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Exploration and Emotional Behavior in Conditional NPY1r KO Mice in Relation to Sex and Early Maternal Environment: a Comprehensive Analysis by Principal Components

Esplorazione e comportamento emozionale in topi KO condizionali per NPY1r in relazione al sesso e all'ambiente materno precoce: un'analisi mediante componenti principali

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ABSTRACT

NPY1r has been largely studied for its capability not only to stimulate appetite-related behaviors, but also to modulate emotional behaviors and stress response. In humans and other animals, NPY1r appears to be involved in some psychopathological disorders such as Major Depression and Post-Traumatic Stress Disorder. The conditional NPY1r knockout mouse model can be a powerful tool to understand the behavioral response to stressful events and the role of this receptor in psychopathological disorders, as well as possible gene-environment interactions since a potential involvement of maternal care in the regulation of NPY expression has been hypothesized.

In the present study, knockout and wild type mice were fostered at birth to one of four different lactating dams' strains characterized by different levels of maternal cares and as adults, were subjected to the Open Field test; some of them, following a stress event (pregnancy for females and Social Stress test for males), underwent a second test session. Mouse behaviors and other data, such as mean velocity or time spent in different areas of the arena, were analyzed. 367 mice from 9 different cohorts were analyzed.

Principal component analysis was conducted with the aim of reducing the dimensionality of the dataset; remarkably, in line with our expectation, all considered dependent variables were grouped in five principal components accordingly to the data's covariance matrix, resulting in groupings in accordance with their inherent characteristics (one category for anxiety-like behaviors, one for vertical exploration, one for general exploration/activity, etc.).

Firstly, by means of a screening of the wild type population, strong sex effects were found, with females generally being more exploratory and less anxious than their male counterparts. This effect was also observed in relation to the foster dams' strain: females reared by CD1dams were more exploratory than males, whereas mice reared by C57, FVB or BALB/c

strains did not show such a sex difference. In a second analysis that took into account the effect of genotype, knockout mice were less exploratory and more anxious than wild type mice, and generally this was not related to sex or foster dams' strain.

Under present conditions, our results showed how early maternal environment and NPY1 receptor are involved in the expression of anxiety-like behaviors. These results may help to better understand the etiopathogenesis of anxiety-related psychopathological disorders and help to develop future therapies to reduce psychopathological disorders.

ABSTRACT (Italiano)

NPY1r è stato ampiamente studiato per via della sua capacità non solo di stimolare comportamenti appetitivi, ma anche di modulare comportamenti emozionali e risposte stressogene. Nell'essere umano e anche in altri animali, NPY1r sembrerebbe essere coinvolto in alcuni disturbi psicopatologici come la depressione maggiore ed il disturbo da stress post-traumatico. Lo studio del modello murino knockout condizionale per NPY1r può essere un valido strumento per comprendere la risposta comportamentale ad eventi stressogeni e il ruolo di questo recettore in disturbi psicopatologici, così come la possibile interazione tra il gene e l'ambiente dato che è stato ipotizzato un potenziale coinvolgimento delle cure materne nella regolazione dell'espressione di NPY.

In questo studio, topi knockout e wild type sono stati adottati alla nascita da uno dei quattro ceppi di madri allattanti caratterizzati da diversi livelli di cure materne e da adulti, sono stati sottoposti al test dell'Open Field, ed alcuni di essi sono stati sottoposti una seconda volta al test a seguito di un evento stressogeno (per le femmine la gravidanza ed i maschi la procedura di Social Stress). Sono stati analizzati vari comportamenti dell'animale e altri dati come la velocità media e il tempo speso nelle diverse aree dell'arena. Sono stati analizzati 367 topi di 9 coorti diverse.

È stata condotta un'analisi delle componenti principali con l'obiettivo di ridurre la dimensionalità del dataset; in linea con le nostre aspettative, le variabili dipendenti sono state accorpate in cinque componenti principali sulla base della matrice di covarianza dei dati di partenza, risultando raggruppate in accordo con le loro caratteristiche intrinseche (una categoria per comportamenti simil-ansiosi, una per esplorazione verticale, una per esplorazione/attività generale, ecc).

In primo luogo, attraverso lo screening sui wild type si sono evidenziati forti effetti del sesso, per cui le femmine, in generale, risultavano essere maggiormente esplorative e meno

ansiose dei maschi. Questo effetto si è presentato anche in relazione all'ambiente materno precoce: le femmine adottate da CD1 risultavano più esplorative dei maschi, mentre topi adottati dai ceppi C57, FVB o Balb/c non hanno mostrato una tale differenza sessuale. In una seconda analisi che ha preso in considerazione anche l'effetto del genotipo si è osservato come i topi knockout siano stati meno esplorativi e più ansiosi rispetto ai wild type; in generale non è risultato essere in relazione al sesso oppure all'ambiente materno precoce.

Al momento, i nostri risultati dimostrano come l'ambiente materno precoce ed NPY1r siano coinvolti nell'espressione di comportamenti simil-ansiogeni. Questo tipo di studi può aiutare a comprendere maggiormente l'eziopatogenesi di disturbi psicopatologici legati all'ansia e contribuire allo sviluppo di future terapie per ridurre questi disturbi.

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1 INTRODUCTION

1.1 Neuropeptide Y

Neuropeptide Y (NPY) is a peptide composed of 36 amino acids, first isolated in the pig brain (Tatemoto et al., 1982). Not only is the neuropeptide present in mammals but also in invertebrates, showing that it has been conserved throughout evolution. NPY together with peptide YY (PYY), and pancreatic polypeptide (PP) belong to the pancreatic peptide family. NPY appears to be mostly present in the central nervous system (CNS), in particular, the limbic system and hypothalamus and appears to be involved in various functions including vasoconstriction, appetite stimulation, circadian rhythm, pain, anxiety, and adrenal hormone release (Baraban et al., 1997; Colmers and Bleakman, 1994; Stanley et al., 1993; Wahlestedt and Rei, 1993).

NPY, PYY, and PP peptides interact with a family of protein-G-coupled receptors belonging to the rhodopsin class 1 receptor-like superfamily. The NPY system is a rather complex network, interacting with a family of receptors termed "Y receptors" (Larhammar et al., 2001; 2004; Michel et al., 1998)

In mammals, the biological action of NPY is mediated by five receptors (Y1, Y2, Y4, Y5, y6), although as many as eight have been described in vertebrates (Sundstrom G. et al., 2013). The Y6 receptor does not act, though, in all mammals, including rats and humans. NPY shows higher affinity for Y1, Y2 and Y5. The Y1 and Y2 receptors are diffusely arranged in various parts of the brain, including the frontal cortex, lateral septum, *nucleus accumbens*, in the bed of the nucleus stria terminalis, arcuate nucleus, the paraventricular nucleus of the hypothalamus, amygdala, hippocampus, *nucleus tractus solitarius* and in the area postrema. The Y5 receptor also acts in various regions of the limbic system but less pronounced than Y1 and Y2; in many cases the expression of this receptor coincides with the expression of Y1.

Finally, the Y4 receptor is expressed in only a few brain areas, including the medial preoptic area, paraventricular nucleus of the hypothalamus, nucleus of the solitary tract and area postrema.

1.2 NPY subtypes

In mammals, five receptor subtypes have been cloned for NPY (Y1, Y2, Y4, Y5, y6). The Y1 receptor (Y1r), Y2 receptor (Y2r) and Y5 receptor (Y5r) preferentially bind NPY and PYY, while the Y4 receptor (Y4r) shows selectivity toward PP. The Y3 receptor subtype was postulated based on pharmacological studies in mammalian tissues. The y6 receptor was assigned this lowercase nomenclature (IUPHAR) because the receptor protein is truncated in most mammals, including humans (Michel et al., 1998). The y6 receptor is functional in mice and rabbits.

In mammals, the five Y receptors for NPY can be divided into three distinct subfamilies based on their amino acid homology: Y1, Y2, Y5 (Larhammar et al., 2001):

- The Y1 subfamily includes the Y1, Y4, and y6 receptors;
- The Y2 subfamily includes the Y2 receptor and the Y7 receptor (found in some fish and frogs);
- The Y5 subfamily includes only the Y5 receptor.

1.2.1 Y1

The first Y receptor to be cloned was the Y1r subtype (Eva et al., 1990; Herzog et al., 1992; Krause et al., 1992; Larhammar et al., 1992). Studies in several mammalian species have revealed a peculiar feature of this receptor; Y1r is able to bind active forms of both PPY and NPY, but not the truncated forms (Berglund et al., 1999). Y1r requires the intact NPY or PPY

peptide for its function, as truncated peptides result in rapid loss of affinity for the receptor (Larhammar and Salaneck, 2004).

Y1r is a 384-amino acid peptide; of all the NPY receptors, it is the one on which studies have primarily focused, particularly because of its ability to stimulate feeding behavior (Kanatani et al., 2000; Kask et al., 1998; Wisialoski et al., 2000), inhibit nociception (Naveilhan et al., 2001; Zhang et al., 1994), regulate hormone secretion (Kalra et al., 1992), and modulate emotional behavior and stress response (Broqua et al., 1995; Heiling, 1995). The Y1 receptor is found expressed in many thalamic, hypothalamic, amygdala, and hippocampal nuclei of mice and rats (Eva et al., 2006), and is mainly localized at the postsynaptic level. However, Y1 is not only present centrally, but also peripherally in various tissues such as heart, kidney, and gastrointestinal tract (Aakerlund et al., 1990). The physiological effects of Y1r were initially discovered through pharmacological studies, using selective agonists and antagonists with high binding affinity for this receptor subtype.

The use of Y1r knockout (KO) animals has provided a viable alternative to the pharmacological approach (Lin et al., 2004). The most common actions of this receptor subtype are inhibition of adenylate cyclase, through binding to the pertussis toxin-sensitive G_i/G_0 GTP protein, mobilization of Ca^{2+} from intracellular reserves (Herzog et al, 1992), stimulation of the cascade of events leading to activation of "mitogen-activated protein kinase" (MAPK), inducing phosphorylation of "extracellularly regulated kinase" (ERK), an effect that has been shown to be dependent on the action of "PI-3-kinase" (Mannon et al.,2000, Nie et al., 1998). Several experimental data support a central role of the Y1 receptor in the development of obesity: pharmacological blockade of the Y1 receptor in rodents leads to significant reduction in food intake, as does the administration of antisense oligodendrocytes against Y1 in the ventromedial hypothalamus in rats (Lopez-Valpuesta et al., 1996). In addition, in mice chronic activation of

the receptor by NPY or by receptor agonists for 6 days results in significant increase in body weight and lipid accumulation, and reduction in lipid oxidation (Henry et al., 2005).

1.2.2 Y2

The Y2 subtype, a 381 amino acid peptide, was first described in 1986 only pharmacologically as a receptor with predominant affinity for NPY and PPY peptides (Wahlestedt, 1986) in the presynapse, where it causes suppression of neurotransmitter release, only later it was cloned in humans (Gerald et al., 1995; Lundell et al., 1995). The Y2 receptor is expressed primarily in the hippocampus, hypothalamus, amygdala, and specific brainstem nuclei (Parker and Herzog, 1999; Dumont et al., 1998). The receptor is involved in numerous physiological functions such as angiogenesis, vasoconstriction, circadian rhythms, gastric emptying, and control of emotional behavior and stress (Heiling, 2004). The Y2 receptor, expressed in the arcuate nucleus of the hypothalamus, has recently gained considerable attention as an appetite inhibitory receptor (Batterham et al., 2002, 2003).

1.2.3 Y4

The second member of the Y1 subfamily is the Y4 subtype (Y4r), which has a higher affinity for the PP peptide (Bard et al., 1995; Gregor et al., 1996) than the NPY and PYY peptides. This affinity is more pronounced in rat and mouse than in other mammalian species, suggesting that there was a more rapid evolution of PP and Y4 in rodents (Larhammar, 1996; Berglund et al., 2001). In humans, in vivo studies conducted with PP hint that Y4 might have a reducing effect on appetite (Betterham et al., 2003).

In situ hybridization and immunohistochemistry techniques have shown that Y4r is expressed primarily in the lateral hypothalamic area, particularly in orexin neurons (Campbell et al., 2003). In Campbell's studies, it is observed that both PP and NPY result in increased food

and water intake when administered directly in the lateral hypothalamic area. In contrast, overexpression of PP peripherally, reduces body weight and adiposity in association with reduced food intake and reduced gastric emptying rate in mice (Ueno et al., 1999). These results suggest that PP, an endogenous ligand for Y4r, represents an anorexigenic signal in the periphery and an orexigenic signal in the central nervous system (Katsuura et al., 2002).

1.2.4 Y5

The discovery of the Y5 receptor was anticipated by several publications describing the possible existence of a receptor involved in appetite stimulation, a receptor different from Y1 (Gerald et al., 1996; Herzog et al., 1997). Its amino acid sequence is related to that of Y1 and Y2 receptors and, although it binds the same ligands, it has an amino acid identity with them of only 30%. The pharmacological peculiarity of Y5 is that it has a high affinity for the truncated forms (the first two amino acids are absent) of the NPY and PYY peptides but has a low affinity toward the shorter fragments (Michel et al, 1998).

Through in situ hybridization studies, we now know that the Y5 receptor is predominantly expressed in the hypothalamic nuclei, dentate gyrus, CA3 area of the hippocampus, cingulate cortex and amygdala (Dumont et al, 1998; Gerald et al, 1996). As mentioned above, Y5r has similar effects to the Y1 receptor; it stimulates food intake (Marsh et al., 1998), causes anxiolytic-like effects in many models of anxiety (Carvajal et al., 2006), causes sedation (Sorensen et al., 2004), and regulates GABA release (Pronchuk et al., 2002). Pharmacological and genetic evidence suggests that the Y5 receptor plays a key role in inhibiting excitatory neurotransmission in mice, including in the CA3 region.

1.2.5 Y6

Y6 was named after in vitro characterization showed that it was a different receptor subtype from Y1 (Borowsky et al., 1998). According to IUPHAR (International Union of Basic and Clinical Pharmacology) nomenclature, its name should be y6 (with a lowercase y), since no physiological activity has been demonstrated, but Larhammar decided to consider it on par with the other subtypes and write it with a capital Y (Y6). The y6 receptor is encoded by a pseudo gene in humans and some primates (Gregor et al., 1996) and is also nonfunctional in other rodents, such as the guinea pig (Wraith et al., 2000). However, a functional y6 has been discovered in a distant relative of the swine, the peccary (Larhammar et al., 2004). This raises the question of whether the y6 gene is nonfunctional in all mammals and thus lost its function in mammalian ancestors or whether it was inactivated independently in different mammalian genera. Although y6 is not important in any mammals, it clearly has an ancient evolutionary origin, as sequences have been cloned in several animal species such as chickens, frogs, and spiny dogfish (Salaneck and Larhammar, 2003).

1.3 Y1 receptor

As mentioned above, Y1r was the first receptor for NPY to be cloned in the rat; it was later isolated in humans and mice as well. Overall, this receptor appears to be largely conserved during evolution (Larhammar and Salaneck, 2004).

Y1r is primarily located at the postsynaptic level, although in some cases it can also be found at pre-synaptic sites. Several studies have analyzed its localization in the CNS of different mammals, finding medium to high levels of binding sites for Y1r in the cerebral cortex, hippocampus (with higher density in the dentate gyrus), mamillary nucleus, geniculate nucleus, and nucleus tractus solitarius (NST). It has also been found at low concentrations in the septum, cerebellum, and paraventricular (PVN) and dorsomedial (DMH) nuclei of the hypothalamus.

More recent studies in rats and mice have found higher amounts of positive nuclei, particularly in the thalamus, limbic system (hippocampus, amygdala and terminal stria bed) and hypothalamus (medial preoptic area, ventromedial and arcuate nucleus) (Figure 1).

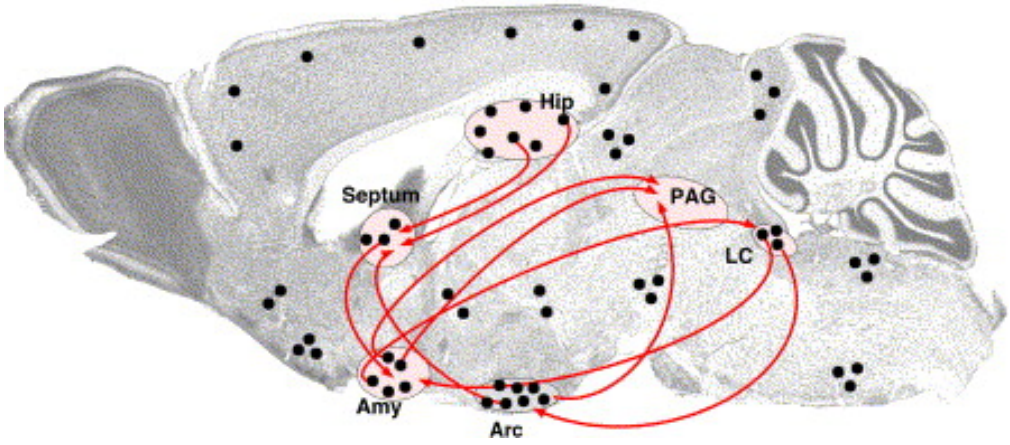


Figure 1. Brain NPY pathways thought to be involved in NPY-effects related to stress and emotionality (modified from Eva et al., 2006).

1.4 NPY in Human psychopathology

Y1r would appear to be the central focus of NPY effects on anxiety mechanisms. Amygdala is strongly implicated in several psychiatric disorders such as depression, post-traumatic stress disorder, anxiety, epilepsy, and eating disorders, and it is in this brain region that the anxiolytic effect of the neuropeptide is recorded.

Given the results obtained through the animal model regarding the role of NPY in affecting anxiety-like behaviors, several authors sought to see how NPY is distributed in human brains and how it is involved in some psychiatric disorders closely related to anxiety and stress (see chapter 1.4.1 and 1.4.2).

Adrian and colleagues (1983) by mean of radioimmunology and immunocytochemistry techniques investigated the distribution of NPY in the human brain. Brains were obtained *post-mortem* from patients with no prior history of psychopathology. A large concentration of NPY

was recorded at the basal ganglia, in the amygdala, and in the nucleus accumbens; the putamen and caudate were found to be particularly dense. Another region in which a modest amount was present was the primary motor cortex (Broadmann's area 4) so, given the presence of NPY also in the basal ganglia, the authors argue how this neuropeptide may play a key role in the control of motor functions in humans.

According to DSM-5, in humans, anxiety disorders, are characterized by irrational worry about everyday life situations and are manifested through physiological symptoms, including increased heart rate, increased concentration to deal with threat, and fight-or-flight response. Similar changes in physiological and behavioral responses to noxious stimuli in humans and other animals suggest the possibility of analogies of ethologically motivated defensive responses (Bailey & Crowley 2009). Using the animal model, it has been shown how NPY is implicated in anxiogenic responses; deletion of the NPY-1r gene in mice causes the onset of anxious behaviors, and administration of agonists for Y1r results in anxiolytic effects (Karl et al., 2006). Heiling (1995) also demonstrated how the anxiolytic action of NPY is prevented when, by injection of antisense oligodendrocytes, Y1r is inhibited in the amygdala in the rat.

Regarding stress responses, several studies have shown that NPY has a modulatory effect on the hypothalamic-pituitary-adrenal axis by inhibiting the anxiogenic effect of corticotropin-releasing hormone (CRH).

Given these assumptions regarding the role of NPY on anxiogenic and stressogenic responses in the animal model, it is interesting to understand whether similar effects can be observed in humans regarding the role of NPY in psychopathologic disorders such as major depressive disorder (MDD) and post-traumatic stress disorder (PTSD).

