mothers showed less closed arm entries' frequency compared to sons of BA mothers (W = 64, p = .009) but not of AP mothers (W = 21, p = .244). No significant differences were found between sons of AP and BA mothers (W = 93.5, p = .094).

Mean values and standard deviations are shown in Table 22S.

Sex effects within each group born to AV, BA and AP mothers on EPM parameters were calculated (p-values are reported in Table 23S; mean values and standard deviations in Table 21S and Table 22S). Overall, a significant sex effect was found within the offspring born to AV mothers with females spending more time in the open and less time in the closed arm compared to male siblings.

Summary of maternal effects on F1:

- **The maternal phenotype did not influence the offspring response to A/A conflict;**
- **The maternal phenotype influenced response to novelty only in female offspring. Namely, the daughters of AP mothers spent more time in contacting the novel object;**
- **The maternal phenotype influenced offspring anxiety levels at the EPM only in males.**

5.2 Paternal effect

5.2.1 Approach/Avoidance Y-Maze

Paternal phenotype significantly influenced offspring's response to A/A conflict.

FEMALE OFFSPRING, paternal effect

Kolmogorov-Smirnov analyses revealed that paternal phenotype influenced female offspring's behaviors at the A/A Y-Maze (Fig. 42). In fact, the distribution of female offspring born to AP fathers was significantly shifted on the AP-side compared to the daughters of AV fathers (D = 0.6, p = .039) and different from the daughter of BA fathers, even if not at a significant level (D = 0.5, p = .054). No significant difference was found between females born to AV fathers and BA fathers (D = 0.14444, p = .999).

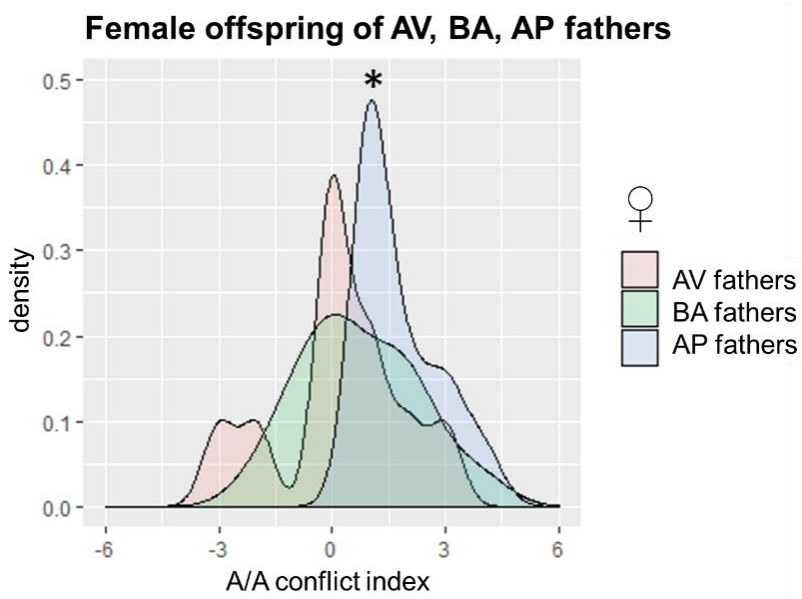

Fig. 42 Approach/Avoidance conflict index in female offspring, paternal effect. Density plots indicate the distributions of Approach/Avoidance (A/A) conflict

index in daughters of avoiding (AV, in pink), balancing (BA, in green) and approaching (AP, in blue) fathers**.**

Paternal phenotype influenced also female offspring's latency to enter the white arm in S1 (H = 10.596, df = 2, p = .005; Table 24S). The daughters of AP fathers were faster in entering the white arm during the S1 of the A/A Y-Maze than daughters of BA (W = 60, p = .044) and AV (W = 9.5, p = .0009) fathers (Fig. 43).

No significant difference was found between daughters born to AV and BA fathers (W = 118, p = .187).

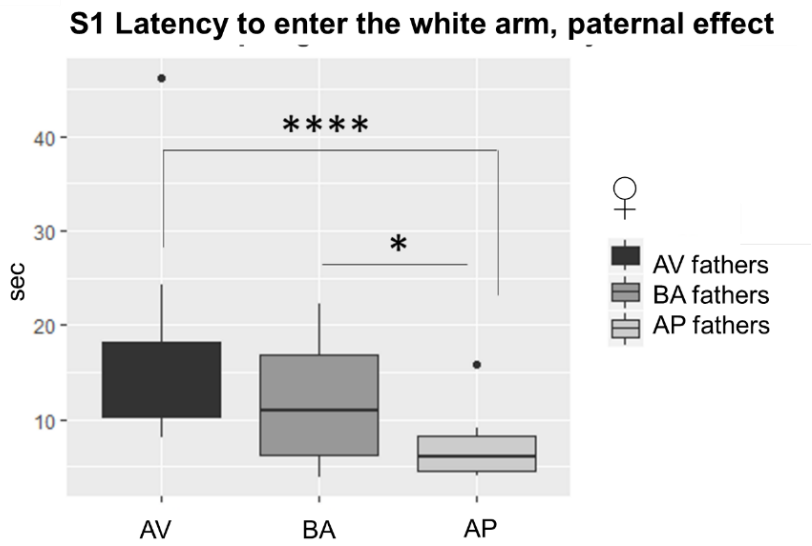

Fig. 43 S1 Latency to enter the white arm in female offspring, paternal effect. Boxplots indicate latencies (seconds -sec) to enter the white arm at the Session 1 (S1) of Approach/Avoidance Y-Maze in female offspring born to avoiding (AV), balancing (BA) and approaching (AP) fathers.

No significant paternal phenotype effects were found in other A/A Y-Maze parameters analyzed (p-values of Kruskal-Wallis comparisons are reported in Table 24S).

Mean values and standard deviations of behavioral parameters of female offspring of AV, BA and AP fathers are reported in Table 25S.

MALE OFFSPRING, paternal effect

Kolmogorov-Smirnov analyses revealed that paternal phenotype influenced male offspring's behaviors at the A/A Y-Maze (Fig. 44). In fact, the distribution of male offspring born to AP fathers was significantly shifted on the AP-side compared to that of the sons of AV fathers (D = 0.7, p = .004). No significant differences between the son born to AP and BA fathers (D = 0.45, p = .095) and between the son born to AV and BA (D = 0.27778, p = .822) were found.

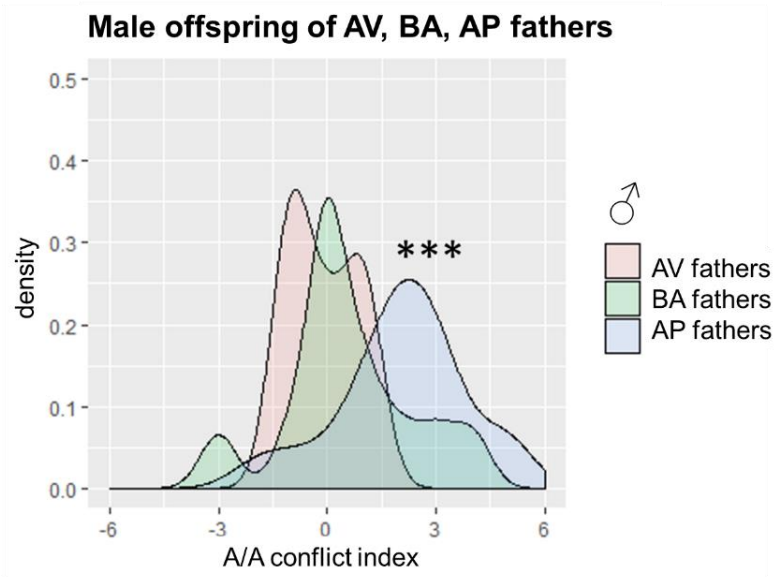

Fig. 44 Approach/Avoidance conflict index in male offspring, paternal effect. Density plots indicate the distributions of Approach/Avoidance (A/A) conflict index in sons of avoiding (AV, in pink), balancing (BA, in green) and approaching (AP, in blue) fathers**.**

Paternal phenotype significantly influenced also male offspring's latency to enter the white arm (H = 10.219, df = 2, p = .006; Table 24S). The sons of AP fathers were faster in entering the white arm during the S1 compared with the sons of BA (W = 64, p-value = .030)

and AV (W = 29, p = .004) fathers. No significant difference was found between sons born to AV and BA fathers (W = 39, p = 0.31) (Fig. 45).