1.4.1 Major depressive disorder

Major depressive disorder consists of a persisting experience of sadness and hopelessness and loss of interest in activities people once enjoyed. Individuals can also present with a physical symptom such as chronic pain or digestive issues.

The DSM-5 outlines nine different criteria to get a diagnosis of depression. The individual must be experiencing five or more symptoms during the same 2-week period and at least one of the symptoms should be either (1) depressed mood or (2) loss of interest or pleasure:

1. Depressed mood most of the day, nearly every day.
2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day.
3. Significant wight loss when not dieting or weight gain (5% change over 1 month) or decrease or increase in appetite nearly every day.
4. Insomnia or hypersomnia, nearly every day
5. Psychomotor retardation or agitation, nearly every day.
6. Loss of energy or fatigue, nearly every day.
7. Worthlessness or guilt
8. Impaired concentration or indecisiveness
9. Thoughts of death or suicidal ideation or attempt.

Neuropeptide Y could be a possible marker for MDD. In several animal model studies, different functional roles have been attributed to NPY, some of which may suggest an association with depressive-like symptoms.

A study by Widerlöv and colleagues (1987) measured neuropeptide Y immunoreactivity in cerebrospinal fluid (CSF) on patients diagnosed with major depression, schizophrenia (drug-free) and a healthy control group. What they found was that subjects with MDD had

significantly lower levels of NPY than both the control group but also than subjects diagnosed with schizophrenia (who had a lower level of peptide YY).

Another study investigated whether there were alterations of neuropeptide Y plasma levels in mood disorder patients with a recent suicide attempt. What they found was that NPY and CRH levels were significantly lower than in the control group, while cortisol was higher (Westrin et al., 1998). It should be kept in mind that in this study significance was found in subjects with depression NOS (not otherwise specified). Within mood disorders the one with a higher rate of suicides is bipolar disorder and given some contradictions in the literature it could be that NPY plasma levels alterations are mainly related to bipolar disorder and not unipolar ones, in fact Caberlotto and Hurd (1999) demonstrated a suppressed NPY expression in human post-mortem brain tissue in bipolar, but not unipolar affective disorder.

It is well known that a proportion of patients diagnosed with unipolar disorder in fact has a genetic vulnerability for bipolar disease but has not yet presented with their first manic episode and may never do so. It is therefore possible that involvement of NPY is primarily related to bipolar traits and that the discrepant CSF-results are partly due to varying proportion of this patient category in the different clinical samples (Heiling, 2004).

1.4.2 Post-traumatic stress disorder

Post-traumatic stress disorder (PTSD) is a mental health condition that's triggered by a terrifying event (either experiencing it or witnessing it). Symptoms may include flashbacks, nightmares, and severe anxiety, as well as uncontrollable thoughts about the event. Most people who go through traumatic events may have temporary difficulty adjusting and coping.

Any traumatic situation can cause PTSD, such as:

- Serious road accidents
- Violent personal assault, such as sexual assault, mugging or robbery

- Serious health problems
- Childbirth experience

It is not said that PTSD develops immediately after the traumatic experience, it can occur days, weeks, months or even years later.

NPY and NPY receptors in the limbic and brainstem areas play an important role in regulating physiological and behavioral responses that may be relevant to PTSD, such as stress and anxiety, fear, learning and memory, and control of blood and sympathetic activity (Sah and Geracioti, 2013) (figure 2).

Rasmusson and colleagues (2000) investigated whether NPY could contribute to the hyperreactivity of the sympathetic nervous system in PTSD. Measuring plasma NPY responses to yohimbine they found out that PTSD subjects had a lower plasma NPY level at baseline and a dampened increase in yohimbine-stimulated plasma NPY compared with healthy control subjects. The suggestion is that decreases in plasma NPY induced by combat stress may partially mediate the hyperreactivity of the noradrenergic system. The persistence of this reduction in plasma NPY may eventually contribute to symptoms of hyperarousal and the expression of exaggerated alarm reactions, anxiety reactions, or both.

Sah (2009) found the same results as Rasmusson, a decrease of plasma NPY in the CSF in PTSD veterans relative to healthy control subjects. She emphasizes that these results could be related to the disorder but even with the extreme stress exposure per se.

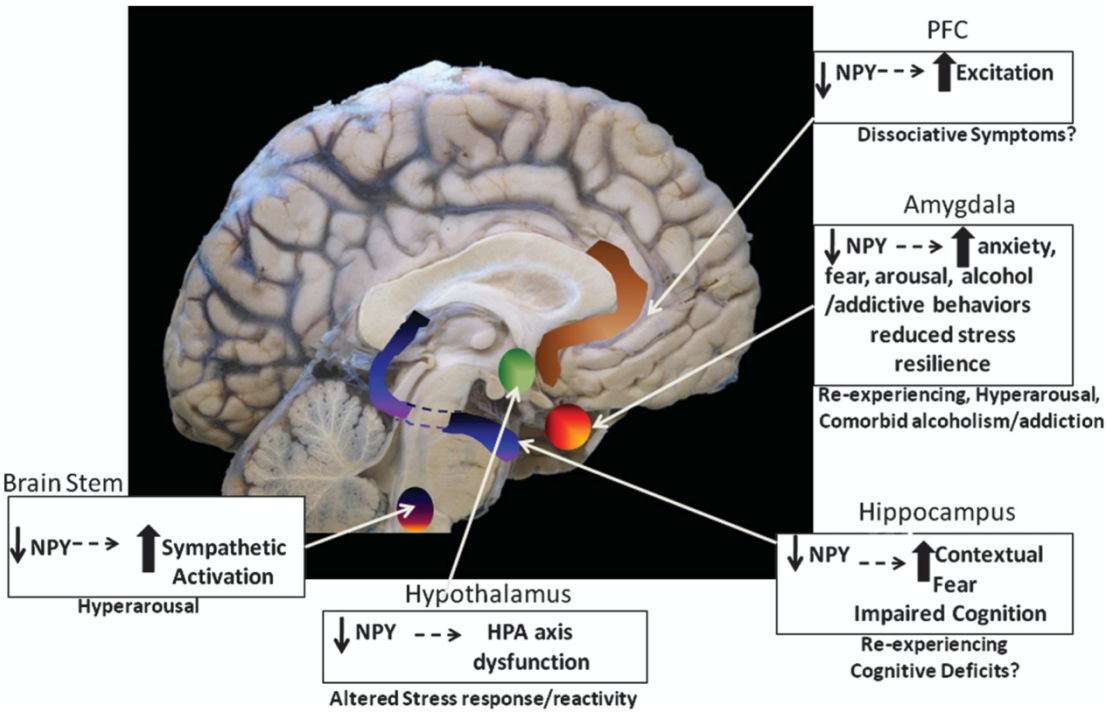


Figure 2. Potential association of NPY to PTSD (Sah and Geracioti, 2013).

1.5 Animal model

In scientific research, physiological parameters, pharmacological effects, behavioral phenomena, and mechanisms underlying sensations (satiety, pain, appetite etc.) or psychological situations (anxiety, depression etc.) are impossible to study without the use of *in vivo* models that exactly reproduce these physiological or pathological states. The animal model also plays a key role in understanding the pathophysiological, genetic and molecular mechanisms of the organism, helping to determine the causes and mechanisms of onset of many diseases. Regarding the study of behavioral, neurological, and psychiatric disorders, the use of laboratory animals offers significant advantages, both for the etiopathogenesis and for what concerns therapy, as it allows the effect of experimental pharmacological substances to be evaluated in a meaningful and comprehensive manner. However, observation of behavior alone makes it difficult to distinguish between the neurological and psychiatric spheres in the animal model.

Several animal models have been used to study neuropeptide Y and the receptors to which it binds: from those phylogenetically most distant from humans such as *drosophila* to those closest to us, primates.

In *drosophila melanogaster*, for instance, knockdown of NPFR1, a homologue of NPY1-r, led the animal to reduce the avoidance of noxious stimuli (food). This homologue of NPY might be part of the neural circuit that regulates risky behaviors, suppressing anxiety and fear (Wu, Zhao and Shen, 2005).

Trough immunohistochemical and tracing techniques Hamassaki and Britto (1990), found a connection between the thalamic NPY-LI (neuropeptide Y-like immunoreactive) and the nucleus of the basal optic root (nBOR) of the accessory optic system in the pigeon. The authors hypothesize that this may play a role in the avian visual system overall.

In rodents, several studies have been carried out on neuropeptide Y and the Y1 receptor. Through KO of NPY-1r, it has been seen that there is an alteration in body weight of animals and increase in anxiogenic behaviors, all of which are also related to the early maternal environment. In section 1.7 I report studies showing these alterations in KO mice.

In primates, Roseboom and colleagues (2014) investigated whether NPY-1r expression in the amygdala was predictive of anxious temperament (AT). AT is assessed as a combination of inhibition of vocalizations, threat-induced freezing behavior, and increased plasma cortisol levels. The AT phenotype was defined as an overall score of behavioral (increased freezing and decreased vocalizations) and hormonal (increased plasma cortisol) measures in response to the mildly threatening No-Eye Contact challenge. Using quantitative real time polymerase chain reaction, they measured central nucleus (Ce) of the amygdala mRNA levels and they found out that, regarding NPY-1r (and NPY-5r), lower mRNA levels in the Ce predicted elevated AT. The metabolic activity underlying the AT-related neural circuit could be altered by decreased

levels of NPY in Ce. This suggests that enhancing NPY signaling might reduce the risk of developing psychopathology.

As suggested by the different examples given, the studies aimed at understanding the role of NPY are manifold and have been carried out in different animal models for different purposes. From now on I am going to investigate the role of NPY and its receptors in relation also to the early maternal environment solely in mice.

1.6 Effects of maternal behavior in the neurobehavioral development of offspring

Attachment studies by Bowlby on humans (1958) and Harlow on macaques (1958) showed how maternal deprivation causes depressive outlines and stress-related responses on infants. Subsequent studies have shown how early postnatal separation induces significant changes on the adult phenotype, characterized by an increased hypothalamic-pituitary-adrenal axis response (Plotsky and Meany, 1993).

Studies have shown that in the rat, low levels of maternal care characterized mainly by "arched back nursing" and "licking" cause behavioral differences in offspring as adults, especially regarding higher levels of anxiety in response to stress caused by a high profile of stress-dependent neuroendocrine response (Champagne, 2001).

These effects are mediated by epigenetic alterations in stress-related genes, which are associated with reduced stress response documented in offspring who received high maternal care (Weaver I.C.G. et al., 2014).

Similar findings concerning humans showed that variations in the quality of maternal care are associated with greater social difficulties and with stress (Hane A.A., 2006). It has been found that poor quality of maternal care leads to stress-related biobehavioral profiles characterized by greater fear during the presentation of new stimuli, lower ability in sharing

attention (join attention) with strangers in following objects, and lower affect effect in mother-child interaction.

Starting from this research and the NPY-1r correlation to anxiety and depression, a possible involvement of maternal care in the regulation of NPY expression was hypothesized.

1.7 Maternal care: regulatory function in Y1 receptor expression

Several studies have shown how the expression of the behavioral and metabolic phenotype due to conditional inactivation of the NPY-Y1r receptor can be influenced by the environment, especially the early environment, represented mainly by maternal care. Bertocchi's 2011 study investigated how various degrees of maternal care, from adoptive mothers of different strains, may influence phenotype development in knock-out mice. To study the function of Y1r expressed in the limbic system and to exclude that early inactivation of the NPY1r gene induced undesirable developmental effects, Bertocchi and colleagues (2011) exploited a conditional KO mouse model against the Y1 receptor and NPY, in which inactivation of the gene was restricted only to excitatory neurons in the forebrain, and only from an advanced stage of the animal's development, using the CRE-loxP system. In addition, to analyze the impact that different degrees of maternal care have toward development and feeding and anxiety behavior in KO (NPY-Y1r^{rfb}) and control (NPY-Y1r^{2lox}) offspring, the offspring were given for adoption to two different strains of adoptive mothers, namely FVB/J and C57BL/6J. One of the most important aspects of this study was the observation of how differences in the molecular, physiological, and behavioral phenotype between KO and control mice became evident only when animals of both genotypes gift were reared to foster mothers of the FVB/J strain, and not from those of the C57/6J strain. First, it was observed that body growth of the KO mice began to slow down around PND 40, a day coinciding with the maximum level of receptor inactivation, and the lower body weight persisted throughout adulthood and was associated with lower white adipose

mass and lower serum leptin levels (Bertocchi et al, 2011). At the behavioral level, it was observed that male NPY1r^{rfb} (KO) mice were characterized by higher levels of anxiety than NPY-Y1r^{2lox} wild-type (WT) mice, and, again, this effect was present only when the animals were reared from FVB/J strain adoptive mothers and not C57/6J. The anxiety-provoking effect of site-specific inactivation of the NPY-1r gene was monitored by Open Field (OF) and Elevated Plus Maze (EPM) tests. Specifically, KO mice reared by FVB dams showed reduced exploration of the OF and increased immobility in the central area (Bertocchi et al., 2011), both behaviors indicative of an increased state of anxiety (Choleris et al., 2001), while in the EPM, they showed a lower frequency of entry and less stay in the open arms (Bertocchi et al., 2011). Control mice reared by FVB/J dams showed lower levels of anxiety, greater body weight gain and lower activation of the hypothalamic-pituitary-adrenal (HPA) axis than those reared by C57/6J dams (Bertocchi et al., 2011). In contrast, no significant differences were found between KOs and controls reared by adoptive mothers of the C57/6J strain, although controls were found to be more anxious than those reared by FVB/Js, because of a lower level of care received. The hypothesis that differences in maternal care of the offspring by adoptive mothers might have long-term consequences for NPY-1r signaling finds strong support in the increased mRNA levels for Y1r in the hippocampal CA1 and CA3 fibers and dentate gyrus of NPY1r mice were shown to be very similar to each other and no significant phenotypic differences between the two genotypes were established: this suggests that CRE-induced inactivation of NPY1r may not further decrease gene expression in excitatory forebrain neurons.

The analysis conducted by Bertocchi and colleagues (2011), therefore, showed that conditional downregulation of hippocampal Y1 receptors leads to an increase in anxiety-related behavior and provided experimental genetic evidence that limbic Y1 receptors were required for body weight regulation. Finally, these data indicate that NPY/Y1r neuronal pathways in the

limbic system are important targets of maternal care-induced programming of anxiety and energy homeostasis.

1.8 Gene and environment interaction in the development of the phenotype

The term genotype refers to the set of all genes that make up the DNA of an organism or population. Each gene, individually and/or cooperatively, contributes differently to the development, physiology, and functional maintenance of the organism. Genotype is the gene construct of an organism (or group of individuals) that presides over the expression of somatic characters. The set of observable traits is called the phenotype. The genotype alone does not define or determine the phenotype, this is possible by an interaction with the environment, internal or external. If two individuals have the same genotype, they may not necessarily have the same phenotype.

This can be explained by epigenetics, a branch of biology, which studies genetic mutations and the transmission of inherited traits that cannot be directly attributed to the DNA sequence. Epigenetic mechanisms enact gene regulation that, through chemical processes, while not directly altering the nucleotide sequence, can result in a change in the phenotype of the individual or its progeny. These phenomena alter the physical accessibility to the genome by molecular complexes deputed to gene expression, thus affecting gene function.

1.9 Generation of a conditional KO mouse model for NPY-1r

To evaluate Y1r-mediated physiological effects on the limbic system, and thus on regions particularly involved in phenomena related to social and emotional behaviors and neuronal excitability, researchers from University of Turin and Max Planck Institute in Heidelberg generated a conditional KO mouse model for NPY1r in which the gene deletion is controlled in space and time. The deletion is selectively restricted to neurons expressing the alpha subunit

of cyclo-calmodulin kinase type II (αCamKII), thus only in certain regions of the forebrain, such as amygdala, striatum, hippocampus, and hypothalamic arcuate nucleus. The gene deletion occurs in absence of Dox (tet-off system) through the doxycycline-dependent CRE recombinase system.

If germinal KO was obtained, compensatory phenomena would occur, causing interference in the phenomena to be investigated. Therefore, the deletion occurs after 40 days and is limited to the postnatal forebrain. (Bertocchi et al., 2011).

1.10 Open Field Test

The Open Field Test (OF) is commonly used for measuring exploratory and anxious-like behaviors, and general activity. This test is used in rodents, originally in rats, afterwards in mice. The activity of the animal can be measured both quantitatively and qualitatively. OF was first introduced by Hall in 1932, while he was studying rats' behavior with or without food.

The OF is an enclosure (arena), generally a square, but it can be also rectangular or circular, with walls preventing escape. A camera is used to monitor movement inside the arena.

Usually, the test lasts from 2 to 10 minutes to assess the animal's exploratory behavior in a new environment rather than its baseline activity. The procedure requires the experimenter to place the animal inside the arena, immediately exposing it to a new environment and thus forcing it to interact with the novel environment. Therefore, the animal cannot choose to enter the arena in order to explore it (as it is the case in the free-exploratory test). Therefore, under present conditions, we can only measure exploration and anxiety-like levels, and not the propensity of the animal to explore.

Different behavioral measures can be assessed, from the more classic ones such as distance traveled, time spent moving, and rearing to less frequent ones such as sniffing, immobility, and grooming.

The OF is not simply a test that assesses motor activity, as the latter could be influenced by other factors such as exploratory drive and thus curiosity or by fear, such as anxiety could be. Especially in cases where the mouse is placed in the arena for the first time, it will tend to spend most of its time near the walls, a phenomenon defined as thigmotaxis; this behavior is indicative of an anxiety-like state (figure 3). Precisely, anxiety plays a key role in case the test is taken for the first time and for a short period of time.

Not surprisingly, the OF along with other tests such as the Elevated Plus Maze (EPM) and the Light/Dark box are used to assess anxiety-like behavior in rodents. The less anxious mice tend to spend more time in the center of the arena than the more anxious ones (Crawley 1985). Bale and colleagues (2000), using mice that had a deficiency for corticotropin-releasing hormone receptor-2, demonstrated, at a neuroendocrine level, how the mice exhibited anxiety-like behavior by administering tests such as OF, EPM and Light/Dark box. This study also shows us how the Open Field test, in concordance with other measurement tests, can be a valid test for the evaluation of anxious-like behavior.

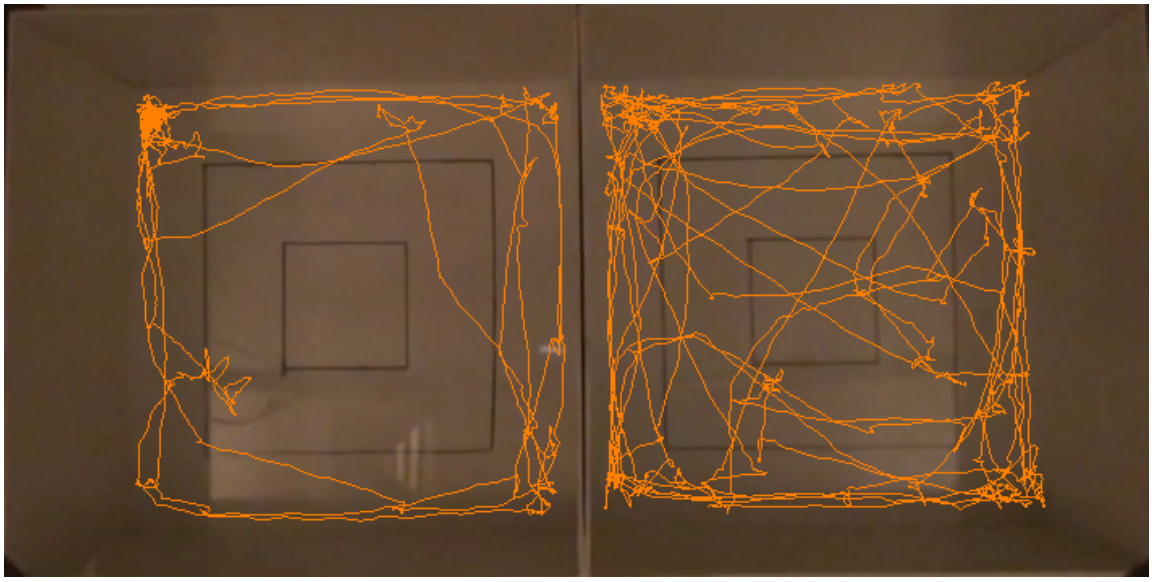


Figure 3. Movement tracking of two mice in the open field test. As can be seen from the left arena, the animal spent almost the entire duration of the test near the walls (thigmotaxis), this is indicative of higher levels of anxiety. The animal on the right explored the central areas of the arena and thus is considered less anxious than the animal on the left.

2 AIM OF THESIS

My undergrad research focused on the comprehensive and detailed analysis of exploratory and anxious-like behaviors as observed during the OF test of conditional KO mice for NPY-Y1r in relation to their genotype, sex and early maternal environment. By means of two specific software (Boris, University of Turin, Italy and Ethovision, Noldus, The Netherlands), I scored the behavior of 376 animals from 9 different cohorts, taking into account 3 experimental variables: genotype (KO, WT), foster dams' strain (CD1, FVB, C57, BALB) and sex (M, F).

I analyzed the behavior during a 5-min OF test of all experimental animals examined so far in our laboratory as part of the behavioral and metabolic phenotyping project of this mouse model. All the mice I analyzed were subjected, in different experimental replicates, to the same experimental procedure.

Immediately after birth (PND 0), Experimental animals (both KO and WT) were given randomly to adoption to lactating dams of 4 different strain, namely FVB/J, BALB/c, CD1 and C57BL/6J. Spontaneous maternal behavior expressed by lactating dams was observed during the first postnatal week (PND 1-7) using ethological observation techniques by means of instantaneous sampling. At weaning (PND 27), animals were genotyped for the presence of tTA, indicative of the NPY1r deletion. As adults, animals underwent a battery of behavioral tests, including the OF test to assess exploratory and anxiety-like behavior. Males … females… tested twice. Some animals underwent a second experimental session after a stressfull event, in particular females while they were pregnant and males during the Chronic Mild Stress test.

I wrote an ethogram specifically for the OF that included all the possible behaviors that the mouse could express in the arena; these mutually exclusive behaviors are described in detail in section 3.9. Afterwards, I analyzed the mouse behavior during the OF test by means of the software "Boris". The other software, "Ethovision", allowed me to derive other information

regarding the animals in the arena such as: total distance moved and speed, elapsed time, distance moved, and latency to enter in the three different areas of the arena (edge, median area and center).

These data can be useful to derive information about the anxious-like behaviors of the mice. The use of these two software programs (Boris and Ethovision) provides a comprehensive view of the animal's behavior regarding the Open Field Test, since different parameters were evaluated using the two different software.

Finally, I scored the number of feces present in the arena at the end of the test.

The large sample size used and the absence of possible variation due to differences in observation allowed principal component analysis (PCA) to be applied to the data produced. PCA is a technique used in multivariate statistics for simplification of source data. The primary purpose of this technique is to reduce a larger or smaller number of variables (representing as many characteristics as possible of the phenomenon being analyzed) into few latent variables. This is done by means of a linear transformation of the variables that projects the original ones into a new Cartesian system in which the variables are sorted in descending order of variance: thus, the variable with the greatest variance is projected onto the first axis, the second onto the second axis, and so on. Complexity reduction is achieved by merely analyzing the mean (by variance) among the new variables.

3 MATERIALS AND METHODS

3.1 Generation of a conditional knock-out, mouse model

To obtain mice with inactivation of the NPY1r gene, which is restricted to the excitatory nuclei of the adult forebrain ($Npy1r^{rfb}$), three different mouse genetic lines were bred: the $NPY1r^{2lox}/Tg^{LC1}$ and $NPY1r^{2lox}/Tg^{\alpha CaMKII\text{-}tTA}$ strain, which are necessary for the production of the experimental line, and the $NPY1r^{2lox}$ strain, which is necessary for the maintenance of heterozygosity of the $NPY1r^{\alpha CaMKII\text{-}tTA}$ line.

The conditional KO for NPY1r was obtained following the protocol described in the study by Bertocchi et al. (2011) by crossing mice belonging to the $NPY1r^{2lox}/Tg^{LC1}$ and $NPY1r^{2lox}/Tg^{\alpha CaMKII\text{-}tTA}$ lines, in order to achieve spatiotemporal controlling of CRE recombinase, through the specific αCaMKII promoter and the Tet-Off system.

Through the doxycycline-dependent Tet-Off system of CRE recombinase, the presence of antibiotic prevents the transcription of recombinase and consequently allows the expression of NPY1r; on the contrary, in the absence of antibiotic, the trans-activator (tTA) can bind TRE (Test Responsive Element), allowing the transcription of recombinase and preventing the expression of NPY1r. Two different genotypes were thus obtained:

- $NPY1r^{2lox}/Tg^{LC1}$;
- $NPY1r^{2lox}/Tg^{\alpha CaMKII\text{-}tTA/LC1}$.

The $NPY1r^{2lox}/Tg^{\alpha CaMKII\text{-}tTA/LC1}$ represents the conditional KO model ($NPY1r^{rfb}$), in which the inactivation of the gene for the Y1 receptor is by CRE recombinase; while the other line, represent the controls ($NPY1r^{2lox}$), i.e., WT mice in which inactivation is not present (figure 4).

In order to maximize the probability of conception, each male animal was mated with two females in a single cage for a period of 10 days, at the end of which the male was removed

from the cage and the two females were isolated in two separate cages and left undisturbed until the time of parturition. At the same time, males and females of the FVB/J, BALB/c, CD1 and C57BL/6J strains were mated so that there would be enough adoptive mothers when the experimental mice were born.

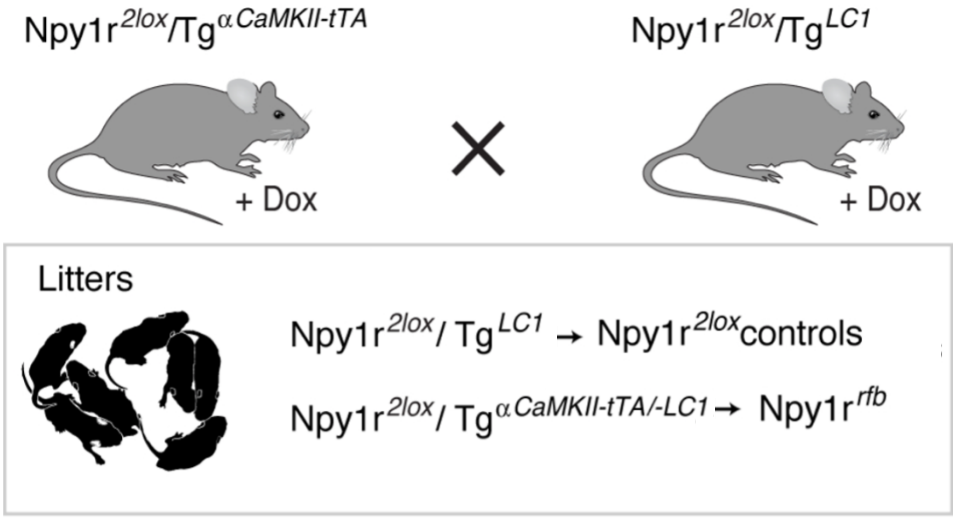


Figure 4. Pairings between different murine lines for obtaining conditional KO

3.2 Dams' doxycycline treatment and offspring fostering procedure

Doxycycline is an antibiotic that belongs to the tetracycline family and interferes with bacterial protein synthesis. This was administered from the first day of mating of the females, which were replaced the water with one containing a sugary solution (1% sucrose), in which doxycycline (50 mg/l; Sigma-Aldrich) was diluted. Because of the photosensitivity of doxycycline, the drinking bottles used were black.

Treatment with this antibiotic was then discontinued at the birth of the pups (Post Natal Day 0, PND 0), which were distinguished by gender, weighed by use of a digital scale and given for adoption to mothers who had never been exposed to doxycycline (dox-naïve) belonging to three different inbred strains, BALB/C, C57BL/J6, and FVB/J, and one outbred strain, CD1, in order to allow the complete elimination of doxycycline in the circulating body

of the young and to allow the tTa transactivator to activate and induce Cre recombinase expression, which occurs around PND 40.

The early environment consisting of maternal care from birth to 7 days was evaluated next.

3.3 Analysis of spontaneous maternal behavior of foster dams of the FVB/J, BALB/c, CD1 and C57BL/6J strains

On the day of birth (after about 21 days of gestation), pups were weighed with a precision scale and given for to foster lactating dams of strains FVB/J, BALB/c, CD1 and C57BL/6J. During the fostering procedure, foster dams were temporarily moved from their cages and the pups to be reared introduced to their nest. Afterwards, foster dams were returned to their cages. In order to have litters as uniform as possible, 6/8 pups were in each litter; when less than 6 pups, pups were added from another litter.

The spontaneous maternal behavior of the adoptive mothers was observed daily, for one week (PND1 to PND7) from 9:00 am to 11:00 am (last two hours of the dark phase). Observations were carried out during the last two hours of dark phase, using bulbs producing red light, as mice are unable to perceive it. Adoptive mothers were lactating females of FVB/J, BALB/c, CD1 and C57BL/6J strains, housed in plexiglass cages (42 cm x 26.5 cm x 15cm), with food and water *ad libitum*, room temperature at 22°C ± 1 and a 12-hour light-dark cycle, with light on at 11:00am. Each mother was observed every 4 min, for a total of 30 observations in 2 hours, by instantaneous sampling. As per Palanza et al. (2002), the behaviors were:

- Arched-back: the mother nurses in the typical arched position above the pup, with the dual function of warming and nursing (figure 5).
- Nursing: the mother nurses at least one pup.
- Licking pups: the mother licks and cleans the pups.

- Nest building: the mother builds or arranges the nest and can be either inside or outside of it.
- Eating/drinking: the mother eats or drinks.
- Grooming: the mother cleans herself.
- Active: the mother is active inside the cage, without exhibiting any specific behavior among those previously listed.
- Out of nest: the mother is motionless, outside or inside the nest, without performing any other activity.

Figure 5. Arched-back nursing (modified from Stern et al., 2002)

3.4 Weaning and genotyping

At weaning (PND27), animals were separated from their mothers and housed in unisexual sibling groups in plexiglass cages closed at the top by a metal grate on which water and food were placed *ad libitum*. Each cage contained bedding, consisting of sawdust (Mucedola) and nesting material, which was changed weekly. The temperature of the experimental room was maintained at 22°C ± 1 and the light-dark cycle was 12 hours with light on at 7:00am. The animals were characterized genomically, so as to distinguish knockout (experimental) mice from WT (controls), in which deletion of the gene of our interest did not occur. The operation is done through the process of DNA extraction from solid tissue and the process of analysis by Polymerase Chain Reaction (PCR).

3.5 Monitoring of body weight growth

From the time of weaning (PND 27) until adulthood (PND 97), all animals regardless of sex were weighed once a week with a digital scale (Sartorius, USA) to monitor their body growth curve. During this time, all animals shared the same cage with their brothers/sisters and were housed under standard conditions.

All animals reentered the body growth monitoring protocol until adulthood. They were then subjected to different behavioral tests.

3.6 Analysis of anxious-like behaviors, exploration and locomotor activity

The procedures described so far have been consistently applied to all cohorts of animals that are part of the research project carried out by the Behavioral Biology Laboratory.

For the analysis of anxiety-like behavior, the following were carried out:

- Elevated Plus Maze (EPM): elevated platform consisting of two arms enclosed by dark walls and two open arms, in which the animal's tendency to expose itself in the less safe areas and thus indicative of higher levels of anxiety is examined.
- Open Field (OF): arena in which the animal is free to explore, visually divided into three areas: an outer one abutting the arena walls, indicative of higher levels of anxiety, a middle one, and a central one (figure 6).

For the analysis of depression-like behavior, the following were performed:

- Anhedonia test, the animal's ability to experience pleasure is assessed through a choice between a drinking bottle containing only water and another containing water and sugar.
- Social Open Field (SOF), in addition to the arena described above, a wire mesh cage is added near one of the arena walls, which demarcates the social zone. The test is divided into two sessions: in the first one the cage is empty, in the second one an intruder is present inside the cage; used to assess social avoidance and reduced contact interest.

For the analysis of autistic-like behavior was carried out:

- Three-chamber test, arena divided into three areas by transparent bulkheads. Sliding doors in the two partitions allow animals to pass from one space to another, forming three "chambers": one central and two laterals. The test is divided into three sessions: in the first one, the animal explores the three sections, in the second one, two metal cages are inserted, an intruder is placed in one, into the two lateral chambers, and in the third one, the intruder is moved to the previously empty cage.

For the analysis of depression-like behavior was carried out:

- MARBLE test, 12 glass marbles are placed in a cage under sawdust, after which the animal is left free to explore for 30 minutes. A high number of marbles buried at the end of the test is indicative of neophobia and impulsivity, while high locomotor activity is indicative of restlessness.

For the assessment of aggressive, social, and defensive behaviors, it was performed:

- Resident/Intruder (RI) test, an intruder is placed inside the cage where an animal is already present for 10 minutes. During this time, the behavior of both animals is observed.
- CMS, Resident/Intruder test performed every day for one month.

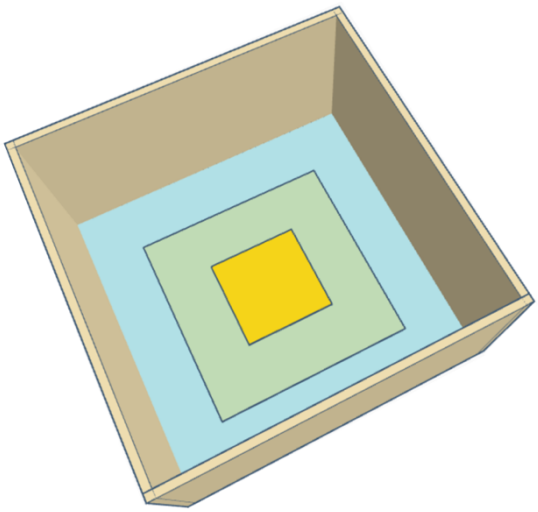


Figure 6. Areas in the Open Field: Edge (light blue), Median area (green) and Center (yellow).

3.7 Experimental subjects

Experimental subjects were 376 (177 males and 199 females) NPY1r conditional KO mice belonging to 9 cohorts. In the first experimental phase (pre-pregnant females and pre-CMS males) with a total of 367 mice; animals reared from C57 dams were 131, 68 females, of which 24 KO and 44 WT, and 63 males, of which 23 KO and 40 WT; 128 animals reared from FVB dams, 60 females, of which 24 KO and 36 WT, and 68 males, of which 26 KO and 42 WT; 34 animals reared from BALB dams, 12 females, of which 4 KO and 8 WT, and 22 males, of which 9 KO and 13 WT; 74 animals reared from CD1 dams, 50 females, of which 22 KO and 28 WT, and 24 males, of which 9 KO and 15 WT.

Seven of the nine cohorts underwent a second experimental phase (post-pregnancy females and post-Social stress test males) with a total of 209 mice; animals reared from C57 dams were 89, 53 females, of which 20 KO and 33 WT, and 36 males, of which 12 KO and 24 WT; 63 animals reared from FVB dams, 39 females, of which 10 KO and 29 WT, and 24 males, of which 6 KO and 18 WT; 34 animals reared from BALB dams, 12 females, of which 4 KO and 8 WT, and 22 males, of which 9 KO and 13 WT; 33 animals reared from CD1 dams, 28 females, of which 12 KO and 16 WT, and 5 males, of which 1 KO and 4 WT.

3.8 Timing of different cohorts

CD1												
VIII cohort	**PND 26**	**PND 90**	**PND 94**	**PND 97**	**PND 120**	**PND 126**	**PND 127**	**PND 142**	**PND 153 - 182**	**PND 170**	**PND 182**	**PND 190**
♀	Weaning	Isolation	EPM	SOF	Basal STD for UCMS	Anhedonia test	in UCMS with STD	Anhedonia test	in UCMS with HFD	Anhedonia test	GTT	Sacrifice
♂	Weaning	Isolation	EPM	SOF								
CD1												
XVI cohort	**PND 27**	**PND 120**	**PND 121-141**	**PND 142**	**PND 148**	**PND 159**	**PND 160**	**PND 161**	**PND164**	**PND 171**		
♀	Weaning	OF	Vaginal smears analysis	Mating	Separation	Anhedonia test	OF	Nesting test	Spontaneous maternal behavior observation	Sacrifice		
C57 FVB												
XVIII cohort	**PND 27**	**PND 105**	**PND 106-113**	**PND 114**	**PND 121**	**PND 128**	**PND 131**	**PND 132**	**PND 133**	**PND140**		
♀	Weaning	OF	Vaginal smears analysis	Mating	Separation	Anhedonia test	OF	Nesting test	Spontaneous maternal behavior observation	Sacrifice		
	PND 27	**PND 96**	**PND 98**	**PND 99-102**	**PND 103**	**PND 118**	**PND 120**	**PND 121**	**PND 125**	**PND 141**	**PND 146**	**PND 152**
♂	Weaning	Anhedonia test	OF	Basal STD	CMS	Anhedonia	OF	Blood sugar	CMS + HFD	Anhedonia	GTT	Sacrifice
C57 FVB Balb/c												
XXI cohort	**PND 27**	**PND 99**	**PND 100**	**PND 103**	**PND 105**	**PND 106**	**PND 110**	**PND 113**	**PND 134**	**PND 139**	**PND 142**	**PND 150**
♂	Weaning	Isolation	Anhedonia test	Marble test	EPM	SOF	Resident-Intruder test	CMS	Anhedonia test	OF	GTT	Sacrifice
	PND 27	**PND 118**	**PND 119**	**PND 120**	**PND 124**	**PND 130**	**PND 136**	**PND 146**	**PND 148**	**PND 149**	**PND 150**	**PND 142**
♀	Weaning	Marble test	EPM	SOF	Vaginal smears analysis	Mating	Separation	Anhedonia test	OF	Nesting test	Spontaneous maternal behavior observation	
C57 FVB												
XXIII cohort	**PND 0-7**	**PND 27**	**PND 120-121**	**PND 121**	**PND 127**	**PND 134**	**PND 138**	**PND 142**	**PND 164**	**PND 165**	**PND 169**	**PND 170**
♂	Spontaneous maternal behavior observation	Weaning	Isolation	Anhedonia test	SOF	3-chamber test	Basal STD	HFD	Fasting	GTT	Fasting	Sacrifice
	PND 0-7	**PND 27**	**PND 133**	**PND 134**	**PND 137**	**PND 143**	**PND 148**	**PND 158**	**PND 165**	**PND 172**	**PND 177**	**PND 186**
♀	Spontaneous maternal behavior observation	Weaning	Isolation	Anhedonia test	SOF	3-chamber test	Vaginal smears analysis	Mating	Separation	out HFD	Spontaneous maternal behavior observation	Sacrifice
C57 FVB Balb/c												
XXIV cohort	**PND 0-7**	**PND 27-35**	**PND 98 -99**	**PND 99-100**	**PND 103-104**	**PND 107**	**PND 111**	**PND 117-118**	**PND 119-120**	**PND 142**	**PND 157-158**	**PND 148**
♂	Spontaneous maternal behavior observation	Weaning	Isolation	EPM	SOF	Locomotor test	CMS + STD diet	EPM	SOF	Anhedonia test	GTT	Sacrifice
	PND 0-7	**PND 27**	**PND 113**	**PND 122**	**PND 158**	**PND 160**	**PND 161**	**PND 162**	**PND 165**	**PND 188**		
♀	Spontaneous maternal behavior observation	Weaning	OF	Vaginal smears analysis	Mating	Separation + anhedonia	OF	Nesting test	Spontaneous maternal behavior observation	Sacrifice		
C57 FVB												
XXV cohort	**PND 0-7**	**PND 27**	**PND 124**	**PND 125**	**PND 127**							
♂	Spontaneous maternal behavior observation	Weaning	Isolation	OF	Marble test							
	PND 0-7	**PND 27**	**PND 124**	**PND 125**	**PND 127**	**PND 128**	**PND 149**	**PND 166**	**PND 167**	**PND 168**	**PND 174**	**PND 182**
♀	Spontaneous maternal behavior observation	Weaning	Isolation	OF	Marble test	Vaginal smears analysis	Mating	Anhedonia test	OF	Nesting test	Spontaneous maternal behavior observation	Sacrifice

CD1												
XXVI cohort	**PND 0-7**	**PND 27**	**PND 90**	**PND 91**	**PND 94**	**PND 96**	**PND 118**	**PND 119**	**PND 122**	**PND 129**	**PND 131**	
♂	Spontaneous maternal behavior observation	Weaning	Isolation	EPM	SOF	CMS + STD diet	EPM	SOF	Anhedonia	GTT	Sacrifice	
	PND 0-7	**PND 27**	**PND 112**	**PND 115**	**PND 136**	**PND 152**	**PND 153**					
♀	Spontaneous maternal behavior observation	Weaning	OF	Vaginal smears analysis	Mating	Anhedonia test	OF					
FVBC57												
XXVIII cohort	**PND 0-7**	**PND 34**	**PND 122**	**PND 147**	**PND 158**							
♂	Spontaneous maternal behavior observation	Weaning	OF	Anhedonia test	Sacrifice							
	PND 0-7	**PND 34**	**PND 90**	**PND 122**	**PND 136**	**PND 137**	**PND 138**	**PND 145**	**PND 150**			
♀	Spontaneous maternal behavior observation	Weaning	OF	Mating	Anhedonia	OF	Nesting test	Spontaneous maternal behavior observation	Sacrifice			

Table 1. Unpredictable Chronic Mild Stress (UCMS), Chronic Mild Stress (CMS), Glucose Tolerance Test (GTT), Open Field (OF), Social Open Field (SOF), Standard Diet (STD), High Fat Diet (HFD), Elevated Plus Maze (EPM), Post Natal Day (PND).