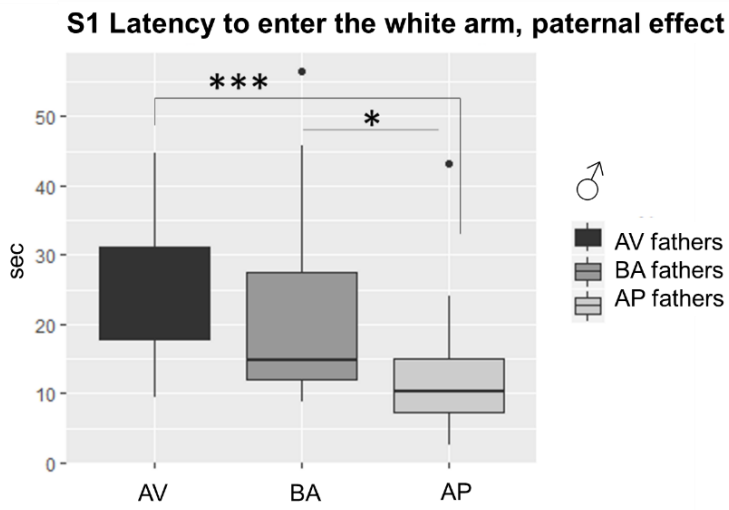

Fig. 45 S1 Latency to enter the white arm in male offspring, paternal effect. Boxplots indicate latencies (seconds -sec) to enter the white arm at the Session 1 (S1) Approach/Avoidance Y-Maze in male offspring born to avoiding (AV), balancing (BA) and approaching (AP) fathers.

No significant paternal phenotype effects were found in other A/A Y-Maze parameters analyzed (p-values of Kruskal-Wallis comparisons are reported in Table 24S).

Mean values and standard deviations of behavioral parameters of male offspring of AV, BA and AP fathers are reported in Table 26S.

Sex effects within each group born to AV, BA and AP fathers on latency to enter the white arm were calculated (p-values are reported in Table 27S).

During the S1 the sons of AP fathers were slower than daughters to enter the white arm (W = 57.5, p = .015).

Mean values and standard deviations are reported in Table 25S and Table 26S.

5.2.2 Open Field with novel object

FEMALE OFFSPRING, paternal effect

No significant paternal effects were found on female offspring's behaviors at the OF (see Table 24S for non-significant Kruskal-Wallis comparisons).

MALE OFFSPRING, paternal effect

Paternal phenotype influenced sons' percentage of time spent in the OF periphery (H = 8.7822, df = 2, p = .012) and novel object contact frequency (H = 9.4212, df = 2, p = .009). Non-significant p-values for the remaining OF parameters are reported in Table 24S.

In S1, the sons of AP fathers spent more time in the arena periphery compared to sons of BA (W = 188, p = .008) but not AV (W = 131, p = .056) fathers. No significant difference was found between the sons of AV and BA fathers (W = 71, p = .246) (Fig. 46).

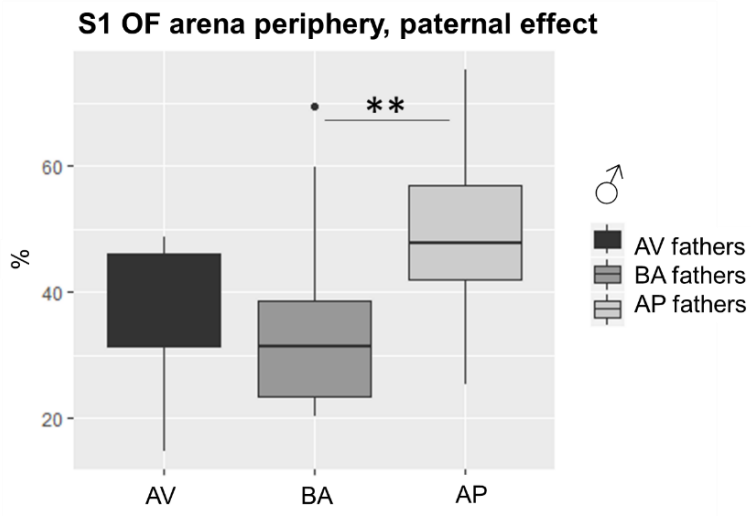

Fig. 46 Arena Periphery, male offspring, paternal effect. Boxplots indicate percentage (%) of time spent in the arena periphery during the Session 1 (S1) in male offspring born to avoiding (AV), balancing (BA) and approaching (AP) fathers.

Furthermore, the sons of BA fathers showed blunted novelty response in the OF S2 compared to the offspring of both AV (W = 83.5, p = .039) and AP (W = 197.5, p = .002) fathers. No significant difference was found between the sons of AV and BA fathers (W = 92.5, p = .924) (Fig. 47).

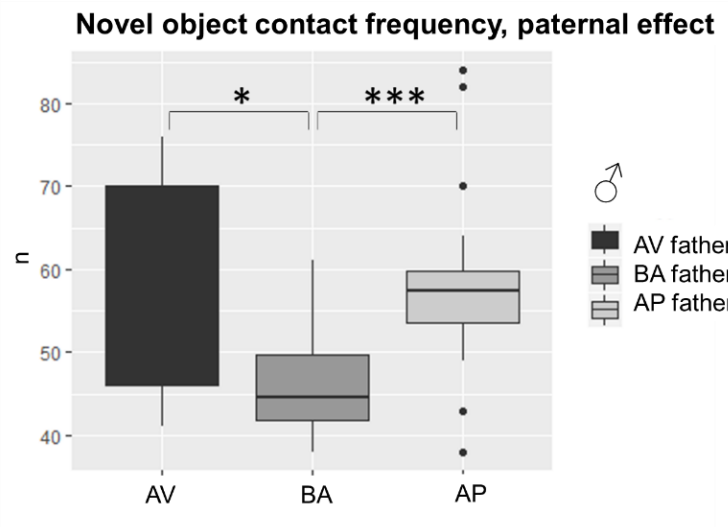

Fig. 47 Novel object contact frequency, male offspring, paternal effect. Boxplots indicate frequency (number -n) of novel object contact in male offspring born to avoiding (AV), balancing (BA) and approaching (AP) fathers.

Sex effects within each group born to AV, BA and AP fathers on OF parameters were calculated (p-values are reported in Table 27S). Mean values and standard deviations are reported in Table 25S and Table 26S.

No sex effects were found in all parameters and within each group, but in percentage of time spent in the arena periphery of offspring born to BA fathers (controls). Namely, the daughters of BA fathers spent more time in the arena periphery compared to their male siblings.

5.2.3 Elevated Plus Maze

FEMALE OFFSPRING, paternal effect

Paternal phenotype significantly influenced female offspring's anxiety measured at the EPM.

Females born to AP, BA, and AP fathers were different in time spent in the open arm ($H = 6.4209$, $df = 2$, $p = .040$), with daughters of AP fathers spending more time in the open arm compared to daughters of BA ($W = 135.5$, $p = .030$) and AV ($W = 80$, $p = .023$) fathers. No significant difference was found between the daughters of AV and BA fathers ($W = 82$, p-value = 0.719) (Fig. 48).

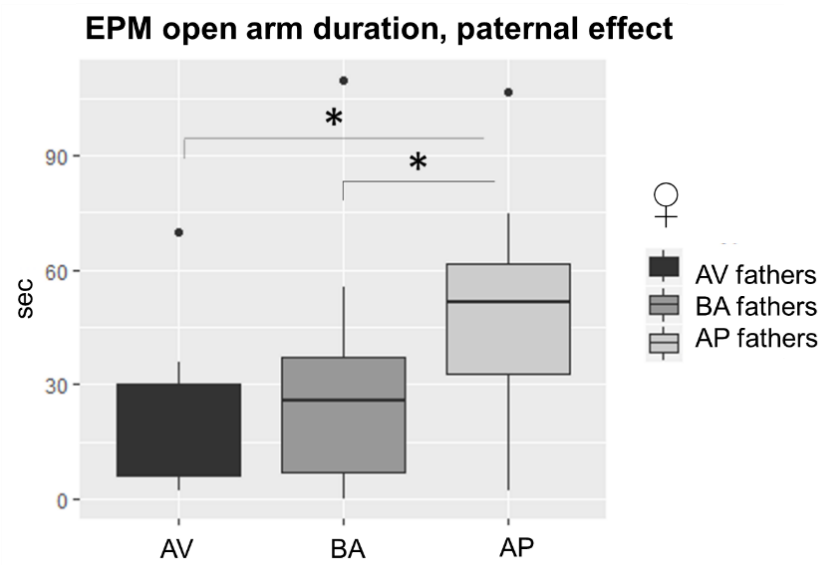

Fig. 48 Elevated plus maze, female offspring, paternal effect. Boxplots indicate time spent (seconds -sec) in the open arm of the EPM in female offspring born to avoiding (AV), balancing (BA) and approaching (AP) fathers.

A paternal phenotype effect was found also in open arm entries frequency ($H = 6.7004$, $df = 2$, $p = .035$), with daughters of AP fathers showing an increased frequency in the open arm visiting compared

to daughters of AV (W = 85.5, p = .007) but not BA (W = 130, p = .057) fathers. No significant difference was found between the female offspring of AV and BA fathers (W = 81, p = .681).

Paternal phenotype influenced also time spent in the closed arm (H = 7.3934, df = 2, p = .02). Daughters of AP fathers spent less time in the closed EPM arm compared to daughters of BA (W = 40, p = .015) and AV (W = 19, p = .018) fathers. No significant difference was found between the female offspring of AV and BA fathers (W = 100, p-value = .654) (Fig. 49).