3.9 Open Field Test: ethogram

For the mouse behavior analysis during the open field test, I wrote a specific ethogram that includes different mutually exclusive behaviors. Specifically, 10 behaviors were identified as follows:

- Walking Behavior: the animal walks exploring the arena while sniffing the environment. The body is compact, and the tail is not taut. The hind legs are under the animal's body.
- Stretch Attend Posture (SAP): the animal stretches its body; its tail is outstretched and at least one hind leg is stationary. The mouse sniffs the environment, tends to stretch and then retreat back to the starting position. At least one hind paw, while performing the behavior, is always stationary.
- Walking with Stretching Behavior: the animal is stretching and exploring the environment by sniffing it. The hind legs during locomotion are spread apart and lateral to the body, the tail is outstretched, and the body is elongated. Most of both hind legs are clearly visible.
- Rearing Behavior: the mouse lifts both front legs without leaning them.

- Wall Rearing Behavior: the mouse raises both front legs by leaning them on the walls of the arena.
- Stationary Sniffing Behavior: the stationary animal sniffs the environment. The body is compact, and the tail is not taut. Rotation, change of direction of body and head may also occur, but always fixed in the same place.
- Grooming Behavior: the stationary animal cleans itself; all body parts are included. The front paws move quickly over the animal's snout; the mouse with its mouth cleans its coat, hind legs and/or tail (figure 7).
- Immobility: locomotor activity is absent; no sniffing or grooming occurs.
- Other Behaviors: behaviors that are not associated with the previous ones.
- Undefined Behavior: confusing and unclear behaviors, usually due to shadowy areas that do not make the image clear or from the position from which the test was filmed (from above), so specific behaviors are not clearly distinguishable.

Video were scored through the software “Boris” (University of Turin, Italy). For each behavior frequency, total time and mean time have been taken into account for statistical data analysis, as well as the number of feces found in the arena after the 5-minute test.

Distance moved, duration, frequency and latency to enter in three different areas (edge, median area and center) and mean velocity (cm/s) were obtained through the software “Ethovision” (Noldus, The Netherlands).

Figure 7. Self-grooming of a mouse. The animal starts from the snout and then concludes the behavior toward the hind legs or tail (modified from Geuther et al., 2021)

3.10 Statistical analysis

For the evaluation of exploratory and anxious-like behaviors related to the data obtained from the OF test, I conducted analysis of variance (ANOVA) and PCA using R (R Development Core Team).

The analysis with the above software was carried out continuously over a brief period of time by a single operator. To make sure that the evaluation of behaviors was consistent over time, an inter-rater reliability analysis (Cohens's kappa coefficient) was carried out randomly; as the coefficient was always higher than the acceptability threshold (0.70), concordance of data was established.

A preliminary analysis was conducted on data from all the experimental animals that underwent the first session of the OF, including also those animals that were not tested a second time. The analysis was performed on the frequency and total time of different behaviors such as: walking behavior, wall rearing behavior, stationary sniffing behavior, stretch attend posture and grooming behavior. Other variables analyzed were defecation, total distance moved in the arena and average speed of the animal in the arena, time spent in, distance moved, frequency and mean velocity in the edge, median area and center; latency to enter in the median area and in the center. In particular, firstly a 3-way ANOVA was performed with the aim of investigating differences between the effects of genotypes, sex and foster dam's strain and their potential interactions. Secondly, I conducted a 2-way ANOVA on WT only to evaluate the effect of sex and early maternal care in the overall population.

All the 367 experimental subjects of the first experimental session were analyzed using the Principal Component Analysis (PCA). I excluded from this analysis variables with very high percentages of zeros such as: Immobility, Other Behaviors, Undefined Behaviors, Walking and Stretching Behavior and Rearing Behavior. Other variables were excluded due to a Measure

of Sampling Adequacy (MSA) less than 0.5 obtained from the Kaiser-Meyer-Olkin (KMO) test such as: grooming behavior, mean time of walking behavior, defecation, frequency observed and time spent in the edge, mean velocity and latency to enter in the median area and in the center of the arena.

I conducted PCA since the variables in the dataset were sufficiently intercorrelated (KMO) and correlated (Bartlett's test) (PCA, Kayser's eigen-value-greater-than-one rule; Kaiser-Meyer-Olkin = 0.81; Bartlett's test: K-squared = 41641, df = 20, $p < 0.001$). The 5 factors that emerged from the unrotated matrix were rotated (Oblimin rotation) to obtain the best approximation to a simple structure model matrix (Fabrigar et al., 1999). As in the preliminary analysis, I conducted a 3-way ANOVA on the 5 principal components obtained from the whole sample and a 2-way ANOVA on data only from WT.

One of the limits of the present study is that there was only one KO male reared by CD1 dams tested in the second OF session. This led to a strong imbalance between groups. Therefore, I anayzed the effect of the testing session only among two well-represented foster strains in relation to the other three factors. Specifically, for this analysis, only mice, who performed both OF sessions, reared by FVB and C57 lactating females were considered. This choice resulted in a reduced sample size (143 experimental subjects), so it was not possible to conduct a second PCA; therefore, I performed a mixed 4-way ANOVA by analyzing sex, genotype, experimental session, and foster dams' strain factors on the same dependent variables as in the preliminary analyses. Finally, I conducted two smaller 3-way ANOVA to assess the effects of sex, genotype and foster strain separately for each experimental session.

In all analyses, the Tukey's honest significance test was performed as a post-hoc analysis to uncover specific differences between three or more group means.

4 RESULTS

4.1 Preliminary analysis on behavioral data during the OF first session

4.1.1 3-way ANOVA on the whole sample size

I conducted a preliminary 3-way ANOVA (factors: sex, genotype and foster dams' strain) on behavioral measures obtained from all experimental subjects as observed during the OF first session: grooming behavior, walking behavior, wall rearing behavior, stretch attend posture, stationary sniffing and their frequency; defecation, distance moved and mean velocity in the arena, edge, median area and center of the arena; time spent in and frequency observed in the edge, median area and center of the arena and their latency to enter median area and center of the arena.

Foster dams' strain was highly significant on time spent in and frequency of observation of SAP (respectively: $F_{(3,218)}= 7.395$, $p<0.001$; $F_{(3,218)}=10.751$, $p<0.001$; Fig.8): in particular, regardless their sex and genotype, mice reared by CD1 dams spent significantly less time and were observed less frequently in SAP compared to mice reared by either C57 or FVB dams (respectively: $p<0.001$ for C57; $p<0.05$ for FVB for time spent in; $p<0.01$ for FVB for frequency observed in).

Interestingly, a highly significant effect of genotype on both time spent in and frequency of grooming behavior was found (respectively: $F_{(1,351)}= 14.349$, $p<0.001$; $F_{(1,351)}= 16.562$, $p<0.001$; Fig.9): in particular, regardless of their sex and foster dams' strain, KO mice spent significantly more time and were observed more frequently in grooming behavior than WT. Besides, 3-way ANOVA yielded a significant effect of the sex by foster dams' strain interaction on time spent in grooming behavior ($F_{(3,351)}= 5.353$, $p<0.01$; Fig. 9A): in particular, female mice reared by Balb dams spent significantly more time in grooming compared to their male counterparts and females reared by CD1 ($p<0.05$); in the other strains, the sex difference observed among mice reared by Balb dams was either reversed (CD1) or null (C57 and FVB).

As shown in Fig.10B, 3-way ANOVA revealed a significant main effect for the genotype on frequency in wall rearing behavior ($F_{(1,351)}$= 5.767, p<0.05;): KO mice spent significantly less time in wall rearing behavior than WT. As pictured in Fig.10B, 3-way ANOVA yielded a significant effect of the sex by foster dams' strain interaction on frequency of observation in wall rearing behavior ($F_{(3,351)}$= 3.617, p<0.05): in particular females reared by CD1 were more active than females reared by Balb, C57 and FVB dams (respectively: p<0.001 for CD1 and FVB; p<0.01 for Balb), whereas no difference was observed among males according to adoption. A significant main effect of foster dams' strain was also found both on time spent in and frequency observed in wall rearing behavior (respectively: $F_{(3,351)}$= 9.354, p<0.001; $F_{(3,351)}$= 12.896, p<0.001; Fig.10): in particular, regardless the sex and the genotype, mice reared by CD1 spent more time and were observed more frequently in wall rearing behavior than mice reared by either by C57 or Balb (p<0.001), and were observed more frequently in wall rearing than FVB (p<0.01); mice reared by FVB dams spent significantly more time and were observed more frequently in wall rearing behavior than mice reared by C57 (p<0.05).

Genotype significantly affected frequency of stationary sniffing behavior ($F_{(3,351)}$= 10.489, p<0.01; Fig.11B): KO showed lower stationary sniffing behavior compared to WT. Foster dams' strain significantly affected on both time spent in and frequency in stationary sniffing behavior (respectively: $F_{(3,351)}$= 3.398, p<0.05; $F_{(3,218)}$= 5.911, p<0.001; Fig.11): mice reared by CD1 dams were observed more frequently in stationary sniffing behavior compared to mice reared by either C57 or FVB (p<0.001; p<0,05). A significant effect of the sex by foster dams' strain interaction on frequency in stationary sniffing was found ($F_{(3,351)}$= 3.089, p<0.05): in particular female mice reared by CD1 dams were observed more frequently in stationary sniffing compared to female mice reared by C57, FVB and Balb dams (respectively: p<0.001; p<0.01; p<0.05), whereas no difference was observed among males according to adoption.

As shown in Fig.12, 3-way ANOVA revealed a highly significant main effect of genotype on both time spent in and frequency of walking behavior (respectively: $F_{(1,351)}$= 10.341, $p<0.01$; $F_{(1,351)}$= 13.105, $p<0.001$): KO mice spent less time in and were observed less frequently in walking behavior than WT. As pictured in Fig.12B, 3-way ANOVA yielded a significant effect of the sex by foster dams' strain interaction on frequency of observation in walking behavior ($F_{(3,351)}$= 3.432, $p<0.05$): in particular females reared by CD1 were more active than females reared by Balb, C57 ($p<0.01$) and FVB dams ($p<0.05$), whereas no difference was observed among males according to adoption. A significant main effect for foster dams' strain was also found on the frequency of walking behavior ($F_{(3,351)}$= 4.954, $p<0.01$; Fig.12B): in particular, regardless the sex and the genotype, mice reared by CD1 were significantly more active than mice reared either by C57 or Balb (respectively: $p<0.01$; $p<0.05$).

3-way ANOVA yielded a highly significant main effect of genotype on distance moved and mean velocity in the arena (respectively: $F_{(1,360)}$= 6.880, $p<0.01$; $F_{(1,360)}$=10.399, $p<0.01$; fig. 13): KO mice were less active and moved slower than WT. As shown in Fig.13, 3-way ANOVA revealed a highly significant main effect of foster dams' strain on distance moved and mean velocity in the arena (respectively: $F_{(3,360)}$= 10.653, $p<0.001$; $F_{(3,360)}$=7.587, $p<0.001$): in particular both males and females reared by CD1 dams were more active and faster in the arena compared to mice reared by Balb, C57 and FVB dams (respectively: $p<0.001$ for distance moved; $p<0.01$, $p<0.001$, $p<0.05$ for mean velocity). A significant effect was also found for the sex by foster dams' strain interaction in activity in the arena (distance moved: $F_{(3,360)}$= 5.473, $p<0.01$; mean velocity: $F_{(3,360)}$=6.106, $p<0.001$): specifically females reared by CD1 moved significantly more and faster than females reared by Balb, C57 dams ($p<0.001$) and FVB ($p<0.001$ for distance moved, $p<0.01$ for mean velocity), whereas no difference was observed among males according to adoption. Females reared by CD1 moved more and faster compared

to their male counterparts (distance moved: $p<0.05$; mean velocity: $p<0.01$), whereas this trend for a sex difference was not observed among other adoptions.

Genotype significantly affected mean velocity in the edge ($F_{(1,360)}=6.286$, $p<0.05$; Fig.14C): KO mice were slower than WT mice. Sex was significant on time spent in the edge ($F_{(1,360)}=17.017$, $p<0.001$;Fig.14A): in particular, regardless of their foster dams' strain and genotype, male mice spent more time in the edge compared to females. Remarkably, a significant effect of the sex by foster dams' strain interaction was found on the distance moved and mean velocity in the edge (respectively: $F_{(3,360)}= 6.046$, $p<0.001$; $F_{(3,360)}=6.055$, $p<0.001$): specifically females reared by CD1 moved more and faster than females reared by Balb, C57 ($p<0.001$) and FVB and (respectively: $p<0.001$ for distance moved, $p<0.01$ for mean velocity), whereas no difference was observed among males according to adoption. Females reared by CD1 moved more and faster compared to their male counterparts (distance moved: $p<0.05$; mean velocity: $p<0.01$), whereas this trend for a sex difference was not observed among other adoptions. In general, foster dams' strain was highly significant on affecting time spent in, distance moved and mean velocity in the edge (respectively: $F_{(3,360)}=3.408$, $p<0.05$; $F_{(3,360)}= 15.285$, $p<0.001$; $F_{(3,360)}= 6.187$, $p<0.001$; Fig.14): in particular mice fostered by CD1 dams moved significantly more in the edge compared to mice reared by Balb, C57 and FVB dams ($p<0.001$), they were also faster compared to Balb, C57 and FVB (respectively: $p<0.01$; $p<0.001$; $p<0.05$) and they spent more time in the edge compared to C57 ($p<0.01$).

Genotype significantly affected distance moved, latency to enter and frequency of observation in the median area (respectively: $F_{(1,360)}=12.163$, $p<0.001$; $F_{(1,360)}=5.121$, $p<0.05$; $F_{(1,360)}=14.918$, $p<0.001$; Fig.15): KO mice moved less, were observed less frequently and were quicker to enter in median area than WT mice. Sex significantly affected time spent in, distance moved, latency to enter and frequency in the median area (respectively: $F_{(1,360)}=14.595$, $p<0.001$; $F_{(1,360)}=12.163$, $p<0.001$; $F_{(1,360)}= 4.197$, $p<0.05$; $F_{(1,360)}=9.900$, $p<0.01$; Fig.15): in

particular, female mice were quicker in entering, spent more time in, moved more and were observed more often in the median area as compared to male mice. As expected, foster dams' strain was highly significant on both time spent in and mean velocity in the median area (respectively: $F_{(3,360)}$= 3,463, $p<0.05$; $F_{(3,360)}$=7.217, $p<0.001$; Fig.15): in particular mice reared by CD1 dams were faster compared to mice reared by C57 and Balb (respectively: $p<0.05$; $p<0.001$) and spent less time than C57 (0.01); FVB mice were faster than C57 ($p<0.05$).

Genotype significantly affected distance moved, latency to enter and frequency of observation in the center (respectively: $F_{(1,360)}$=9.295, $p<0.01$; $F_{(1,360)}$=15.254, $p<0.001$; $F_{(1,360)}$=11.631, $p<0.001$; Fig.16): KO mice moved less, were observed less frequently and were quicker to enter in the center than WT mice. 3-way ANOVA yielded a highly significant effect for sex by genotype interaction on time spent in the center of the arena ($F_{(1,360)}$= 4.414, $p<0.05$; Fig.16B): specifically female KO mice spent less time in the center of the arena compared to female WT ($p<0.05$), whereas male KO spent more time in the center than male WT ($p<0.01$). Distance moved was significant for sex by foster dams' strain interaction in the center ($F_{(1,360)}$= 2.740, $p<0.05$; Fig.16C): in particular, females reared by CD1 dams moved more than their male counterparts and females reared by Balb dams ($p<0.05$), whereas no sex difference was observed among the other strains and no difference was observed in males according to adoption. In line with our expectations, sex was significant on time spent in, distance moved, latency to enter and frequency of observation in the center of the arena (respectively: $F_{(1,360)}$=7.429, $p<0.01$; $F_{(1,\ 360)}$=12.905, $p<0.001$; $F_{(1,\ 360)}$= 8.236, $p<0.01$; $F_{(1,\ 360)}$=13.112, $p<0.001$; Fig.16): in particular, female mice had smaller latencies in entering, spent more time, moved more and were observed more often in the center of the arena compared to male mice.

As pictured in Fig.17, 3-way ANOVA yielded a significant effect of the sex by genotype and foster dams' strain interaction in defecation ($F_{(3,351)}$= 3.938, $p<0.01$): in particular, the number of feces in the arena of KO females reared by CD1 dams was lower compared to KO

females fostered by C57, FVB and Balb dams (respectively: $p<0.1$; $p<0.05$; $p<0.001$), whereas no difference was observed among WT females, neither in the group of the WT males nor in the KO males; interestingly, the only difference observed in relation to genotype was among males reared by FVB, the number of feces left in the arena by KO males reared by FVB was lower than their WT counterparts ($p<0.05$). A significant effect was found for the genotype by foster dams' strain interaction in defecation ($F_{(3,351)}=2.899$, $p<0.05$): in KO mice reared by CD1 and FVB dams the number of feces was lower than in KO mice reared by Balb and C57 dams (respectively: $p<0.01$; $p=0.056$), whereas no difference was observed in their WT counterparts. Also, a sex by genotype interaction was significant in defecation ($F_{(1,351)}= 4.247$, $p<0.05$): in KO males the number of feces in the arena was lower than KO females, whereas in WT sex difference was reversed. A significant result was also found for the sex by foster dams' strain interaction in defecation ($F_{(3,351)}= 4.968$, $p<0.01$): in particular in female mice reared by CD1 dams the number of feces in the arena was lower than in females mice reared by either C57 or Balb (respectively: $p<0.05$; $p<0.001$), whereas no difference was observed in males according to adoption. Lastly, I found a significant main effect of foster strain in defecation ($F_{(1,351)}= 3.076$, $p<0.05$): specifically, in mice reared by CD1 dams the number of feces in the arena was lower than in mice reared by Balb dams ($p<0.05$).

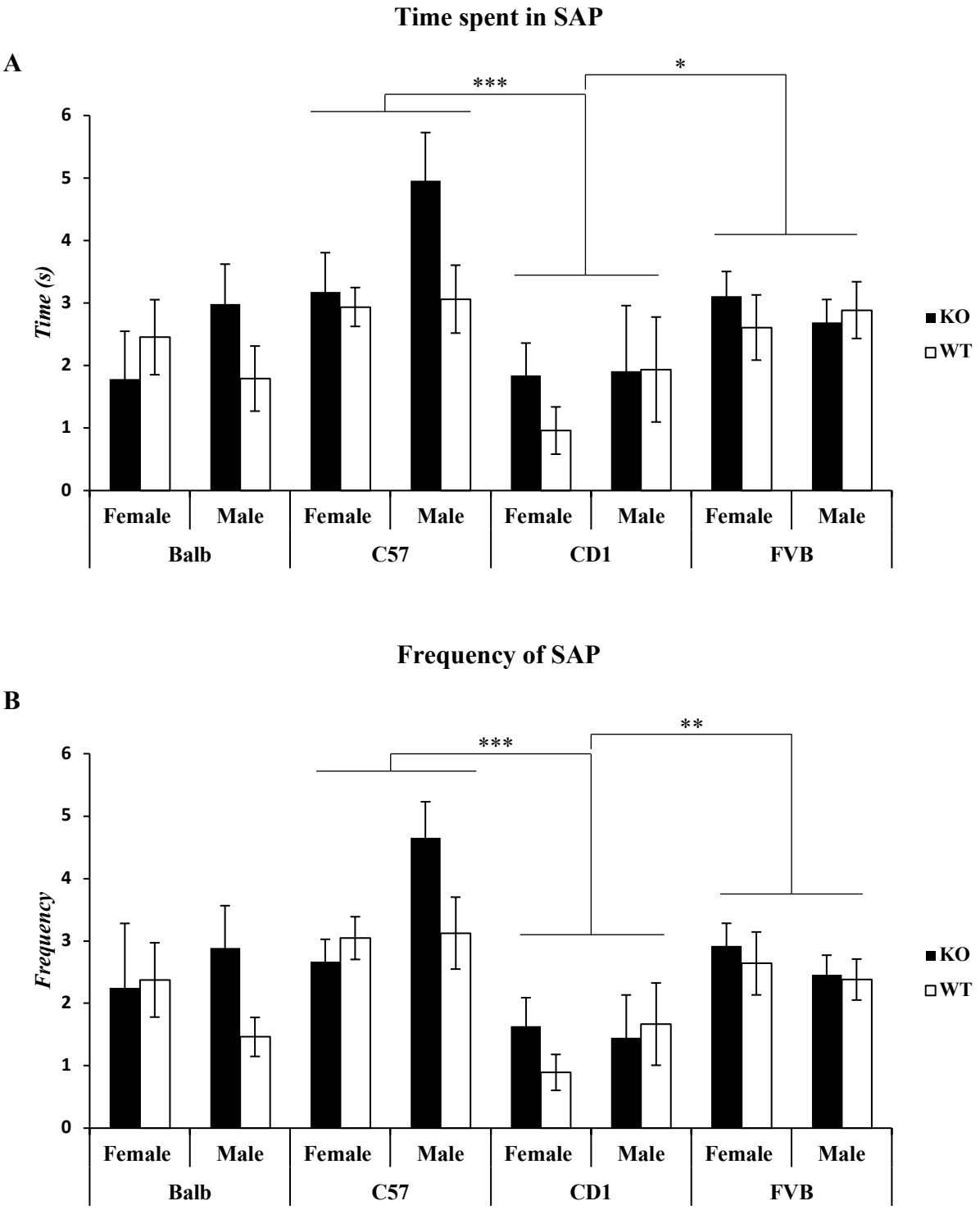


Figure 8. **Time spent in and frequency of SAP of KO and WT showed by male and female mice reared by Balb, C57, CD1 and FVB dams**. Data are shown as mean ± SEM. Mice reared by CD1 dams displayed less SAP than mice reared by both FVB and C57 on both time spent in (A) and frequency (B). * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

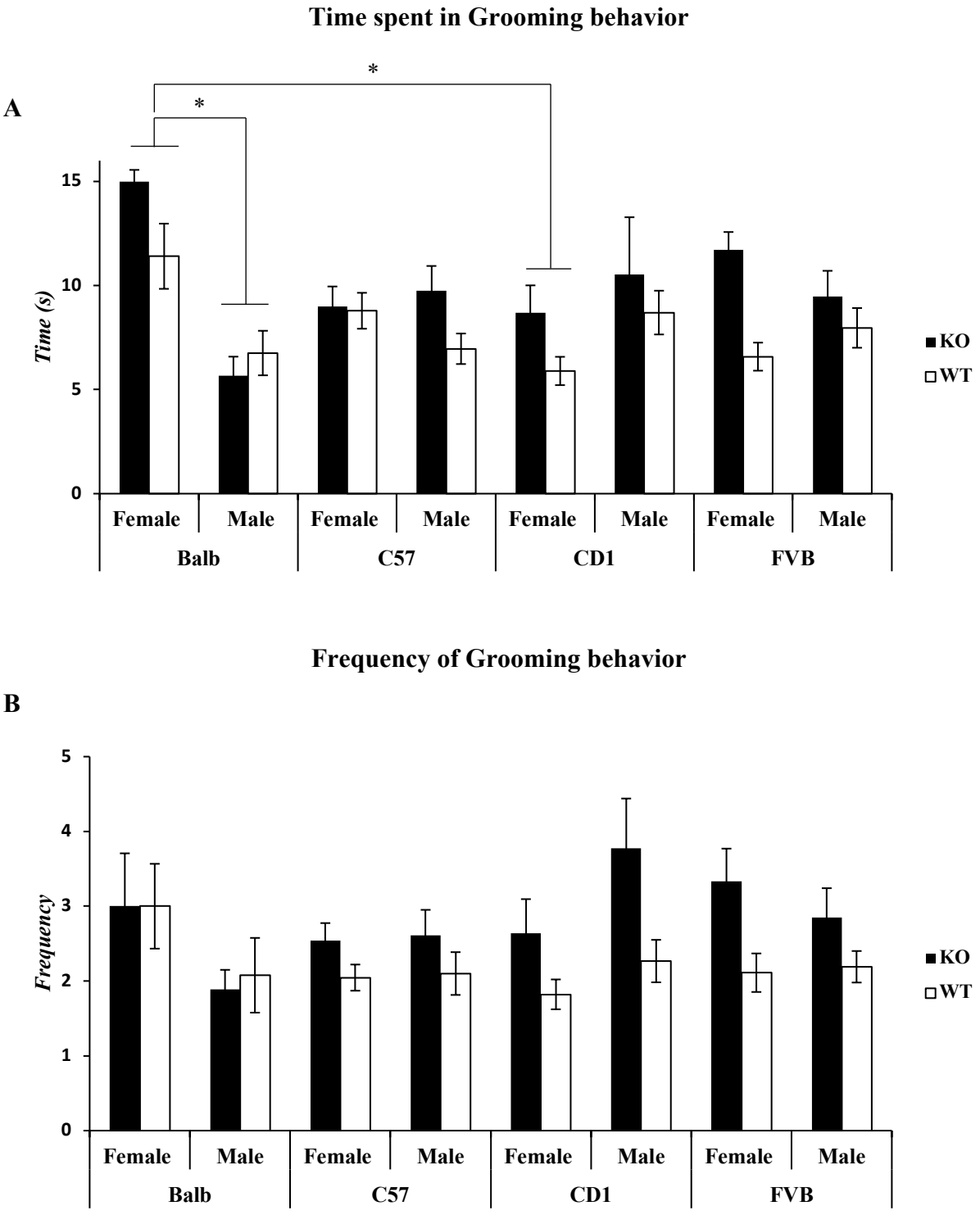


Figure 9. Time spent in and frequency of Grooming behavior of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. KO mice spent more time in (A) and were observed more frequently (B) in grooming behavior than WT mice. Female mice reared by Balb dams spent more time in grooming behavior than their male counterparts and female mice reared by CD1 dams. *p<0.05.

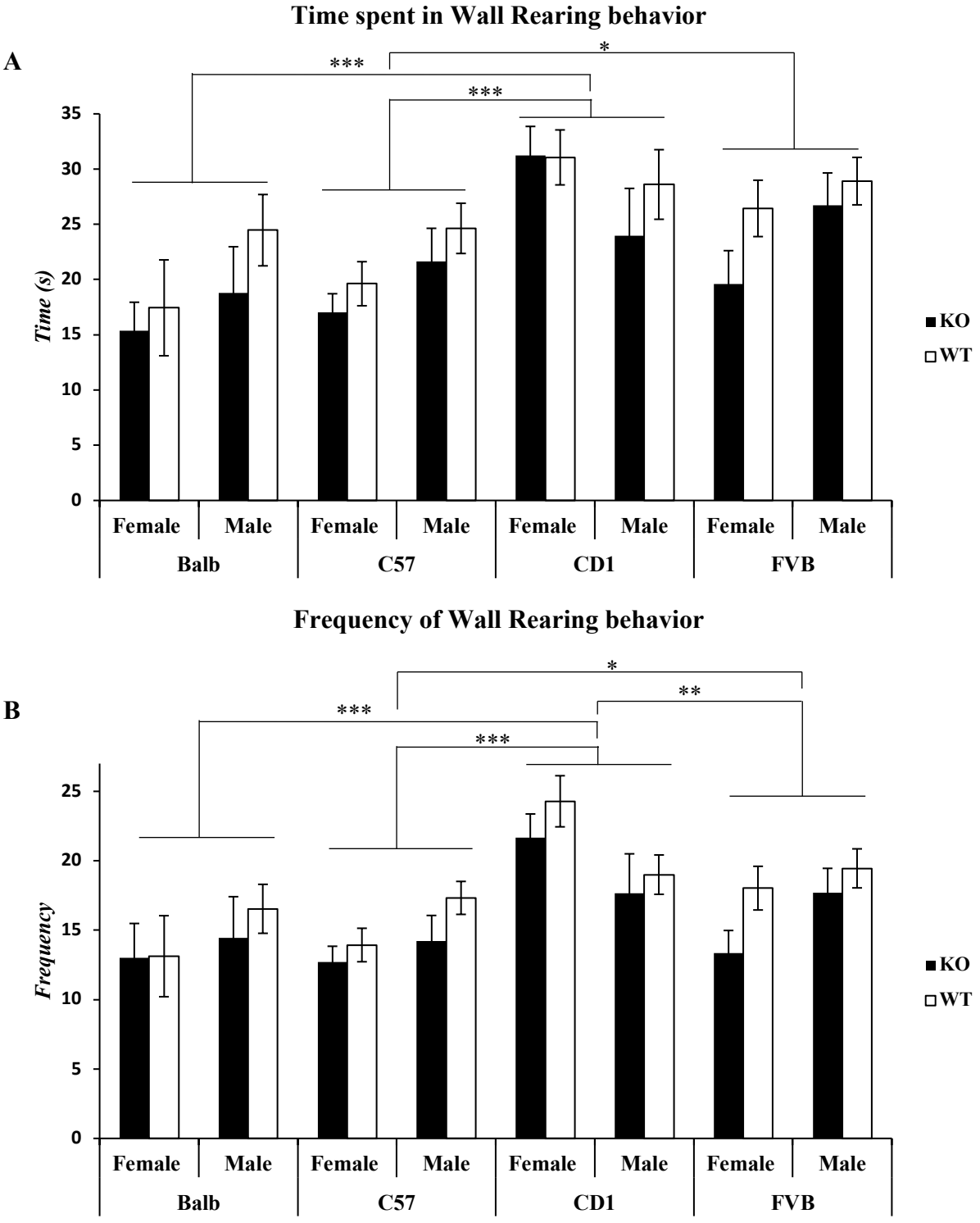


Figure 10. Time spent in and frequency of Wall rearing behavior of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. KO mice spent less time in (A) and were observed less frequently (B) in wall rearing behavior than WT mice; mice reared by CD1 dams spent more time in and were observed more frequently in wall rearing than mice reared by C57 and Balb and mice reared by CD1 dams were observed more frequently in wall rearing behavior than mice reared by FVB dams; mice reared by FVB dams spent more time in and were observed more frequently in wall rearing behavior than mice reared by C57 dams. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

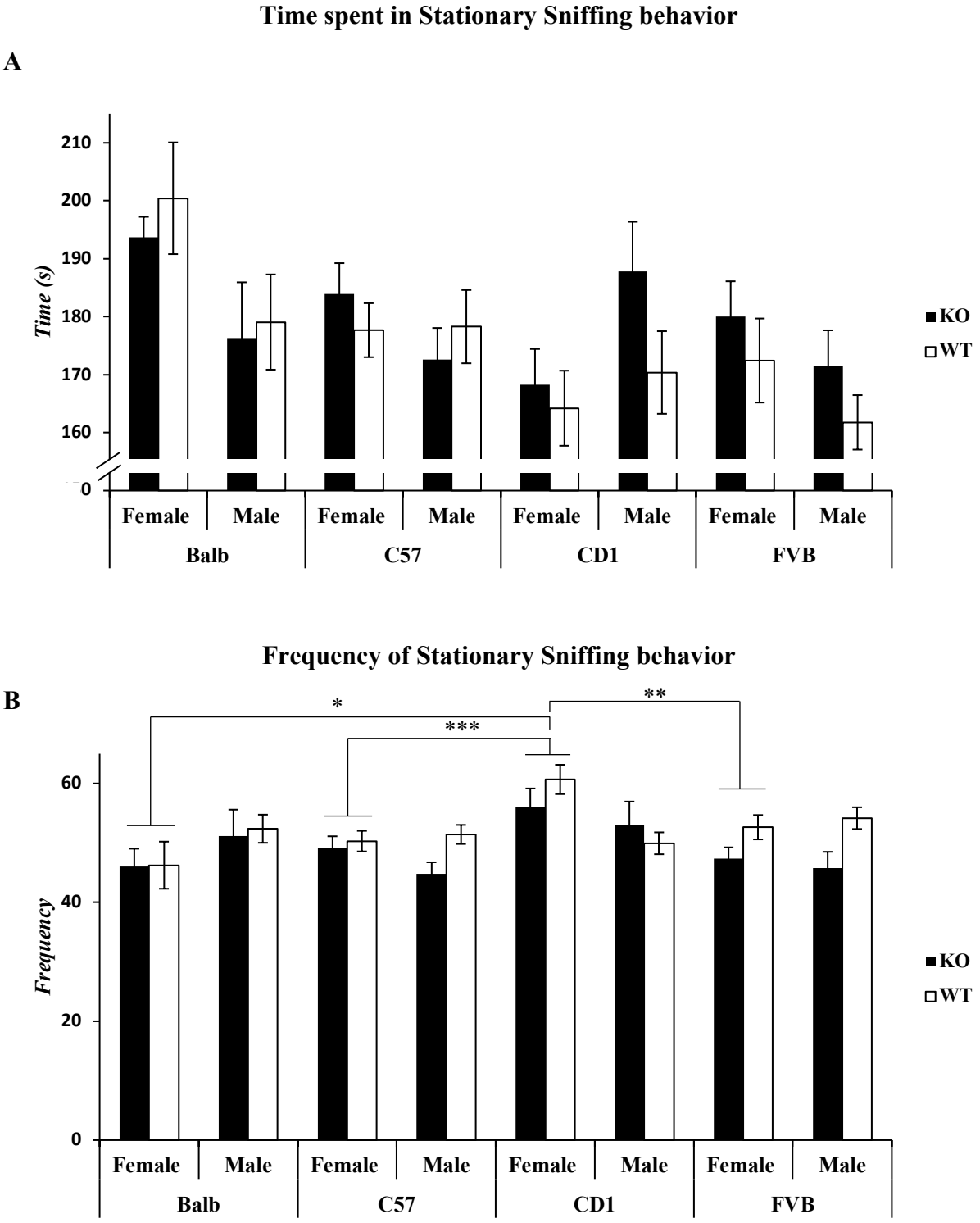


Figure 11. Time spent in and frequency of Stationary Sniffing behavior of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. Foster dams' strain was significant on both time spent in (A) and frequency (B) observed in stationary sniffing behavior: mice reared by CD1 dams were observed more frequently in stationary sniffing than mice reared by either C57 or FVB. Female mice reared by CD1 dams were observed more frequently in stationary sniffing than female mice reared by the other strains, whereas no difference was observed among males. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

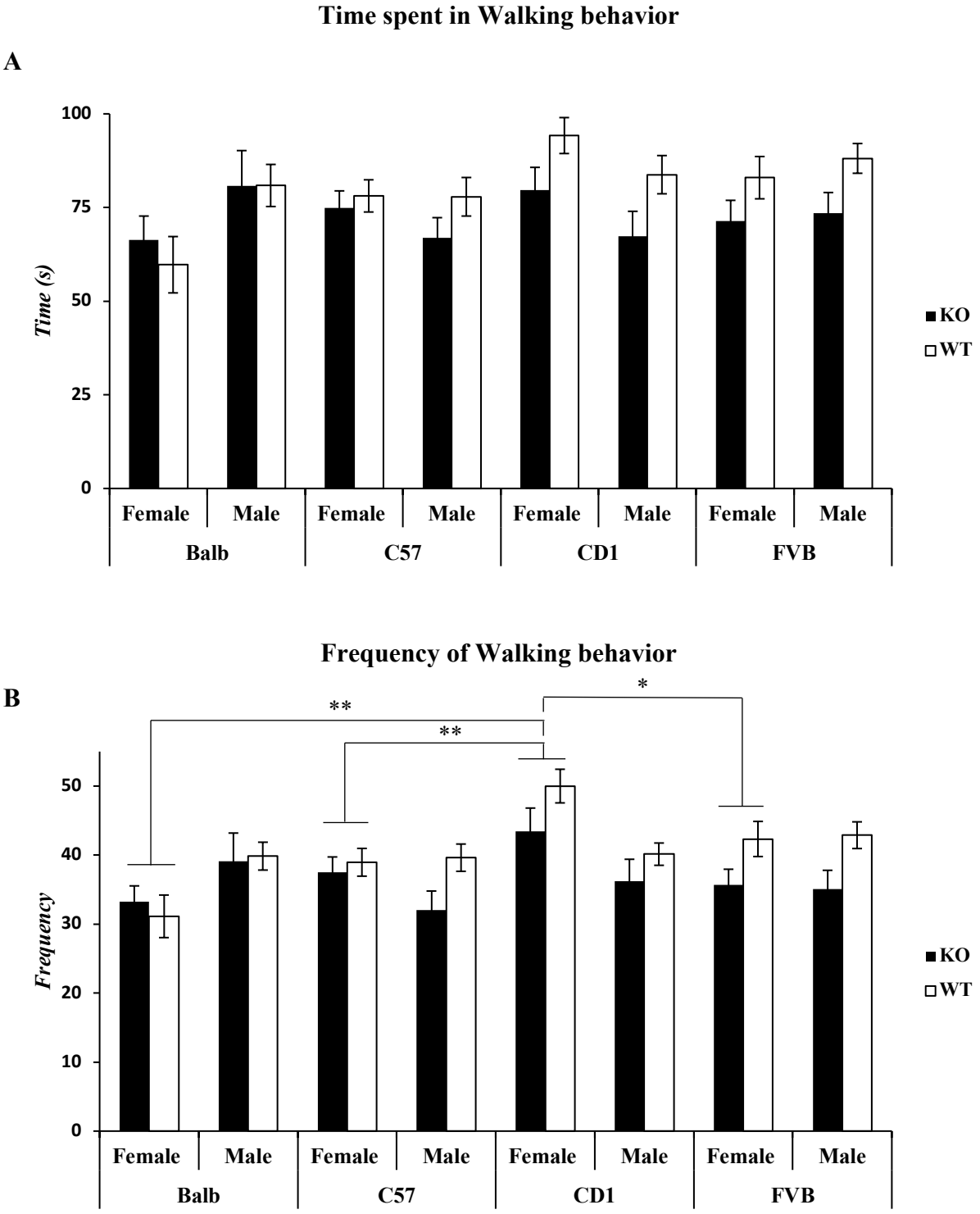


Figure 12. Time spent in and frequency of Walking behavior of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. KO mice spent less time in (A) and were observed less frequently (B) in wall rearing behavior than WT mice; females reared by CD1 were significantly more active than females reared by Balb, C57 and FVB dams, whereas no difference was observed among males according to adoption. Besides, mice fostered by CD1 dams were observed more frequently in walking than mice reared by either C57 or Balb. * $p<0.05$; ** $p<0.01$.