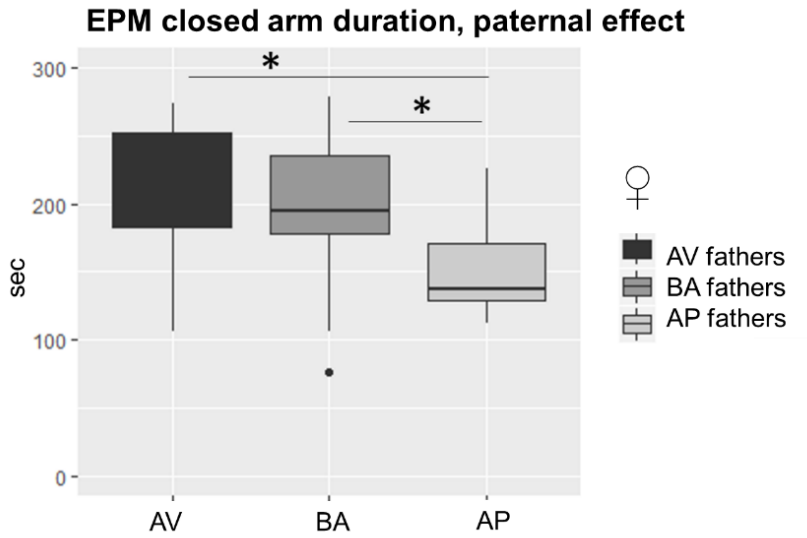

Fig. 49 Elevated plus maze, female offspring, paternal effect. Boxplots indicate time spent (seconds -sec) in the closed arm of the EPM in female offspring born to avoiding (AV), balancing (BA) and approaching (AP) fathers.

No paternal effect on closed arm entries frequency was found (H = 0.3465, df = 2, p = .840).

P-values of paternal effect are shown in Table 24S; mean values and standard deviation in Table 25S.

MALE OFFSPRING, paternal effect

Paternal phenotype significantly influenced male offspring's anxiety measured at the EPM. Namely, males born to AP, BA, and AP fathers were different in time spent in the open arm (H =17.31, df = 2, p = .0001). Sons of AP fathers spent more time in the open arm compared to sons of BA (W = 202, p = .001) and AV (W = 165, p = .0004) fathers, while no significant difference was found between the male offspring of AV and BA fathers (W = 42, p = .412) (Fig. 50).

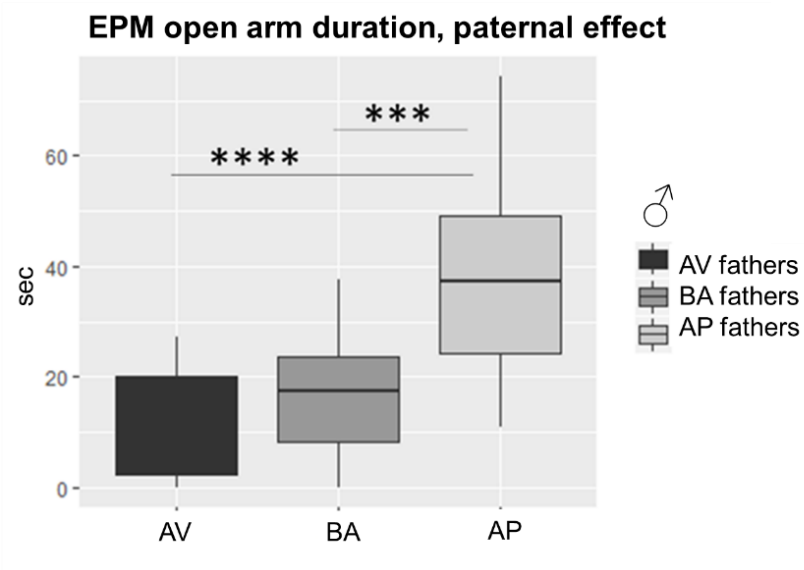

Fig. 50 Elevated plus maze, male offspring, paternal effect. Boxplots indicate time spent (seconds -sec) in the open arm of the EPM in male offspring born to avoiding (AV), balancing (BA) and approaching (AP) fathers.

Paternal phenotype affected also open arm entries frequency (H = 13.622, df = 2, p = .001). Sons of AP fathers enter more frequently in the open arm of the EPM compared to daughters of BA (W = 191.5, p = .005) and AV (W = 156, p = .001) fathers and no significant difference was found between the male offspring of AV and BA fathers (W = 41.5, p = .383).

No paternal effects on closed arm duration (H = 18.972, df = 2, p = .075) and closed arm entries (H = 3.8559, df = 2, p = .145) were found.
P-values of paternal effect are shown in Table 24S; mean values and standard deviation in Table 26S.

Sex effects within each group born to AV, BA and AP fathers on EPM parameters were calculated (p-values are reported in Table 27S). No significant sex effect within phenotypes were observed for all parameters, but for closed arm entries in the control groups. Female offspring of BA parents entered more frequently in the closed arm compare to their male siblings (mean values and standard deviations are shown in Table 25S and Table 26S).

Summary of paternal effects on F1:

- **The paternal AP phenotype significantly affected both female and male offspring response to conflict. In particular, the offspring born to AP fathers were more approaching and faster when tested at the A/A Y-Maze;**
- **The paternal phenotype specifically influenced response to novelty only in male offspring: sons of AV and AP fathers contacted more frequently the novel object at the OF compared to respectively controls;**
- **Paternal phenotype affected both female and male anxiety. Male offspring of AP mothers and male and female offspring of AP fathers showed less anxiety in the EPM compared to controls.**

6 Summary of main F0 and F1 results

To summarize, no significant difference was found in the A/A conflict index distributions of male and female mice.

Females were slower in entering the white arm during the conflicting S2 of the A/A Y-maze and exhibited reduced general locomotor and exploratory activity in the OF S1 and enhanced anxiety levels at the EPM in comparison to males, regardless their phenotype.

A/A parental phenotype did not influence maternal and paternal care measured with the PCO. Nonetheless, paternal phenotype significantly influenced RTV behaviors of fathers (AV fathers were the only group showing maternal-like retrieval) and of BA mothers coupled to them (mothers paired with AV compared to BA fathers, showed minor latency to reach separated pups) (Table 3).

Table 3: Sex effect, F0. Table shows main sex differences found between males and females in the A/A Y-Maze, latency to enter the white arm in Session 1 (S1) and Session 2 (S2); Open Field with novel object (OF), Arena Periphery, Arena Center; Distance traveled and velocity; Retrieval (RTV), Latency to reach separated pups, Retrieval of pups to the nest, Pup-directed behaviors, Digging, Self-grooming, Rearing.

F0		Males	Females
Y	Latency S1	–	–
	Latency S2	↓	↑
OF	Arena Periphery	–	–
	Arena Center	–	–
	Distance traveled	↑	↓
	Velocity	↑	↓
RTV	Latency to pups	–	–
	Retrieval	↓	↑
	Pup-directed behav.	–	–
	Digging	–	–
	Self-grooming	↑	↓
	Rearing	↑	↓

AV females were characterized by lower THY cell density compared to controls (Table 4), but maternal phenotype did not impact offspring's response to A/A conflict. Maternal phenotype effects on progenies were sex-specific, affecting response to novelty only in female (female offspring of AV mothers showed a blunted response to novelty at the OF compared to the daughters of AP mothers) and latency and anxiety levels only in males (sons of AP mothers were faster at the A/A Y-Maze S2 and less anxious at the EPM compared to controls) (Table 4).

AP male mice were characterized by a greater number of VTA-DA neurons, enriched in OTR, compared to controls (Table 5). The AP paternal phenotype was able to bias descendants' behaviors. In fact, daughters and sons of AP fathers were faster and more approaching

at the A/A Y-Maze compared to the offspring of AV fathers and less anxious at the EPM compared to controls. Furthermore, sons of AP fathers more frequently contacted the novel object in the S2 of the OF compared to sons of BA fathers (Table 5).

Table 4: M**aternal effect**. Table shows main maternal effects found in neuronal correlates (Brain): paraventricular hypothalamic nucleus (PVN) oxytocin (OXT), ventral tegmental area (VTA) dopamine (DA), oxytocin receptors/DA colocalization (OTR/DA), and in offspring's (F1) A/A Y-Maze (Y), A/A conflict index, latency to enter the white arm in Session 1 (S1) and Session 2 (S2); Open Field with novel object (OF), Arena Periphery, Arena Center; Distance traveled and velocity, novel object duration (dur.), frequency (freq.); Elevated plus Maze (EPM) Open and Closed arm dur. and freq. Symbols indicate specific effect for male (♂) or female (♀) offspring.