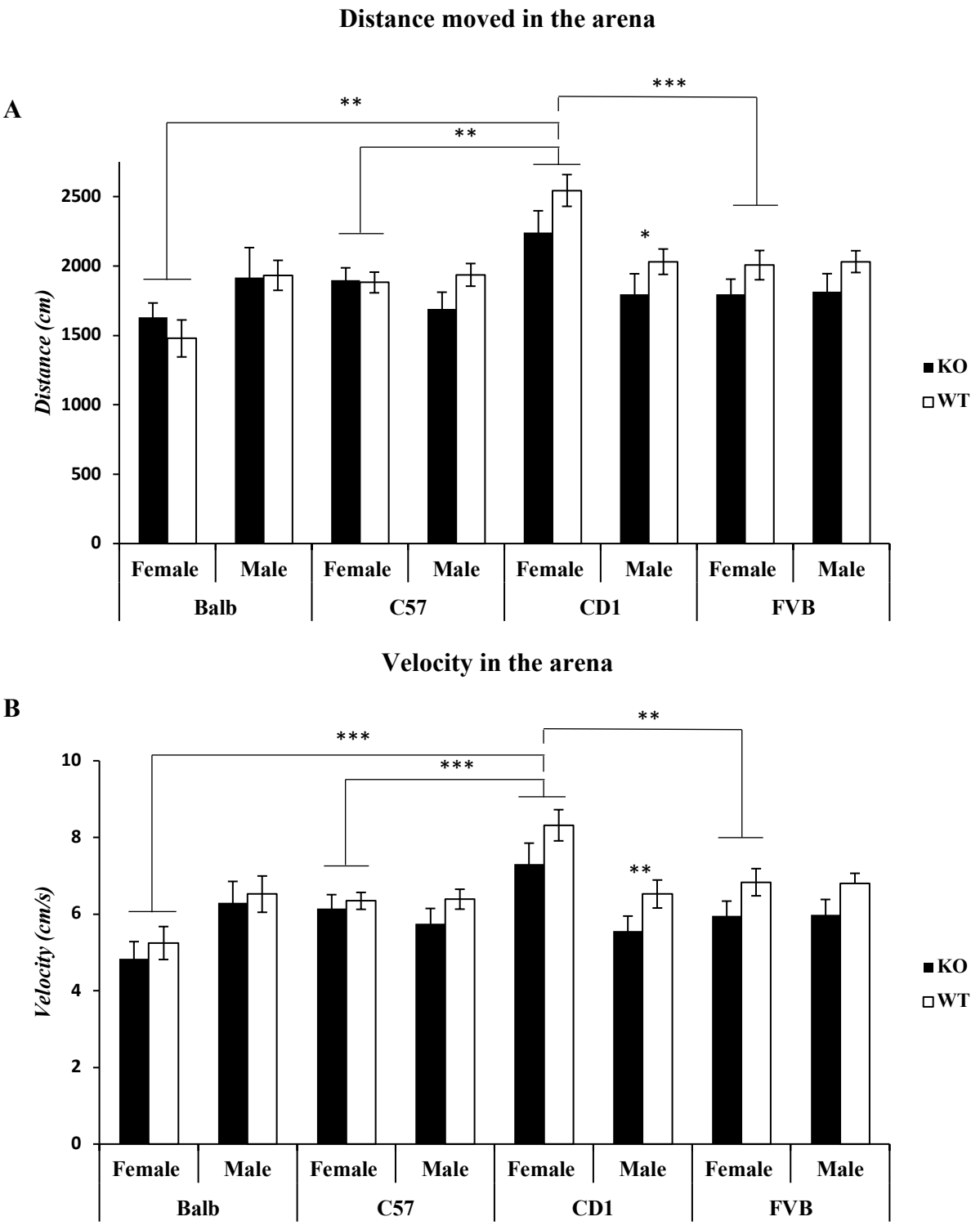
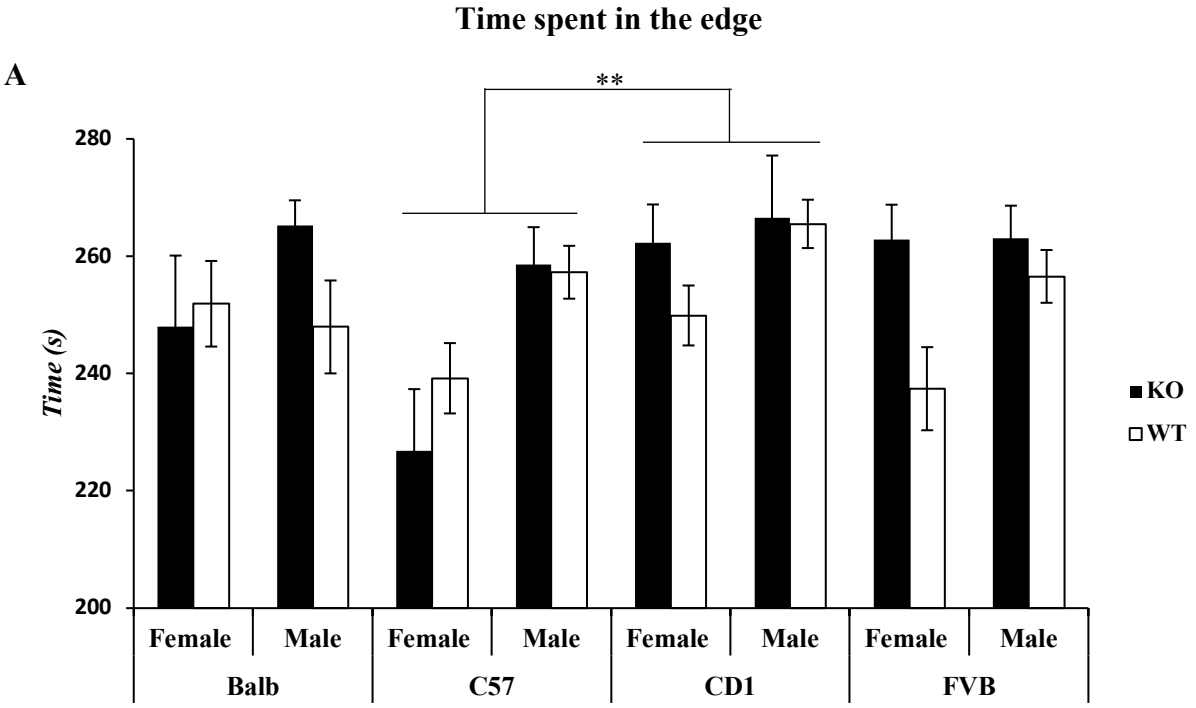
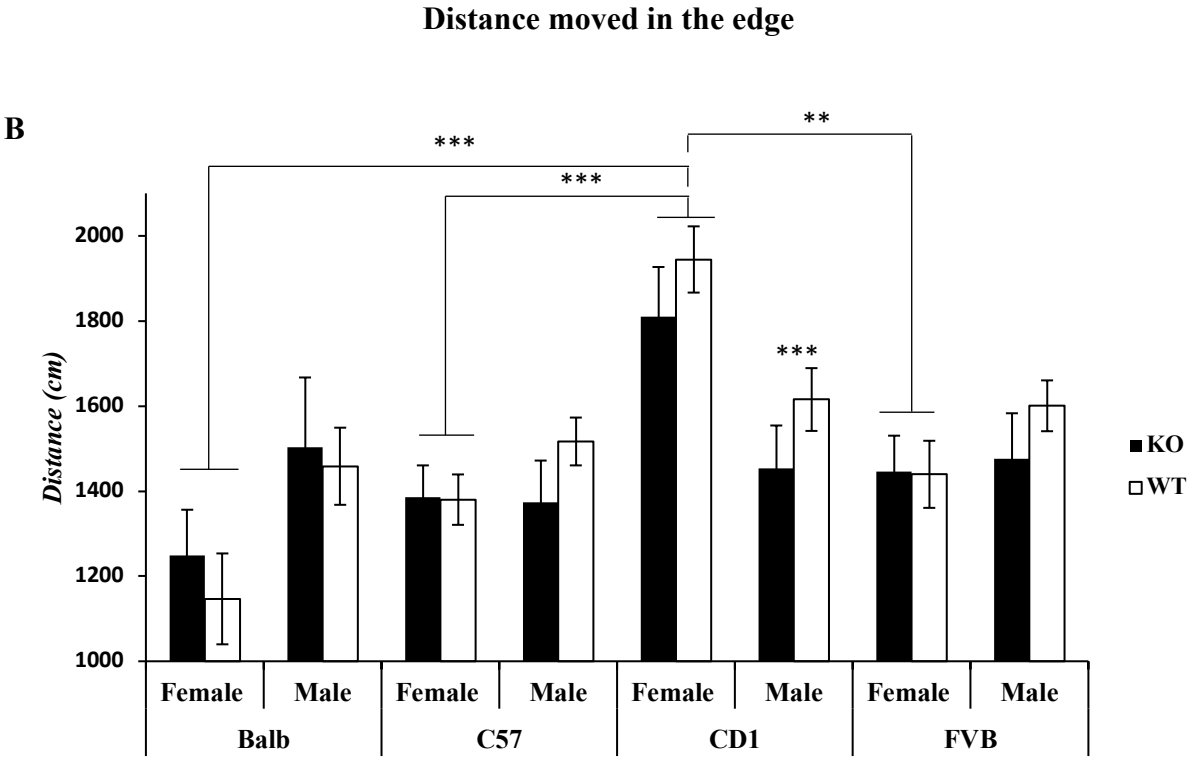


Figure 13. Distance moved and velocity in the arena of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. KO mice were significantly less active (A) and slower (B) than WT mice. Females reared by CD1 moved significantly more and faster than females reared by Balb, C57 and FVB dams, whereas no difference was observed among males according to adoption. Furthermore, CD1-fostered females moved significantly more and faster than their male counterparts. Besides, mice fostered by CD1 dams were significantly more active in the arena than mice reared by FVB, C57 and Balb. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.


Time spent in the edge
A
**
280
260
240
220
200
Time (s)
Female
Male
Female
Male
Female
Male
Female
Male
Balb
C57
CD1
FVB
KO
WT


Distance moved in the edge
B
***
***
**
***
2000
1800
1600
1400
1200
1000
Distance (cm)
Female
Male
Female
Male
Female
Male
Female
Male
Balb
C57
CD1
FVB
KO
WT

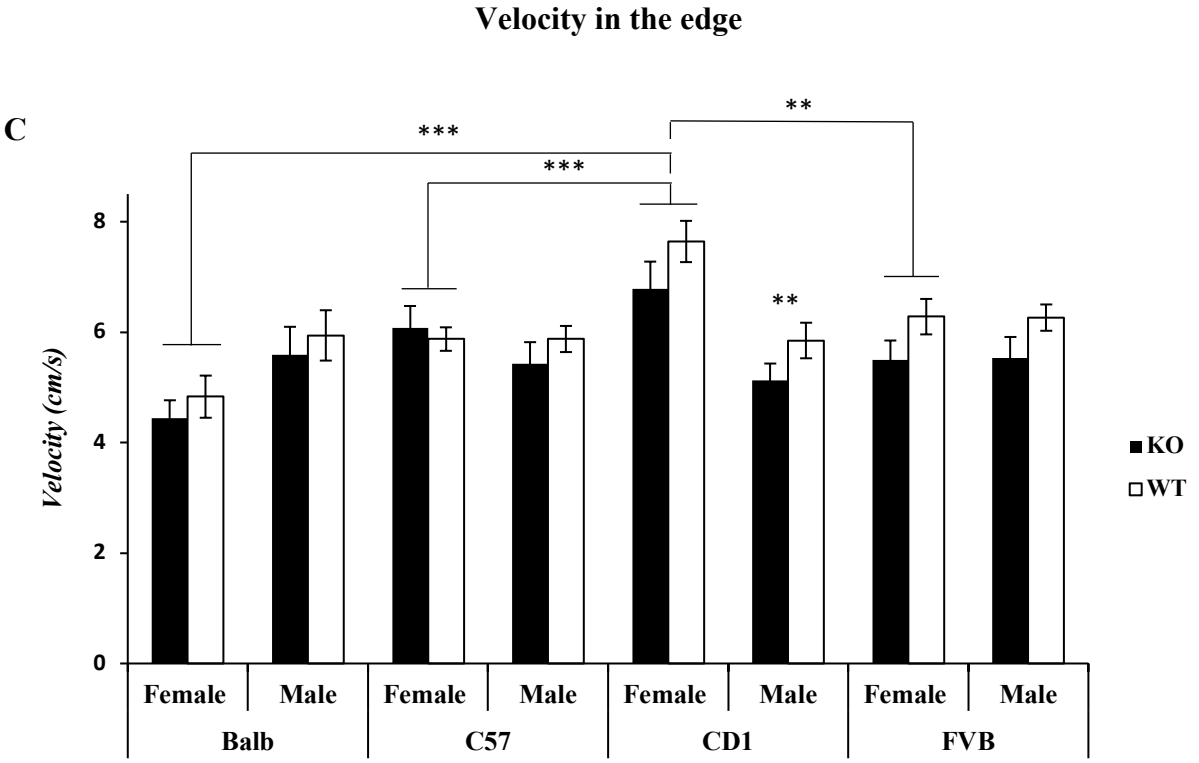
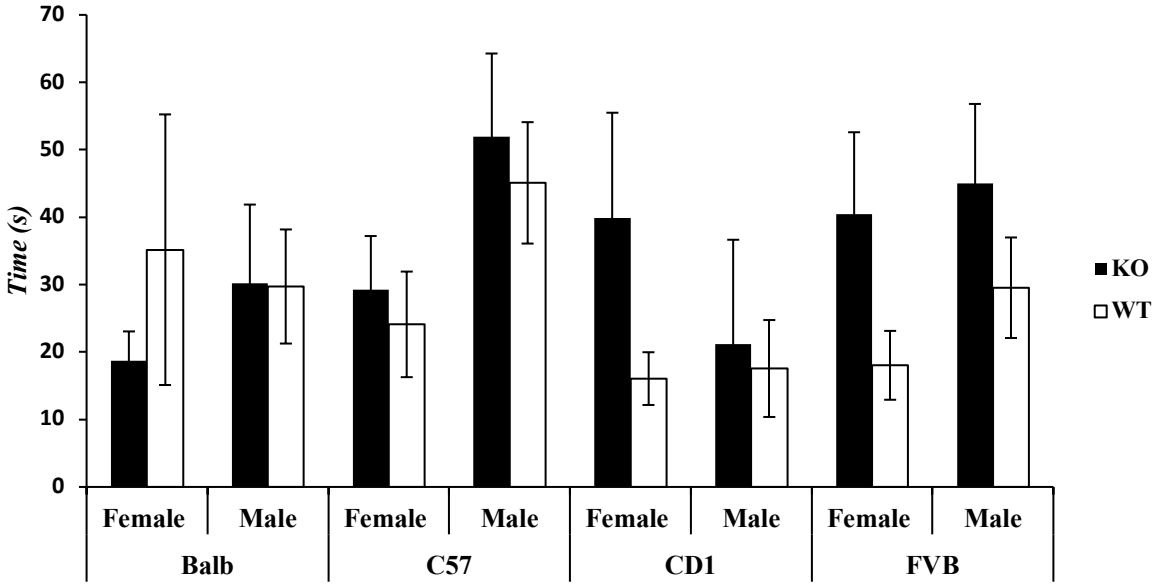


Figure 14. Time spent in, distance moved and velocity in the Edge of the arena of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. KO mice were slower than WT mice (C); male mice spent more time in the edge compared to females (A). Females reared by CD1 significantly moved more (B) and faster (C) than females reared by Balb, C57 and FVB dams, whereas no difference was observed among males according to adoption. Furthermore, CD1-fostered females moved significantly more and faster than their male counterparts. Besides, mice fostered by CD1 dams were significantly more active in the arena than mice reared by FVB, C57 and Balb. Mice fostered by CD1 dams moved significantly more and were faster in the edge compared to mice reared by Balb, C57 and FVB dams, and they spent more time in the edge compared to C57. ** $p<0.01$; *** $p<0.001$.

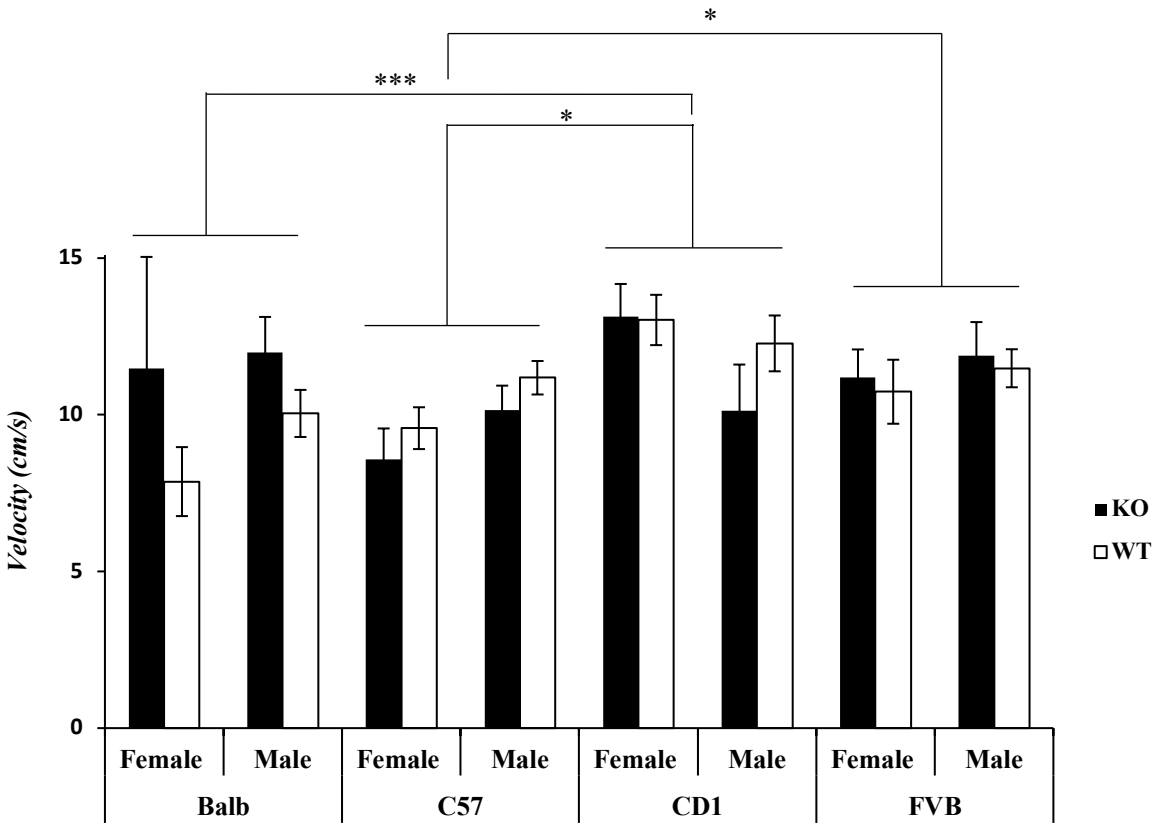
Latency in entering the Median area

A



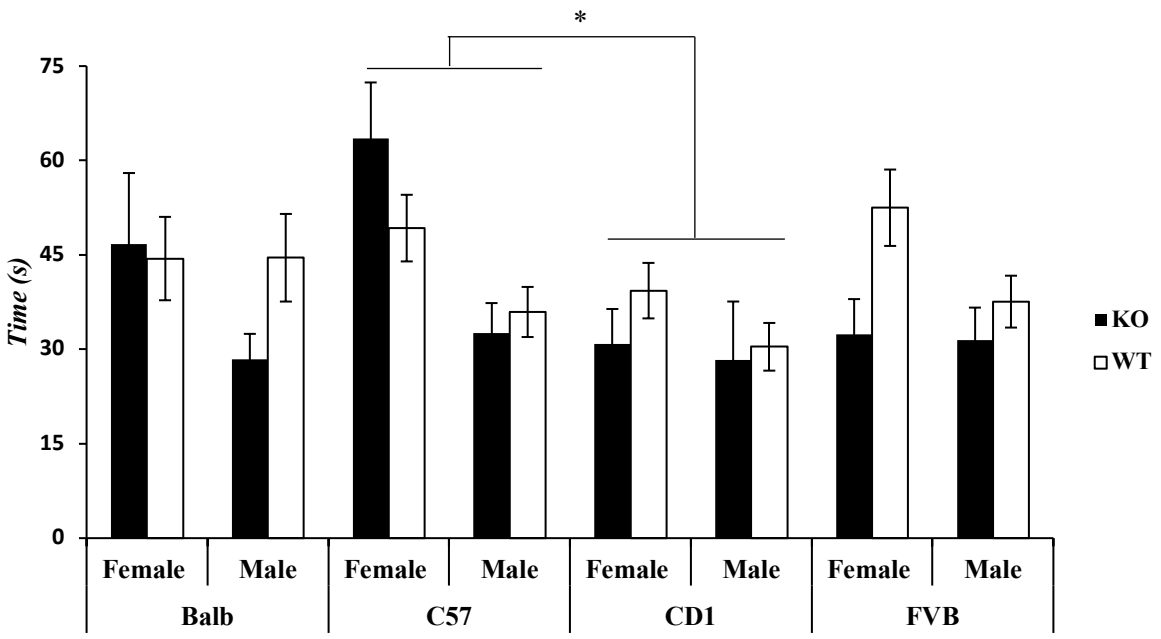
Velocity in the Median area

B



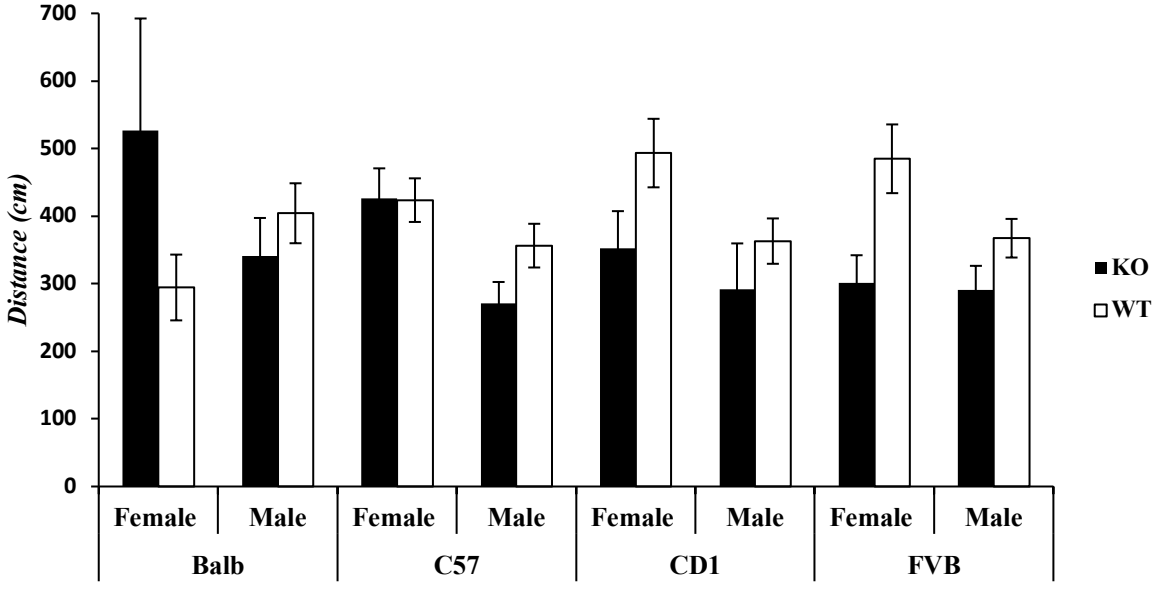
Time spent the Median area

C



Distance moved in the Median area

D



Frequency in the Median area

E

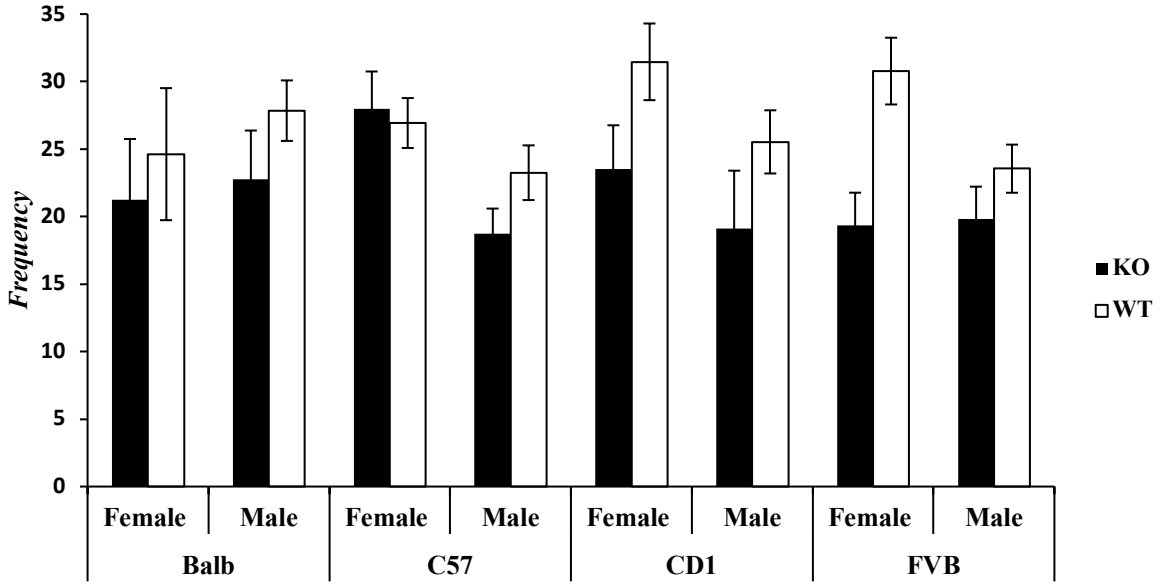
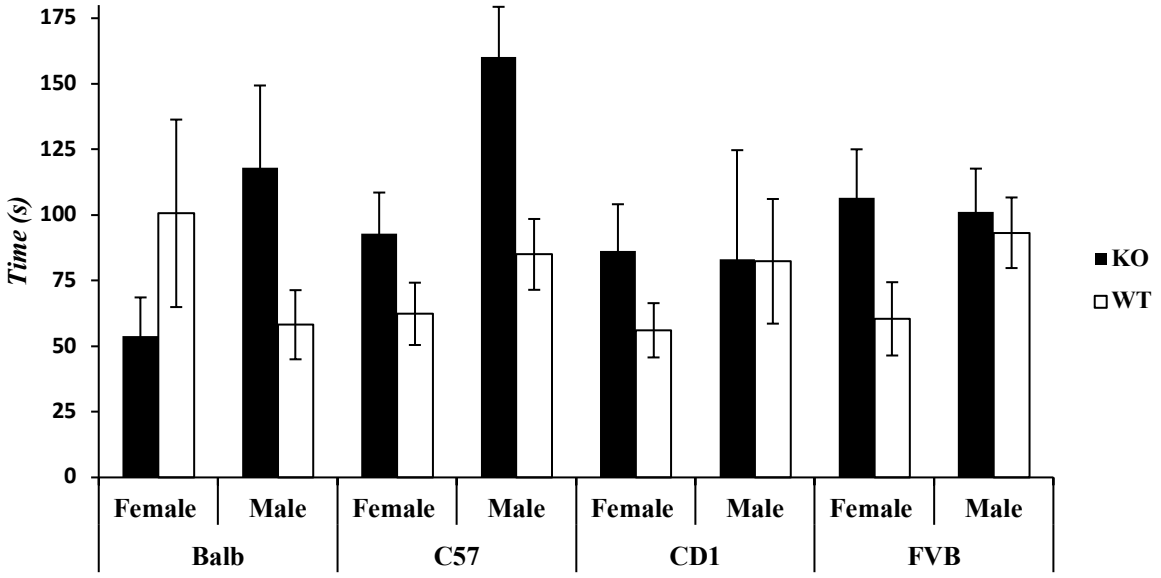


Figure 15. Latency to enter in, velocity, time spent in, distance moved and frequency in the Median area of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. KO mice moved less, were observed less frequently and were quicker to enter in median area than WT mice. Female mice were quicker in entering (A), spent more time in (C), moved more (D) and were observed more often (E) in the median area as compared to male mice. Mice reared by CD1 dams were faster compared to mice reared by C57 and Balb (B) and spent less time than C57; FVB mice were faster than C57. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

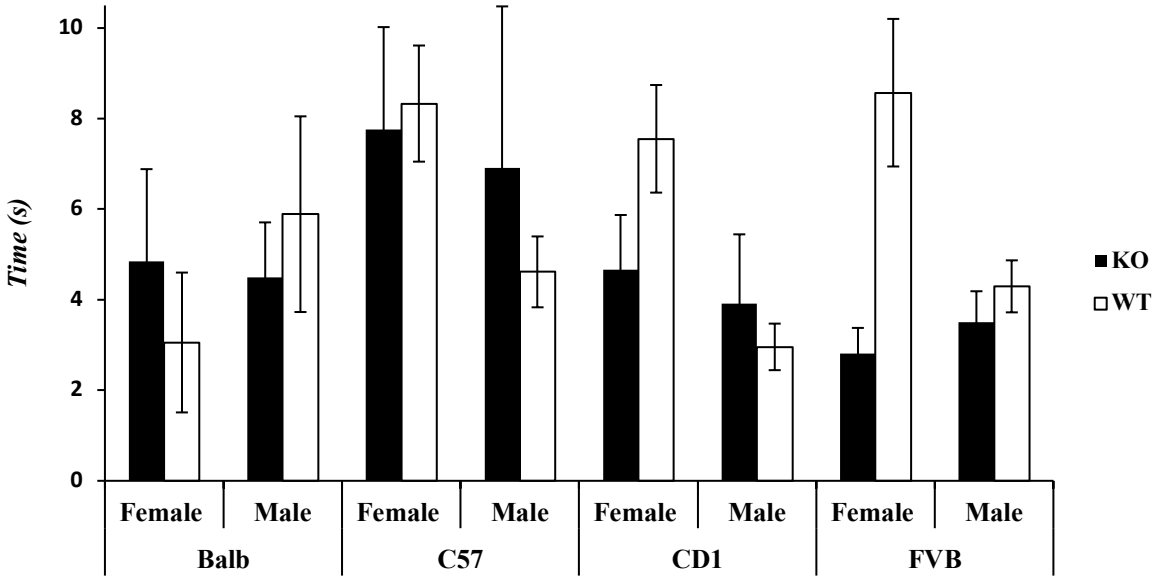
Latency in entering the Center

A



Time spent the Center

B



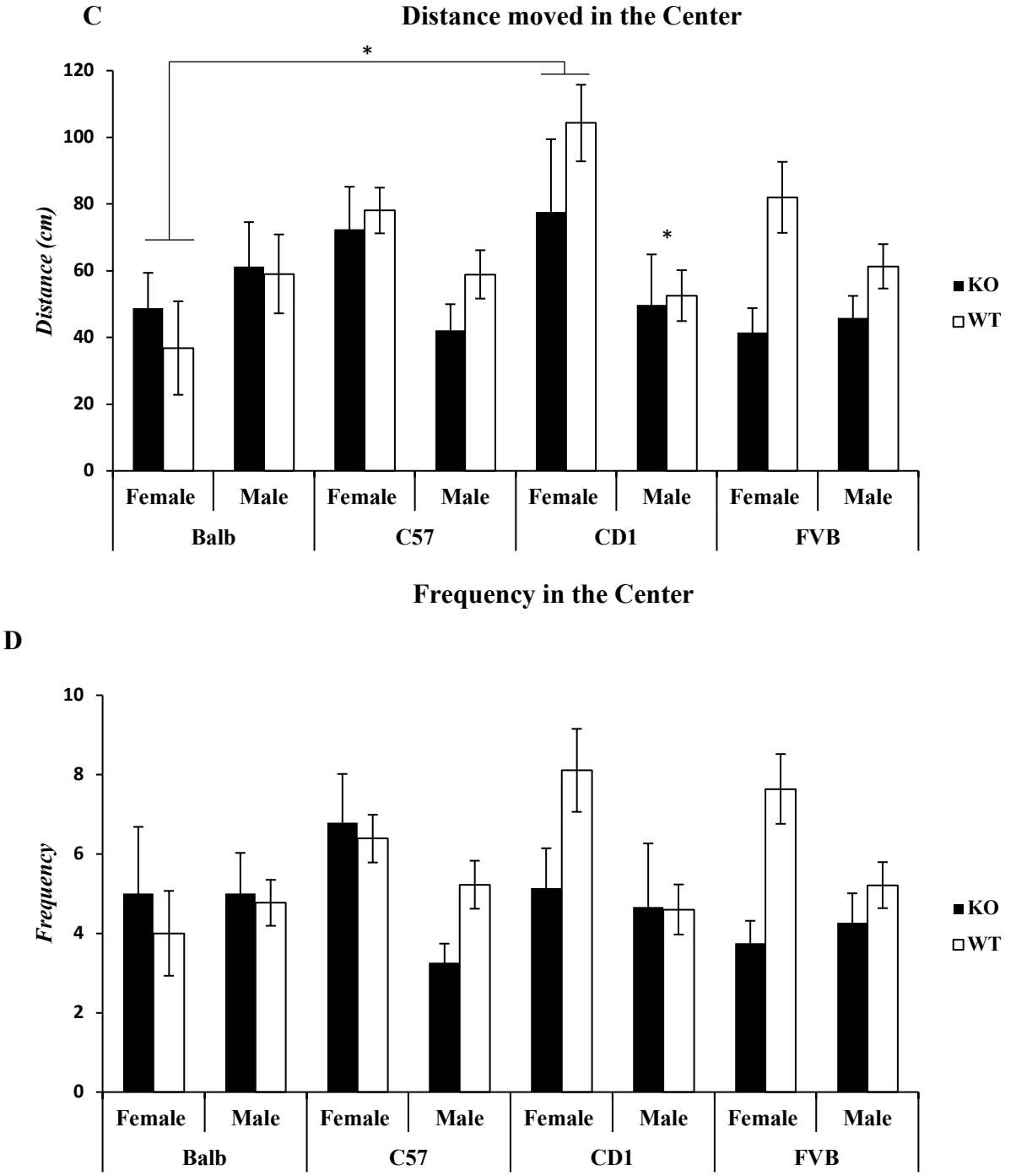


Figure 16. Latency to enter in, time spent in, distance moved and frequency in the Center of the arena of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. KO mice were significantly quicker to enter in (A), moved less (C), were observed less frequently (D) at the center than WT mice. Female KO mice spent less time in the center of the arena compared to female WTs, whereas male KO spent more time in the center than male WT (B). Females reared by CD1 dams moved more than their male counterparts and females reared by Balb dams, whereas no sex difference was observed among the other strains and no difference was observed in males according to adoption. Female mice had smaller latencies in entering, spent more time, moved more, and were observed more often in the center of the arena compared to male mice. * $p<0.05$

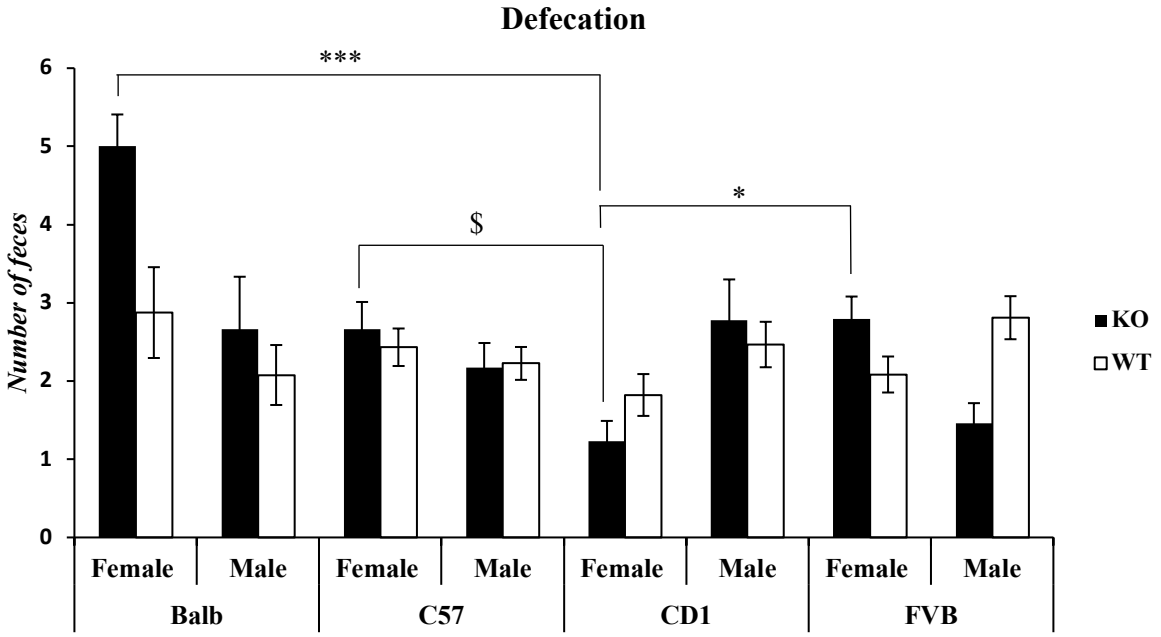


Figure 17. Number of feces in the arena after the five-minute OF test of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. The number of feces in the arena of KO reared by CD1 dams was lower compared to KO females reared by C57, FVB and Balb dams whereas no difference was observed among WT females, neither in the group of the WT males nor in the KO males; interestingly, the only difference observed in relation to genotype was among males reared by FVB, the number of feces left in the arena by KO males reared by FVB was lower than their WT counterparts, in KO mice reared by CD1 and FVB dams the number of feces was lower than in KO mice reared by Balb dams ,whereas no difference was observed in their WT counterparts. In KO males, the number of feces in the arena was lower than KO females, whereas in WT sex difference was reversed. in female mice reared by CD1 dams the number of feces in the arena was lower than in female mice reared by either C57 or Balb, whereas no difference was observed in males according to adoption. $ $p<0.1$; * $p<0.05$; *** $p<0.001$.