		Mothers		
		AV	BA	AP
Brain	PVN OTX	–	–	–
	VTA DA	↓	↑	–
	OTR/DA	–	–	–
F1 Y	A/A conflict index	–	–	–
	Latency S1	–	–	–
	Latency S2	↑♂	↑♂	↓♂
F1 OF	Arena Periphery	↑♂	↓♂	↑♂
	Arena Center	–	–	–
	distance traveled	–	–	–
	velocity	–	↑♂	↓♂
	Novel object dur.	↓♀	–	↑♀
	Novel object freq.	–	–	–
F1 EPM	Open arm dur.	↓♂	↓♂	↑♂
	Closed arm dur	↓♂	↑♂	↓♂
	Open arm freq	–	↓♂	↑♂
	Closed arm freq	↓♂	↑♂	–

Table 5: Paternal effect. Table shows main paternal effects found in neuronal correlates (Brain): paraventricular hypothalamic nucleus (PVN) oxytocin (OXT), ventral tegmental area (VTA) dopamine (DA), oxytocin receptors/DA colocalization (OTR/DA), and in offspring's (F1) A/A Y-Maze (Y), A/A conflict index,

latency to enter the white arm in Session 1 (S1) and Session 2 (S2); Open Field with novel object (OF), Arena Periphery, Arena Center; Distance traveled and velocity, novel object duration (dur.), frequency (freq.); Elevated plus Maze (EPM) Open and Closed arm dur. and freq. Symbols indicate specific effect for male (♂) or female (♀) offspring.

		Fathers		
		AV	BA	AP
Brain	PVN OTX	–	–	–
	VTA DA	–	↓	↑
	OTR/DA	–	–	–
F1 Y	A/A conflict index	↓	↓	↑
	Latency S1	↑	↑	↓
	Latency S2	–	–	–
F1 OF	Arena Periphery	–	↓♂	↑♂
	Arena Center	–	–	–
	distance traveled	–	–	–
	velocity	–	–	–
	Novel object dur.	–	–	–
	Novel object freq.	↑♂	↓♂	↑♂
F1 EPM	Open arm dur.	↓	↓	↑
	Closed arm dur	↑♀	↑♀	↓♀
	Open arm freq	↓	↓	↑
	Closed arm freq	–	–	–

7 Discussion

The present study demonstrated that parental A/A phenotypes were able to bias descendants' behaviors, depending on parents' and offspring's sex.

Regardless the A/A phenotype, gender differences among C57 mice have been found. In fact, female mice showed reduced general locomotor activity and increased anxiety levels compared to males. These differences have been already found in studies that stressed the importance of including females in experimental designs, to overcome the still widespread sex-bias present in preclinical research (An et al., 2011; Berkley, 1992; Karp et al., 2017; Palanza, 2001; Palanza and Parmigiani, 2017; Van Swearingen et al., 2013). An and colleagues (2011), comparing the behaviors of BALB/c and C57BL/6 inbred mice for both genetic background and gender, demonstrated that C57 males were less anxious in the EPM (more time spent in the open arms) and more active in a novel cage (more distance walked and more wall-rearing) compared to females. These findings fit with the present results of higher number of closed arm visits in the EPM, lower general locomotor activity in the OF, and infrequent rearing behaviors in the RTV, in female compared to male mice.

Some sex-differences in mice behaviors can be attributed to the effects of hormones, but no significant influence of the estrous phase on female A/A performances was noticed. Notwithstanding, steroid hormones exert both fluctuating and organizational permanent effects on the central nervous system and differences between males and females could result from gender-specific evolutionary pressures (Geary, 2017; Kelly et al., 1999; Palanza, 2001).

Furthermore, sex differences found in animal models parallel sex differences found in clinical literature evidencing gender-dependent vulnerability, incidence, course and response to pharmacological treatment in several disorders (*e.g.*, depression, anxiety; Blear, 1995; Earls, 1987; Gater et al., 1998; Hanna and Grant, 1997).

Despite the sex-difference observed, within both males and females, individual differences occurred, and AV, BA and AP phenotypes were present in the whole sample. In fact, the A/A conflict task, was able to reveal individual differences in the response to conflict and, the increased latency to enter the white arm from S1 to S2 suggested that mice, especially females, were sensitive to the contention between opposing A/A drives. The S2 hesitation to enter the white arm could reflect the involvement of higher order cognitive processes to resolve the conflict and choose between advancing and withdrawing response (Claes et al., 2016).

Notably, AP male mice showed minor S1 latency at the A/A Y-Maze compared to AV. This phenotype effect reproduces the finding of Laricchiuta and colleagues (2012a) and corroborates the hypothesis that the AP phenotype in particular, is associated with reward seeking behavior and impulsivity/exploration (Pickering and Gray, 2001).

No significant effect on parental care was found with respect of the parental phenotype.

Notably, the protocol used for assessing parental behaviors was planned to let parents and offspring in the most undisturbed conditions possible, with a long period of acclimatization and inside the animal facility.

Nevertheless, when parents were tested at the retrieval test, in which a separation from the pups was experimentally induced, the significant influence of the paternal phenotype emerged.

Despite pup-retrieval behavior belongs to the typically feminine repertoire of care, AV fathers exhibited maternal-like retrieval behaviors. Furthermore, also the BA females coupled with AV mates, showed more responsive behaviors to the separated pups, reaching them more quickly than mothers mated with BA males.

In an elegant experiment, Liu et al. (2013), demonstrated that not-spontaneously-parental sires of laboratory mice, can show maternal-like pup-retrieval if continuously housed with their mates and if allowed to communicate with the dams during the pup-separation test. The authors registered ultrasonic vocalizations between partners and noticed maternal 38-kHz vocalizations to encourage paternal pup-care. Using the same RTV protocol, I showed pup-retrieval behaviors in AV male mice, stably coupled with BA females.

“Paternal via maternal” modifications have been already described in rodents (reviewed in Braun and Champagne; 2014). In fact, females can adjust reproductive investment depending on mate attractiveness and availability and can modify their maternal behaviors also to counteract disadvantages inherited by their offspring to the father. These maternal alterations are more likely when females do not freely choose their partners (Curley et al., 2011; Mashoodh et al., 2012) and such a role of mothers could explain why no specific intergenerational impact of the paternal AV phenotype was found.

In fact, although the offspring of AV and AP fathers were different in many of the investigated behaviors, in no parameter the offspring of AV fathers were different from the BA-controls.

On the contrary, the AP paternal phenotype significantly shaped descendants' behaviors.
AP males were characterized by a significantly greater VTA-DA cell density compared to BA males.
DA transmission from VTA to nucleus accumbens is critical for motivated behaviors (Baik, 2013; Di Chiara et al., 2004; Goto and Grace, 2005; Nestler and Carlezon, 2006; Volkow, Wise and Baler, 2017¸ Wise and Rompre, 1989) and OXT can enhance DA flow in the mesolimbic pathway via OTR, abundantly present, although not significantly, on the DA neurons of AP mice.
Recently, the group of Vanderschuren, (Verharen et al., 2018) linked the hyperactivity of ascending projections from the VTA to the nucleus accumbens and prefrontal cortex to impaired flexible decision making, in rats. Such increased forebrain dopamine signaling is peculiar of aberrant behaviors seen in substance abuse and gambling problem. Furthermore, the VTA activation under high naturalistic conflict scenarios, measured by functional magnetic resonance, has been used for discriminating between approach/avoidance personality profiles also in humans (Gonen et al., 2016), corroborating the relationship between individual differences in response to A/A conflict and the mesostriatal pathway.

AP fathers were not different from BA in paternal care and pup-retrieval, and nor even "paternal via maternal" care modifications were observed.

Interestingly, despite the lack of altered biparental care, the offspring of AP fathers were significantly faster and more advancing in response to A/A conflict and were more willing to explore the aversive open arm of the EPM compared to the offspring of both AV and BA fathers.

This behavioral pattern of greater advancing response to reward, despite negative contingencies, and reduced anxiety in the exploration of novel environment, adapts well with the characterization of approaching/seeking traits described by Alcaro and Panksepp (2011).

Here I demonstrated that spontaneously occurred individual differences in A/A paternal phenotype can influence A/A dispositions in descendants.

Recently, some authors have begun to focus on the transmission of acquired or spontaneous traits via the paternal line, and paternal transgenerational effects have been linked to epigenetic mechanisms as DNA methylation and small non-coding RNAs in the sperm (Alter et al., 2009; Chen et al., 2016; Gapp et al., 2014; Pang et al., 2017; reviewed in Braun and Champagne, 2014 and Yeshurun and Hannan, 2018). In humans, a significant correlation between fathers' and sons' methylation of the dopamine transporter (DAT) gene and their risk for psychological problem (*i.e.*, somatic complaint, internalizing and attention problems and withdraw), has been recently demonstrated (Cimino et al., 2018). Such a finding encourages to speculate on the possible involvement of epigenetic mechanisms also in the transmission of the phenotype here observed.

On the other side, no specific effects of maternal A/A phenotype on descendants' response to A/A conflict were observed. AV females, characterized by lower levels of VTA-DA neurons compared to controls, influenced descendants in a sex-dependent manner.

Notably, maternal phenotype affected response to novelty only in females (female offspring of AV mothers showed a blunted response to novelty compared to the daughters of AP mothers) and latency and anxiety levels only in males (sons of AP mothers were faster at the A/A Y-Maze S2 and less anxious compared to controls).

Sex-specificity of maternal effects was demonstrated also regarding transgenerational epigenetic programming (Dunn et al., 2011; Gabory et al., 2009; Vigé et al., 2008). Sex-chromosomal and sex-determining genes together with sex differences in placental gene expression patterns can interact with maternal entails giving rise to multifaceted effects.

8 Conclusion and outlooks

Individual differences in response to the A/A conflict occur even within inbred strain of mice and influence the A/A motivation and anxiety levels of the subsequent generation basing on paternal *vs.* maternal line and offspring sex.

A specific intergenerational effect, not mediated by obvious altered parental care, was found from AP fathers to descendants.

Influences from parents to offspring can occur at several levels. Genetic inheritance, social/behavioral transfer, epigenetic germline and somatic transmission could all be involved in the transmission of traits and vulnerability to disease across generations (Jirtle and Skinner, 2007; Richards, 2006). In addition, maternal effects can occur during pregnancy and lactation (Liu et al, 2014; Mitchell et al., 2016) and the gestational maternal environment could normalize/exacerbate a gametically programmed phenotype.

A blend of all these components together increases the complexity of phenotypic trajectories across generations and account for pleiotropic parental effects (Mitchell et al., 2016).

In the present work parents and offspring cohabited and it is not possible to disentangle the single mechanisms of transmission accounting for the results observed.

To clearly understand the way of transmission of the AP phenotype, cross-fostering studies, germ cells analysis and assisted reproductive techniques (ARTs, as in vitro fertilization -IVF or embryo transfer) are required.

Understanding these pathway could help in outlining windows for interventions at many levels.

The epigenome is indeed plastic and sensitive to parental effects (Stroud et al., 2017) not only on the vulnerability-side, but also in response to positive experiences, as shown for instance by the effectiveness of environmental enrichment in protecting from the intergenerational transmission of adverse consequences of stress (Gapp et al., 2016).

The present findings on the transmission of spontaneous A/A individual differences in healthy mice lay the foundations to understand and intervene on phenotypes potentially at risk even before disorders associated with an aberrant processing of positive and negative stimuli, and their conflict, becomes evident.

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Supplementary materials

S1 Antibodies' details

Table 1S. Antibodies' details. Table shows the details (Target, Code, Host, Dilution and Brand) of Primary and Secondary antibodies used.

Primary antibody

Target	Code	Host	Dilution	Brand
Anti-Tyrosine Hydroxylase	MAB318	Mouse	1:700	Millipore
Oxytocin	PS38	Mouse	1:1000	Gift from Dr. Hal Gainer
OTR	AVR-013	Rabbit	1:1000	Alomone lab

Secondary antibody

Target	Code	Host	Dilution	Brand
Anti-Mouse Cy2	715-225-150	Donkey	1:200	Jackson Immunoresearch
Anti-Rabbit Cy5	111-175-144	Goat	1:200	Jackson Immunoresearch

S2 Estrous phase determination

Determination of the estrous phase was performed after behavioral testing by direct microscopic evaluation of vaginal smears (Cora et al., 2015; Goldman et al., 2007)

Procedure: Vaginal smears were collected through a micropipette gently inserted into the vaginal orifice at a depth of approximately 1–2 mm rinsed with deionized water and mounted on slides. The detection of estrous phase was conducted by the microscopic evaluation (Zeiss optical microscope, 20x) of the types of cells present in the unstained wet mounted vaginal smears of mice. The stages of estrous cycle (proestrus, estrus, metestrus, and diestrus) were defined by the absence, presence, or proportion of four basic cell types as well as by the cell density and arrangement of the cells on the slide (*i.e*., neutrophils; small nucleated epithelial cells; large nucleated epithelial cells; anucleated keratinized epithelial cells).

ESTROUS CYCLE PHASES

The analysis of variance revealed no significant effect of cycle phase on female A/A conflict index ($F = 0.244$, $df = 3$, $p = .865$; $N = 83$).

S3 Tables

Table 2S. Variable measured in different tests in males and females. Table shows mean values (μ) the standard deviations (σ) in Males and Females. From top to bottom (rows):
Test = Approach/Avoidance Y-Maze (Y); Open Field with novel object (OF); Elevated Plus Maze (EPM).
Parameter = white arm choices (wc) (number) and latency (lat) (seconds) in Session 1 (S1); Session 2 (S2); Approach/Avoidance conflict index (A/A index); Periphery and center of the arena (percentage values); distance (cm); velocity (cm/sec); novel object (Obj) duration (dur) (seconds); frequency (freq) (number); latency (lat) (seconds).

Test	Parameter	Males		Females	
		μ	σ	μ	σ
Y	S1 wc	3.51	1.02	3.63	1.01
	S2 wc	3.98	1.36	4.03	1.28
	S1 lat	14.13	10.35	15.59	12.17
	S2 lat	18.86	17.01	25.5	23.89
	A/Aindex	0.47	1.77	0.40	1.56
OF	Periphery	53.68	17.62	53.16	21.14
	Center	7.17	4.67	8.62	6.28
	Distance	4902.54	1011.45	4104.25	913.05
	Velocity	9.28	1.67	7.74	2.58
	Obj dur	135.37	36.03	121.35	52.89
	Obj freq	52.94	13.51	51.46	15.45
	Obj lat	7.25	7.27	15.52	20.36
EPM	Open dur	29.91	22.3	20	15.87
	Open freq	16	3.31	4	3.5
	Closed dur	178.46	68.38	192.36	57.16
	Closed freq	8.38	1.63	8.38	1.63

Table 3S. A/A Y-Maze latency among phenotypes. Tables show mean values (μ) and standard deviations (σ) of latency to enter the white arm in male (A) and female (B) avoiding (AV), balancing (BA) and approaching (AP) mice, during the Session 1 (S1) and Session 2 (S2) of the Approach/Avoidance Y-Maze. Values are expressed in seconds.

A Males	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
S1	17.62	8.62	15.57	9.73	6.87	3.13
S2	13.18	5.16	20.64	11.7	9.53	3.71

B Females	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
S1	9.6	4.83	14.58	10.41	20.42	15.85
S2	31.8	23.77	29.18	37.34	40.98	47.33

Table 4S. Sex effect on A/A Y-Maze latency.
Table shows non-significant p-values obtained comparing by Mann-Whitney-Wilcoxon tests male and female latency at the Y-Maze of avoiding (AV), balancing (BA) and approaching (AP) mice during the Session 1 (S1) and Session 2 (S2).

	S1	S2
AV	.4	.4
BA	.936	.937
AP	.087	.114

Table 5S. Open Field parameters among phenotypes. Tables show mean values (μ) and standard deviations (σ) of time spent in arena periphery and arena center (percentage); distance traveled (cm); velocity (cm/sec); time spent contacting the novel object, expressed in seconds (Obj dur); frequency of novel object contact, expressed in number (Obj freq); latency of contact with the novel object (Obj lat), expressed in seconds, in male (A) and female (B) avoiding (AV), balancing (BA) and approaching (AP) mice.

A Males	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
Periphery	57.16	55.71	47.87	24.26	57.18	15.13
Center	4.52	1.45	7.96	4.01	8.15	6.36
Distance	5667.1	399.4	4291.8	1071.5	5003.5	947.4
Velocity	10.69	1.44	8.82	1.64	8.79	1.51
Obj dur	126.85	33.11	140.83	37.3	135.59	41.86
Obj freq	52.25	10.34	49.67	10.54	56.67	18.51
Obj lat	6.67	5.26	6.89	6.85	8.01	9.69

B Females	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
Periphery	65.92	16.13	46.57	22.91	53.47	22.1
Center	4.33	5.3	9.17	4.83	11.01	7.82
Distance	3675	276.6	4338.9	1285	4074.1	482
Velocity	6.14	0.45	8.93	3.48	7.17	0.69
Obj dur	110.6	37.65	139.55	48.02	102.08	71.6
Obj freq	43	6	57.17	21.21	49.25	6.18
Obj lat	30.93	24.49	11.02	23.04	10.71	8.06

Table 6S. Phenotype effect on Open Field parameters. Table shows non-significant p-values obtained comparing by Kruskal-Wallis tests avoiding, balancing and approaching mice within males and females. Parameters considered = percentage of time spent in arena periphery and arena center; distance traveled; velocity; time spent contacting the novel object (Obj dur); frequency of novel object contact, (Obj freq); latency of contact with the novel object (Obj lat).

	Males	Females
Periphery	.677	.4459
Center	.448	.306
Distance	.078	.735
Velocity	.151	.118
Obj dur	.990	.561
Obj freq	.713	.409
Obj lat	.962	.203

Table 7S. Sex effect among phenotypes on Open Field parameters. Table shows non-significant p-values obtained comparing by Mann-Whitney-Wilcoxon tests male and female avoiding (AV), balancing (BA) and approaching (AP) mice. Parameters considered = percentage of time spent in arena periphery and arena center; distance traveled; velocity; time spent contacting the novel object (Obj dur); frequency of novel object contact (Obj freq); latency of contact with the novel object (Obj lat).

	AV	BA	AP
Periphery	.628	.937	.914
Center	.628	.8 18	.476
Distance	.057	.937	.171
Velocity	.057	1	.114
Obj dur	.857	.818	.352
Obj freq	.4	.484	.668
Obj lat	.228	.568	.589

Table 8S. Sex effect on Elevated Plus Maze parameters. Table shows non-significant p-values obtained comparing by Kruskal-Wallis tests avoiding, balancing and approaching mice within males and females.
Parameters considered = open and closed arm duration (dur); open and closed arm frequencies (freq).

	Males	Females
Open arm dur	.832	.440
Closed arm dur	.490	.662
Open arm freq	.662	.144
Closed arm freq	.255	.096

Table 9S. EPM parameters among phenotypes. Tables show mean values (μ) and standard deviations (σ) of open arm duration (Open arm) and closed arm duration (Closed arm), expressed in seconds, and of open arm frequency (Open freq) and closed arm frequency (Closed freq), expressed in number, in male (A) and female (B) avoiding (AV), balancing (BA) and approaching (AP) mice.

A Males	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
Open arm	25.25	14.11	27.22	20.67	35.72	29.65
Closed arm	186	80.51	197.8	59.88	154.08	73.13
Open freq	3.75	2.06	2.83	2.32	5.33	4.59
Closed freq	9.25	1.26	7.67	0.82	8.5	2.26

B Females	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
Open arm	12.63	16.9	17.28	18.6	29.61	6.57
Closed arm	212.9	30.9	192.6	81.3	176.46	25.43
Open freq	2.33	2.52	2.67	2.58	5.25	0.5
Closed freq	13	1.73	11	2.1	14	2.71

Table 10S. Sex effect among phenotypes on EPM parameters. Table shows p-values obtained comparing by Mann-Whitney-Wilcoxon tests male and female avoiding (AV), balancing (BA) and approaching (AP) mice. Parameters considered = open arm duration (Open arm) and closed arm duration (Closed arm); open arm frequency (Open freq) and closed arm frequency (Closed freq). In bold, significant p-values.

	AV	BA	AP
Open arm	.4	.393	1
Closed arm	.857	1	.914
Open freq	.592	.805	1
Closed freq	**.047**	**.033**	**.013**

Table 11S. Variable measured in fathers and mothers. Table shows mean values (μ) and standard deviations (σ) in fathers and mothers. From top to bottom (rows):
Test = undisturbed parental care observation (PCO); retrieval test (RTV). Parameter = pups contact duration (Pups dur) (seconds), pups contact's frequency (Pups freq) (number); latency to reach separated pups (seconds); latency to retrieve all pups to the nest (Rtv all) (seconds), time spent in digging, self-grooming (seconds) and rearing (number).

Test	Parameter	Males		Females	
		μ	σ	μ	σ
PCO	Pups dur	1245.53	686.14	1354.92	526.14
	Pups freq	5.82	4.85	9.73	11.06
RTV	First pup	7.71	14.17	3.19	2.4
	Rtv all	566.45	119.24	145.5	212.7
	Pups dur	214.25	103.13	249.17	120.59
	Digging	71.4	39.44	130.06	104.11
	Grooming	11.21	15.81	1.39	2.28
	Rearing	64.83	18.9	38.11	18.92

Table 12S. Undisturbed Parental Care Observation. Table shows mean values (μ) and standard deviations (σ) of pups contact duration (seconds) and frequency (number) of avoiding (AV), balancing (BA) and approaching (AP) mothers and fathers.
Table also shows indirect effect: BA mothers paired with AV (BA♀AV♂), BA (BA♀BA♂), AP (BA♀AP♂) fathers and BA fathers paired with AV (BA♂AV♀), BA (BA♂BA♀), AP (BA♂AP♀) mothers (D).

		Duration		Frequency	
		μ	σ	μ	σ
mothers	AV	1578.39	329.18	5.67	3.79
	AP	1416.47	586.85	7	7.35
fathers	AV	1239.24	959.86	9	9.54
	AP	1193.69	668.15	6.17	4.22
BA mothers	BA♀AV♂	1395.64	519.66	22.33	28.36
	BA♀BA♂	1169.47	613.79	10.5	5.09
	BA♀AP♂	1367.23	593.52	6.5	1.97
BA fathers	BA♂AV♀	1789.14	13.95	3.33	0.58
	BA♂BA♀	939.97	664.64	5.17	4.45
	BA♂AP♀	1378.64	830.42	5.75	4.86

Table 13S. Sex-effect among phenotypes at the PCO. Table shows non-significant p-values obtained comparing by Mann-Whitney-Wilcoxon tests avoiding (AV), balancing (BA) and approaching (AP) male and female mice in time spent in pup-directed behaviors (Pups contact) and pup-contact frequency (Contact freq).

	AV	BA	AP
Pups contact	1	.588	.476
Contact freq	1	.122	.913

Table 14S. RTV parameters among phenotypes. Tables show mean values (μ) and standard deviations (σ) of retrieval parameters = latency to reach separated pups (First pup), expressed in seconds; latency to retrieve all pups to the nest (Rtv all), expressed in seconds; time spent in digging, self-grooming (seconds) and frequency of rearing (number) in avoiding (AV), balancing (BA), approaching (AP) mothers (A) and fathers (B). Tables also show indirect effect: BA mothers paired with AV (BA♀AV♂), BA (BA♀BA♂), AP (BA♀AP♂) fathers (C) and BA fathers paired with AV (BA♂AV♀), BA (BA♂BA♀), AP (BA♂AP♀) mothers (D). In bold, scores that have reached the cut-off.

A	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
First pup	3.35	2.5	4.26	1.45	3.34	2.05
Rtv all	79.22	50.29	278.87	297.21	59.59	53.15
Pups dur	306.91	204.61	234.73	53.2	314.75	192.67
Digging	115.72	135.45	123.65	68.56	84.34	87.52
Grooming	1.59	1.95	1.25	1.73	0.69	1.19
Rearing	24.33	15.95	44.8	18.78	29.67	21.78

B	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
First pup	5.27	6.38	9.62	9.44	2.26	0.71
Rtv all	398.71	264.98	**600**	**0**	**600**	**0**
Pups dur	235.15	121.52	191.47	114.38	169.81	27.49
Digging	74.25	19.72	69.96	47.61	63.14	23.46
Grooming	17.3	29.23	6.75	7.58	7.95	3.57
Rearing	59.33	25.01	71.4	21.13	72.75	17.33

C	BA♀AV♂		BA♀BA♂		BA♀AP♂	
	μ	σ	μ	σ	μ	σ
First pup	1.21	0.43	4.26	1.45	3.09	4.11
Rtv all	222.31	327.13	278.87	297.21	35.32	13.48
Pups dur	252.42	21.49	234.73	53.2	172.3	98.99
Digging	89.91	28.95	123.65	68.56	210.41	159.27
Grooming	1.07	1.6	1.25	1.73	2.16	4.32
Rearing	56.67	11.68	44.8	18.78	32.5	16.11

D	BA♂AV♀		BA♂BA♀		BA♂AP♀	
	μ	σ	μ	σ	μ	σ
First pup	20.82	32.94	9.62	9.44	1.12	0.97
Rtv all	**600**	**0**	**600**	**0**	**600**	**0**
Pups dur	277.24	123.41	191.47	114.38	227.59	144.88
Digging	81.69	58.42	69.96	47.61	71.65	63.01
Grooming	22.89	26.25	6.75	7.58	5.23	5.74
Rearing	53.67	23.01	71.4	21.13	60	6.08

Table 15S. Sex effect among phenotypes on RTV parameters. Table shows p-values obtained comparing by Mann-Whitney-Wilcoxon tests male and female avoiding (AV), balancing (BA) and approaching (AP) mice. Parameters considered = latency to reach separated pups (First pup); latency to retrieve all pups to the nest (Rtv all); time spent in digging and self-grooming and frequency of rearing. In bold, significant p-values.

	AV	BA	AP
First pup	1	.420	.592
Rtv all	.4	.072	**.031**
Pups dur	1	.547	.114
Digging	1	.309	.857
Grooming	1	.204	**.049**
Rearing	.4	.055	.057

Table 16S. Immunofluorescence in males and females. Table shows mean values (μ) and standard deviations (σ) of cell density of oxytocin (OXT) in the paraventricular hypothalamic nucleus and dopamine (tyrosine hydroxylase, THY), and oxytocin receptors (OTR) density in the ventral tegmental area in males and females. Values indicates number of cells per mm^2 (OXT and THY) and receptors/mm^2 (OTR).

	Males		Females	
	μ	σ	μ	σ
OXT	21.76	11.79	18.36	5.47
THY	94.56	55.88	62.49	17.01
OTR	4.32	2.53	2.79	1.15

Table 17S. Immunofluorescence staining. Tables show mean values (μ) and standard deviations (σ) of cell density of oxytocin (OXT) in the paraventricular hypothalamic nucleus and dopamine (tyrosine hydroxylase, THY) and oxytocin receptors (OTR) density in the ventral tegmental area in avoiding (AV), balancing (BA) and approaching (AP) males (A) and females (B). Values indicates number of cells per mm^2 (OXT and THY) and receptors/mm^2 (OTR).

A Males	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
OXT	30.48	20.14	19.6	7.02	19.11	10.64
THY	58.99	26.43	67	10.51	155.69	57.64
OTR	3.21	1.18	4.01	2.07	5.73	3.87

B Females	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
OXT	14.47	5.04	18.39	4.31	22.19	6.06
THY	44.52	20.89	71.39	4.94	69.29	9.83
OTR	2.43	1.49	3.33	1.4	2.62	0.65

Table 18S. Sex effect among phenotypes on immunofluorescence. Table shows p-values obtained comparing by Mann-Whitney-Wilcoxon tests male and female cell density of oxytocin (OXT) in the paraventricular hypothalamic nucleus and of dopamine (tyrosine hydroxylase, THY) and oxytocin receptors (OTR) in the ventral tegmental area in avoiding (AV), balancing (BA) and approaching (AP) mice.

	AV	BA	AP
OXT	.4	.914	.785
THY	.7	.730	.114
OTR	.4	1	.7

Table 19S. Control of selected couples. Table shows the non-significant Kruskal-Wallis analyses run to avoid a single-couple effect on F1 results in male and female offspring A/A conflict index. In (from top to bottom, rows) both balancing (BA) parents (controls CTR) (BA♂BA♀); avoiding (AV) fathers + BA mothers (AV♂ BA♀); approaching (AP) fathers + BA mothers (AP♂ BA♀); AV mothers + BA fathers (AV♀BA♂); AP mothers + BA fathers (AP♀ BA♂).

Male offspring	p-value	Female offspring	p-value
BA♀♂ (CTR)	.367	BA♀♂ (CTR)	.415
AV♂ BA♀	.367	AV♂ BA♀	.367
AP♂ BA♀	.415	AP♂ BA♀	.391
AV♀ BA♂	.317	AV♀ BA♂	.367
AP♀ BA♂	.367	AP♀ BA♂	.391

Table 20S. **Maternal effect in male and female offspring.** Kruskal-Wallis comparisons (p-values) of son (Males) and daughters (Females) born to avoiding (AV), balancing (BA) and approaching (AP) mothers. From top to bottom (rows):
Test = Approach/Avoidance Y-Maze (Y); Open Field with novel object (OF); Elevated Plus Maze (EPM).
Parameter = white arm latency (lat) in Session 1 (S1) and Session 2 (S2); percentage of time spent in the arena Periphery and arena Center; total Distance traveled; mean Velocity; novel Object (Obj) duration (dur), frequency (freq) and latency (lat); Open and Closed arm duration (dur) and frequency (freq). In bold, significant p-values.

Test	Parameter	Males	Females
Y	S1 lat	.078	.195
	S2 lat	**.019**	.081
OF	Periphery	**.027**	.555
	Center	.889	.974
	Distance	.138	.406
	Velocity	**.007**	.081
	Obj dur	.336	**.014**
	Obj freq	.096	.136
	Obj lat	.116	.317
EPM	Open dur	**.003**	.088
	Open freq	**.034**	.067
	Closed dur	**.002**	.112
	Closed freq	**.022**	.242

Table 21S Maternal effect on female offspring behavioral parameters. Table shows mean values (μ) the standard deviations (σ) of daughters born to avoiding (AV), balancing (BA) and approaching (AP) mothers. From top to bottom (rows): Parameter = white arm latency (lat) in Session 1 (S1) and Session 2 (S2); percentage of time spent in the arena Periphery and arena Center; total Distance traveled; mean Velocity; novel Object (Obj) duration (dur), frequency (freq) and latency (lat); Open and Closed arm duration (dur) and frequency (freq).

	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
S1 lat	8.92	5.67	11.76	6.12	13.23	6.69
S2 lat	11.72	5.72	24.41	16.66	13.96	12.02
Periphery	55.36	12.5	52.32	19.82	51.46	16.13
Center	12.48	9.85	10.93	7.9	11.81	9.48
Distance	4326.3	1083.7	4654.1	979.36	4570.5	1025.84
Velocity	8.15	1.85	9.68	1.94	8.33	1.83
Obj dur	115.42	38	136.25	49.59	170.27	53
Obj freq	63.18	13.06	53.64	15.49	63.65	12.38
Obj lat	5.37	8.17	14.58	29.19	2.12	3.33
Open dur	43.01	21.59	28.25	28.3	42.16	20.45
Open freq	8.1	3.96	5.06	4.98	7.4	3.46
Close dur	160.05	32.95	197.24	56.82	161.58	56.1
Close freq	14	1.94	12.47	3.54	13.6	3.22

Table 22S. Maternal effect on male offspring behavioral parameters. Table shows mean values (μ) the standard deviations (σ) of sons born to avoiding (AV), balancing (BA) and approaching (AP) mothers. From top to bottom (rows): Parameter = white arm latency (lat) in Session 1 (S1) and Session 2 (S2); percentage of time spent in the arena Periphery and arena Center; total Distance traveled; mean Velocity; novel Object (Obj) duration (dur), frequency (freq) and latency (lat); Open and Closed arm duration (dur) and frequency (freq).

	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
S1 lat	12.42	6.15	22.39	15.4	11.88	8.76
S2 lat	14.73	8.78	16.32	12.41	6.48	3.36
Periphery	53.24	18.35	34.77	15.41	50.52	13.12
Center	10.02	6.75	8.96	5.08	8.37	5.58
Distance	3517	622.83	4235.4	1370.3	4366.5	601.7
Velocity	8.58	1.01	10.01	1.88	7.71	1.19
Obj dur	123.82	54.28	146.84	42.96	160.07	50.79
Obj freq	61.67	18.47	46.5	7.03	52.08	11.6
Obj lat	2.93	4.97	4.05	5.96	2.52	5.52
Open dur	21.29	19.18	16.17	11.71	43.41	18.69
Open freq	3.67	2.58	2.58	1.83	6.45	3.72
Close dur	181.35	31.17	227.54	35.97	147.03	51.9
Close freq	12.67	2.07	9.08	2.43	10.91	2.7

Table 23S. **Sex effect within groups, maternal effect.** Table shows p-values obtained comparing by Mann-Whitney-Wilcoxon tests male and female offspring born to avoiding (AV), balancing (BA) and approaching (AP) mothers. In bold, significant p-values.

Test	Parameter	AV	BA	AP
Y	S1 lat	.216	**.048**	.426
	S2 lat	.614	.244	**.036**
OF	Periphery	**.0006**	**.007**	**.003**
	Center	**.001**	**.0001**	.058
	Distance	**.0001**	**.00002**	.967
	Velocity	**.0001**	**.00002**	.447
	Obj dur	.660	.630	.967
	Obj freq	.880	.303	**.027**
	Obj lat	.839	.696	.447
EPM	Open dur	**.041**	.239	.798
	Open freq	**.042**	.219	.434
	Closed dur	.180	.227	.54
	Closed fre	.247	**.009**	.063

Table 24S Paternal effect in male and female offspring. Kruskal-Wallis comparisons (p-values) of son (Males) and daughters (Females) born to avoiding (AV), balancing (BA) and approaching (AP) fathers. From top to bottom (rows):
Test = Approach/Avoidance Y-Maze (Y); Open Field with novel object (OF); Elevated Plus Maze (EPM).
Parameter = white arm latency (lat) in Session 1 (S1) and Session 2 (S2); percentage of time spent in the arena Periphery and arena Center; total Distance traveled; Velocity; novel Object (Obj) duration (dur), frequency (freq) and latency (lat); Open and Closed arm duration (dur) and frequency (freq). In bold, significant p-values.

Test	Parameter	Males	Females
Y	S1 lat	**.006**	**.005**
	S2 lat	.973	.202
OF	Periphery	**.012**	.146
	Center	.618	.744
	Distance	.343	.428
	Velocity	.524	.578
	Obj dur	.319	.445
	Obj freq	**.009**	.095
	Obj lat	.789	.290
EPM	Open dur	**.0001**	**.040**
	Open freq	**.001**	**.035**
	Closed dur	.075	**.024**
	Closed fre	.145	.840

Table 25S. Paternal effect on female offspring behavioral parameters. Table shows mean values (μ) the standard deviations (σ) of daughters born to avoiding (AV), balancing (BA) and approaching (AP) fathers. From top to bottom (rows): Parameter = white arm latency (lat) in Session 1 (S1) and Session 2 (S2); percentage of time spent in the arena Periphery and arena Center; total Distance traveled; mean Velocity; novel Object (Obj) duration (dur), frequency (freq) and latency (lat); Open and Closed arm duration (dur) and frequency (freq).

	AV		BA		AP	
	μ	σ	μ	σ	μ	σ
S1 lat	17.38	11.32	11.76	6.12	6.9	3.31
S2 lat	22.1	13.9	24.41	16.66	13.45	6.04
Periphery	42.67	11.01	52.32	19.82	52.9	9.36
Center	10.85	7.42	10.93	7.9	8	4.16
Distance	4512.8	378.94	4654.1	979.36	5126.8	1238.9
Velocity	8.86	1.56	9.68	1.94	9.29	1.87
Obj dur	166.95	51.34	139.61	56.17	153.91	69.41
Obj freq	63.3	14.95	54.28	13.92	64.83	19.63
Obj lat	5.01	7.38	14.2	26.14	9.53	9.65
Open dur	21.34	20.86	27.64	27.58	49.75	29.44
Open freq	3.9	3.84	4.94	4.86	8.2	4.59
Close dur	211.79	53.11	197.59	55.15	151.78	36.59
Close freq	12.8	4.44	12.44	3.43	13.1	3.31

Table 26S. Paternal effect on male offspring behavioral parameters. Table shows mean values (μ) the standard deviations (σ) of sons born to avoiding (AV), balancing (BA) and approaching (AP) fathers. From top to bottom (rows): Parameter = white arm latency (lat) in Session 1 (S1) and Session 2 (S2); percentage of time spent in the arena Periphery and arena Center; total Distance traveled; mean Velocity; novel Object (Obj) duration (dur), frequency (freq) and latency (lat); Open and Closed arm duration (dur) and frequency (freq).

	AV		BA		AP	
Parameter	μ	σ	μ	σ	μ	σ
S1 lat	25.02	11.09	22.39	15.4	12.67	9.08
S2 lat	15.89	13.3	16.32	12.41	14.07	10
Periphery	37.85	11.39	34.77	15.41	49.13	13.55
Center	11.94	7.35	8.96	5.08	10.11	3.33
Distance	4813.1	1918.8	4235.4	1370.3	5152.3	1406.1
Velocity	11.49	4.64	10.01	1.88	10.11	3.33
Obj dur	130.28	57.66	146.84	42.97	170.33	63.31
Obj freq	58.33	13.38	46.5	7.03	58.1	11.02
Obj lat	4.03	7.07	4.05	5.96	6.34	12.57
Open dur	11.7	10.9	16.17	11.71	38.82	17.55
Open freq	1.89	1.45	2.58	1.83	5.65	3.36
Close dur	226.51	46.33	227.54	35.97	141.87	55.67
Close freq	10.89	2.2	9.08	2.43	11.05	3.1

Table 27S. **Sex effect within groups, paternal effect.** Table shows p-values obtained comparing by Mann-Whitney-Wilcoxon tests male and female offspring born to avoiding (AV), balancing (BA) and approaching (AP) fathers. In bold, significant p-values.

Test	Parameter	AV	BA	AP
Y	S1 lat	.094	**.048**	**.015**
	S2 lat	.156	.244	.726
OF	Periphery	.356	**.017**	.424
	Center	.719	.723	.255
	Distance	.660	.368	.687
	Velocity	.315	.490	.923
	Obj dur	.315	.631	.604
	Obj freq	.487	.112	.101
	Obj lat	.835	.296	.311
EPM	Open dur	.252	.260	.248
	Open freq	.134	.237	.114
	Closed dur	.660	.2	.845
	Closed fre	.386	**.007**	.149

S4 Figures

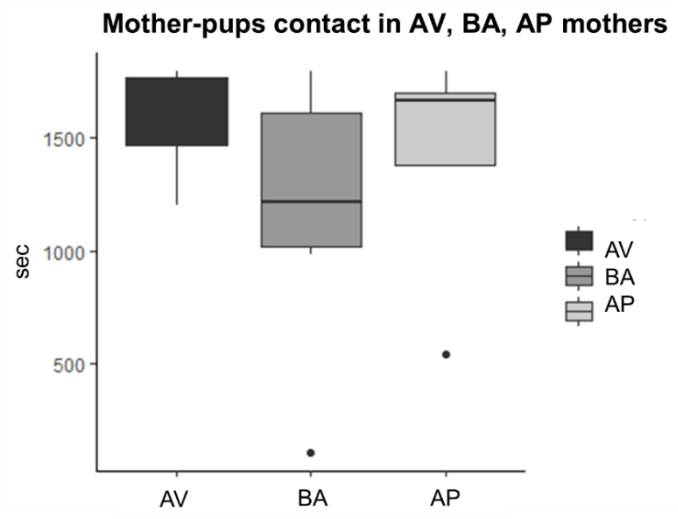

Fig. 1S: Undisturbed parental care. Maternal effect. Boxplots indicate time spent in pup-directed behaviors (seconds) in avoiding (AV), balancing (BA) and approaching (AP) mothers coupled with BA mates, during 30-min undisturbed observation.

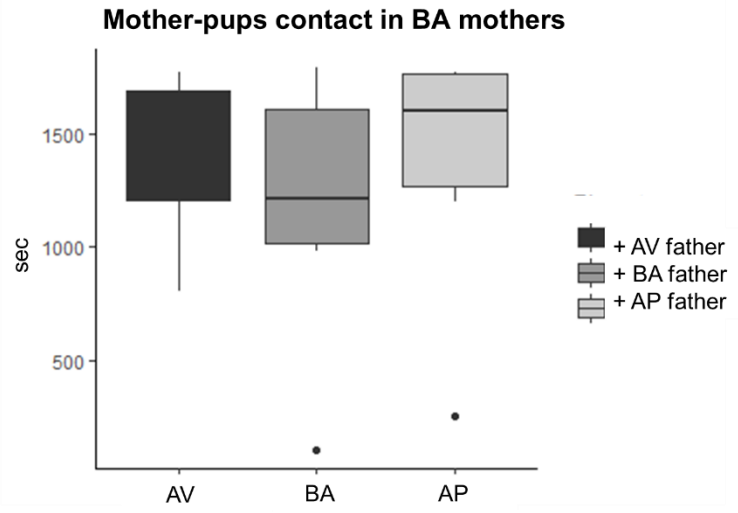

Fig. 2S: Undisturbed parental care. Maternal effect. Boxplots indicate time spent in pup-directed behaviors (seconds) in balancing (BA) mothers coupled with avoiding (AV), BA and approaching (AP) mates, during 30-min undisturbed observation.

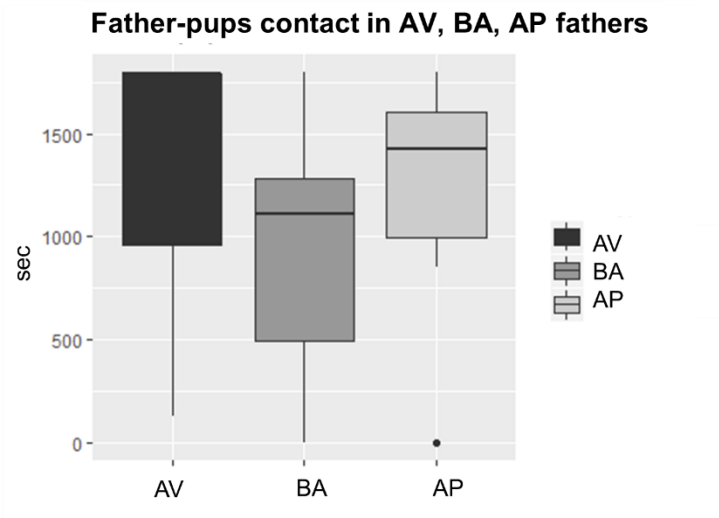

Fig. 3S: Undisturbed parental care. Paternal effect. Boxplots indicate time spent in pup-directed behaviors (seconds) in avoiding (AV), balancing (BA) and approaching (AP) fathers coupled with BA mothers, during 30-min undisturbed observation.

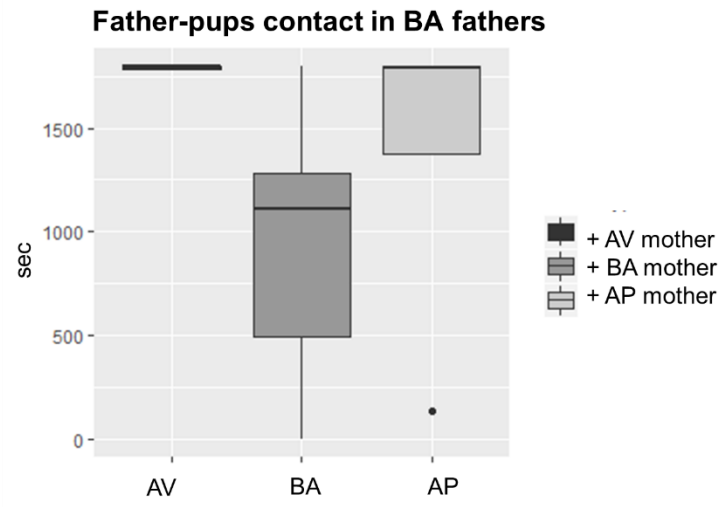

Fig. 4S: Undisturbed parental care. Paternal effect. Boxplots indicate time spent in pup-directed behaviors (seconds) in balancing (BA) fathers coupled with avoiding (AV), BA and approaching (AP) mothers, during 30-min undisturbed observation.

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It ain't what you don't know that gets you into trouble.
It's what you know for sure that just ain't so.

Mark Twain