4.1.2 2-way ANOVA on data from WT mice

Without taking into account the effect of gene inactivation, I conducted a preliminary 2-way ANOVA on the wild type group alone to assess the effects of sex and foster dams' strain on the overall population on the same behavioral measures as in chapter 4.1.1.

2-way ANOVA yielded a significant main effect of foster dams' strain on both time spent in and frequency of observation of SAP (respectively: $F_{(3,218)}= 3.864$, $p<0.05$; $F_{(3,218)}= 5.703$, $p<0.001$; Fig.18): in particular regardless their sex, mice reared by CD1 dams spent significantly less time and were observed less frequently in SAP compared to mice reared by either FVB or C57 dams (respectively: $p<0.05$ for FVB; $p<0.01$ for C57 for time spent in; $p<0.001$ for C57 for frequency observed in).

2-way ANOVA yielded a significant effect of the sex by foster dams' strain interaction on time spent in grooming behavior ($F_{(3,218)}= 3.99$, $p<0.01$; Fig. 19): in particular as pictured in Fig. 8, female mice reared by Balb dams spent more time in grooming compared to their male counterparts, while in the other strains this sex difference was either reversed (CD1) or null (C57 and FVB).

Foster dams' strain was highly significant on both time spent in and frequency of wall rearing behavior (respectively: $F_{(3,218)}= 4.97$, $p<0.01$; $F_{(3,218)}= 7.612$, $p<0.001$; Fig.20). In particular mice reared by CD1 dams spent more time in wall rearing compared to mice reared by either Balb or C57 dams ($p<0.01$). Similarly, mice reared by CD1 were observed more frequently in wall rearing compared to mice reared by either C57, Balb, FVB (respectively: $p<0.001$; $p<0.01$; $p<0.1$). Mice reared by FVB spent more time in wall rearing behavior compared to the mice reared by C57 ($p<0.05$).

Foster dams' strain was significant on both time spent in and frequency of stationary sniffing behavior (respectively: $F_{(3,218)}= 3.197$, $p<0.05$; $F_{(3,218)}= 3.185$, $p<0.05$; Fig.21): mice reared by Balb dams tended to spend more time in stationary sniffing compared to mice reared

by either FVB or CD1($p<0.1$) and mice reared by CD1 dams were observed more frequently in stationary sniffing behavior compared to mice reared by C57 ($p<0.05$). A significant effect was also found for the sex by foster dams' strain interaction on the frequency of observation in stationary sniffing ($F_{(3,218)}= 3.605$, $p<0.05$; Fig.21B): specifically the observation of the frequency of stationary sniffing was higher in females reared by CD1 dams than their male counterparts ($p<0.1$) and females reared by either Balb or C57 dams (respectively: $p<0.05$; $p<0.01$), whereas no difference was observed among males according to adoption.

As shown in Fig.22, 2-way ANOVA revealed a significant main effect of foster dams' strain on both time spent in and frequency of observation of walking behavior (respectively: $F_{(3,218)}= 3.095$, $p<0.05$; $F_{(3,218)}= 4.361$, $p<0.01$): in particular both males and females reared by CD1 dams were more active compared to mice reared by either Balb or C57 dams ($p<0.10$ for time spent in walking; $p<0.05$ for frequency of walking). As pictured in Fig.22B, 2-way ANOVA yielded a significant effect of the sex by foster dams' strain interaction on frequency of observation of walking behavior ($F_{(3,218)}= 2.794$, $p<0.05$): in particular females reared by CD1 were more active than females reared by either Balb or C57 dams ($p<0.01$), whereas no difference was observed among males according to adoption.

As shown in Fig.23, 2-way ANOVA revealed a highly significant main effect of foster dams' strain on distance moved and mean velocity in the arena (respectively: $F_{(3,218)}= 8.941$, $p<0.001$; $F_{(3,218)}= 6.276$, $p<0.001$): in particular both males and females reared by CD1 dams were more active and faster in the arena compared to mice reared by either Balb or C57 dams ($p<0.001$) and FVB ($p<0.01$ for distance moved; $p<0.1$ for mean velocity). A significant effect was also found for the sex by foster dams' strain interaction (distance moved: $F_{(3,218)}= 4.420$, $p<0.01$; mean velocity: $F_{(3,218)}=4.037$, $p<0.01$): specifically females reared by CD1 moved more and faster than females reared by either Balb or C57 dams ($p<0.001$) and FVB ($p<0.01$ for distance moved), whereas no difference was observed among males according to adoption.

Females reared by CD1 tended to move more and faster compared to their male counterparts ($p<0.1$), whereas this trend for a sex difference was not observed among other adoptions.

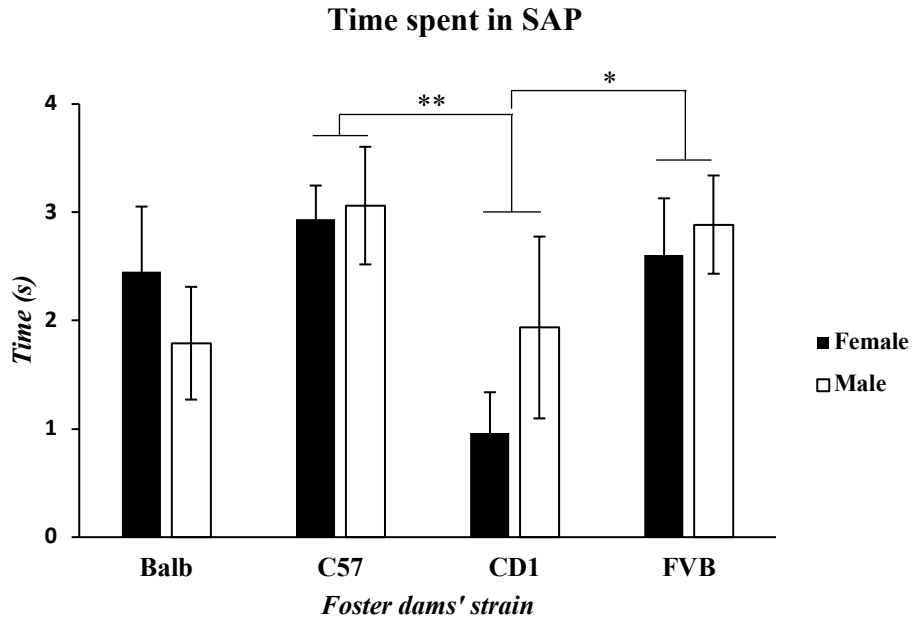
Sex was significant on time spent in the edge ($F_{(1,218)}=11.713$, $p<0.001$;Fig.24X): in particular, regardless of their foster dams' strain, male mice spent more time in the edge compared to females. Remarkably, a significant effect of the sex by foster dams' strain interaction was found on the distance moved and mean velocity in the edge (respectively: $F_{(3,218)}= 4.558$, $p<0.01$; $F_{(3,218)}=4.44$, $p<0.01$; Fig.24): female mice reared by CD1 dams moved faster than their male counterparts ($p<0.05$), whereas no sex difference was observed among other adoptions. Besides, females reared by CD1 moved more and faster than females reared by either Balb or C57 dams ($p<0.001$) and FVB ($p<0.001$ for distance moved; $p<0.05$ for mean velocity); whereas no difference was observed among males according to their adoption. In general, foster dams' strain was highly significant on affecting distance moved and mean velocity in the edge (respectively: $F_{(3,218)}= 11.999$, $p<0.001$; $F_{(3,218)}= 5.67$, $p<0.001$; Fig.24): in particular mice reared by CD1 dams moved significantly more in the edge compared to mice reared by Balb, C57 and FVB dams ($p<0.001$) and they were also faster compared to C57 and Balb ($p<0.001$).

Sex was significant on time spent, distance moved, latency to enter and frequency in the median area (respectively: $F_{(1,218)}=7.791$, $p<0.01$; $F_{(1,218)}= 7.873$, $p<0.01$; $F_{(1,218)}= 4.531$, $p<0.05$; $F_{(1,218)}=8.149$, $p<0.01$; Fig.25): in particular, female mice were quicker (smaller latencies) in entering, spent more time, moved more and were observed more often in the median area compared to male mice. As expected, foster dams' strain was highly significant on mean velocity in the median area ($F_{(3,218)}= 4.717$, $p<0.01$; Fig.25B): in particular mice reared by CD1 dams were faster compared to mice reared by C57 and Balb ($p<0.05$; $p<0.01$).

Sex was significant on time spent in, distance moved, mean velocity, latency to enter and frequency of observation in the center of the arena (respectively: $F_{(1,218)}=14.587$, $p<0.001$;

$F_{(1,218)}$=13.044, $p<0.001$; $F_{(1,218)}$=5.057, $p<0.05$; $F_{(1,218)}$=4.136, $p<0.05$; $F_{(1,218)}$=12.128, $p<0.001$; Fig.26): in particular, female mice had smaller latencies in entering, spent more time, moved more, faster and were observed more often in the center of the arena compared to male mice.

A



B

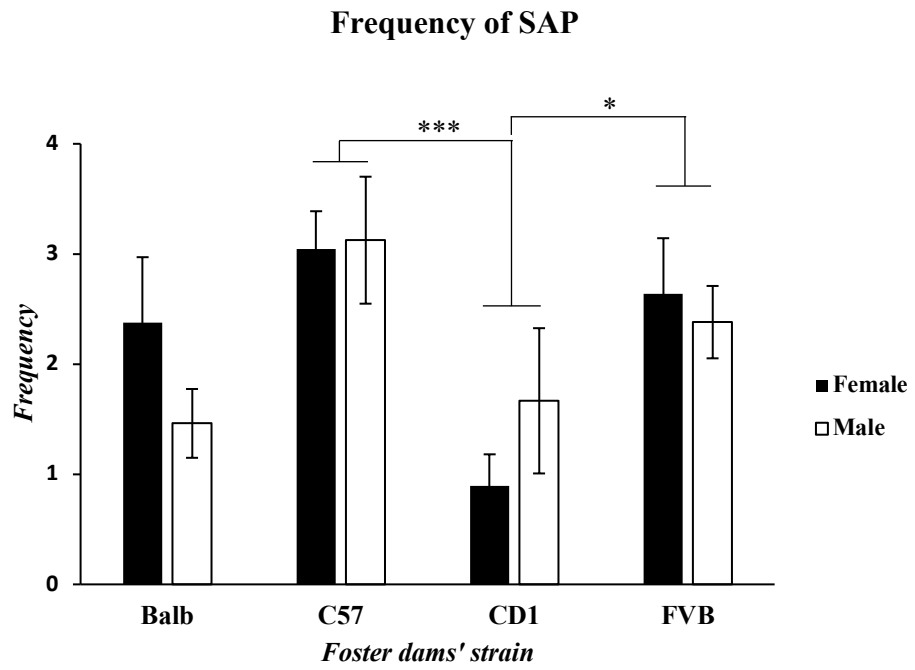


Figure 18. Time spent in and frequency of SAP male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. Mice reared by CD1 dams spent significantly less time (A) and were observed less frequently (B) in SAP compared to mice reared by either FVB or C57 dams. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

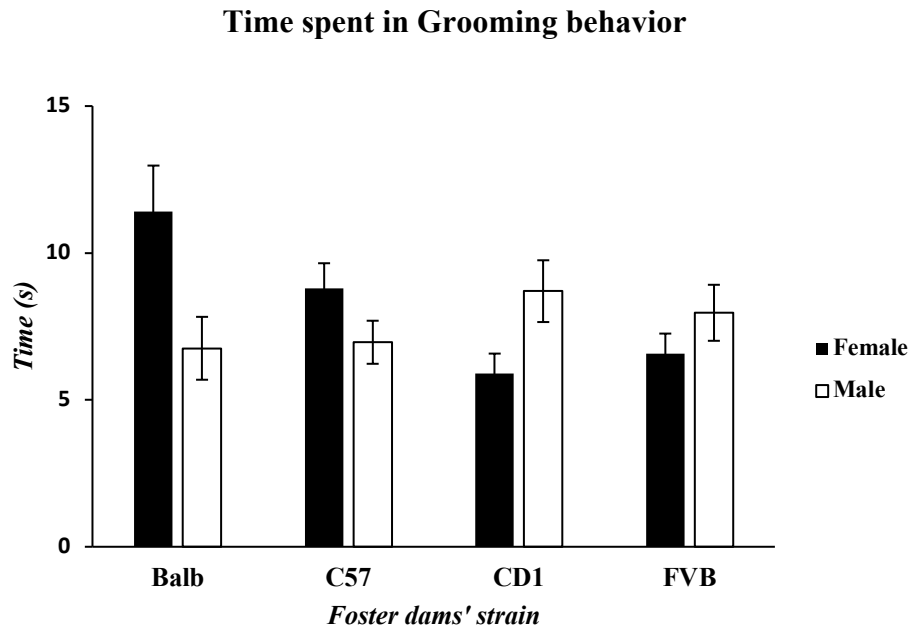
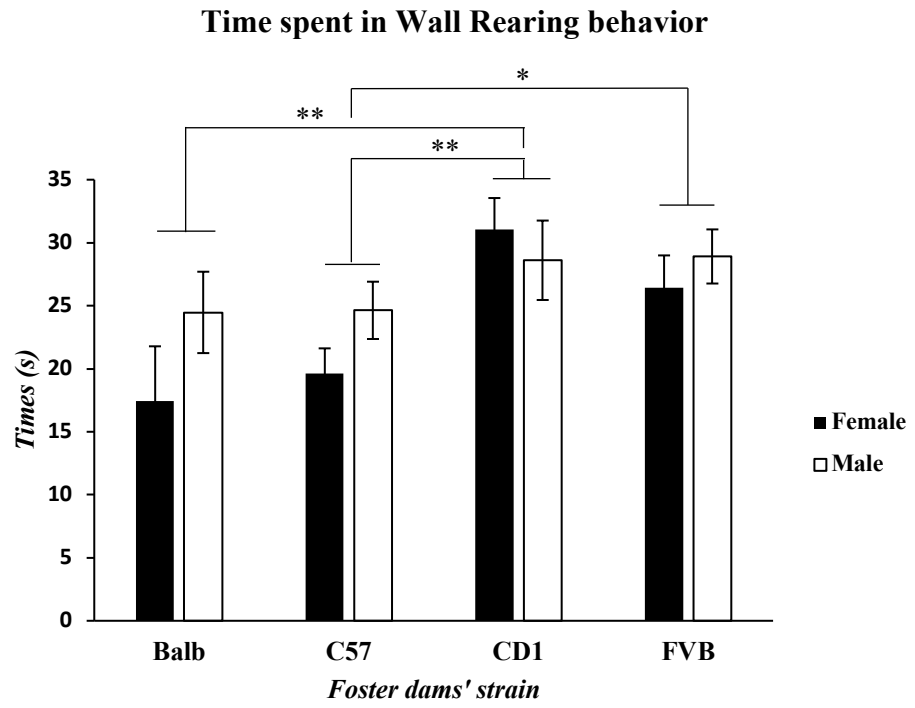


Figure 19. Time spent in Grooming behavior of male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. female mice reared by Balb dams spent more time in grooming compared to their male counterparts, while in the other strains this sex difference was either reversed (CD1) or null (C57 and FVB).

A



B

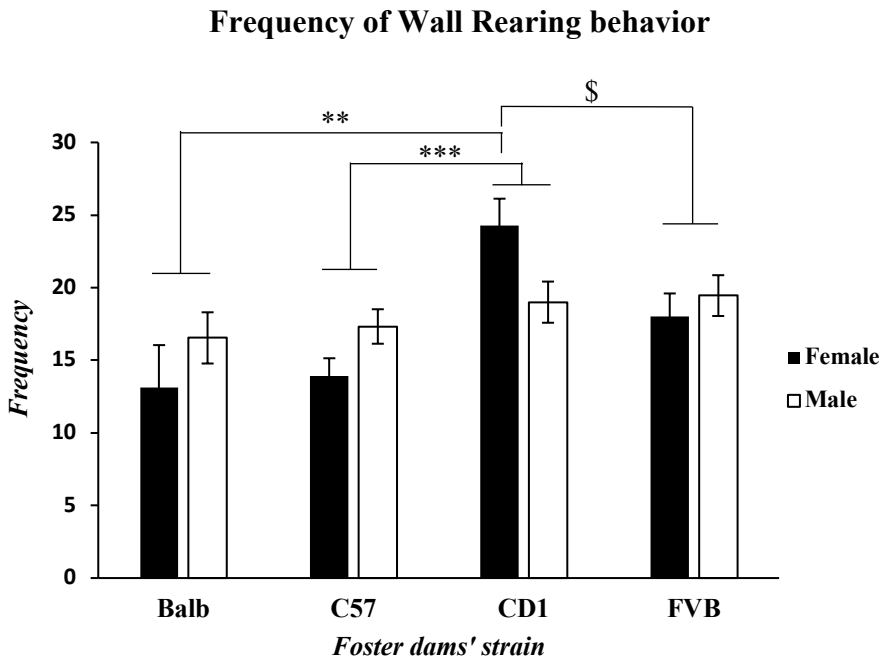
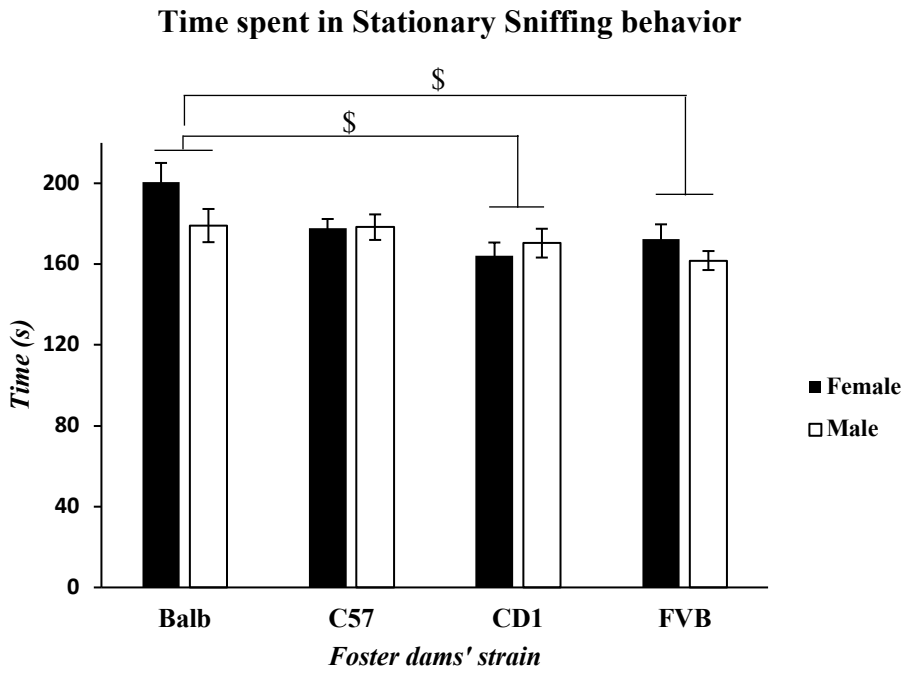


Figure 20. Time spent in and frequency of Wall Rearing behavior of male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. Mice reared by CD1 dams spent more time (A) in wall rearing compared to mice reared by either Balb or C57 dams. Similarly, mice reared by CD1 were observed more frequently (B) in wall rearing compared to mice reared by either C57, Balb, FVB. Mice reared by FVB spent more time in wall rearing behavior compared to the mice reared by C57. $ $p<0.1$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

A



B

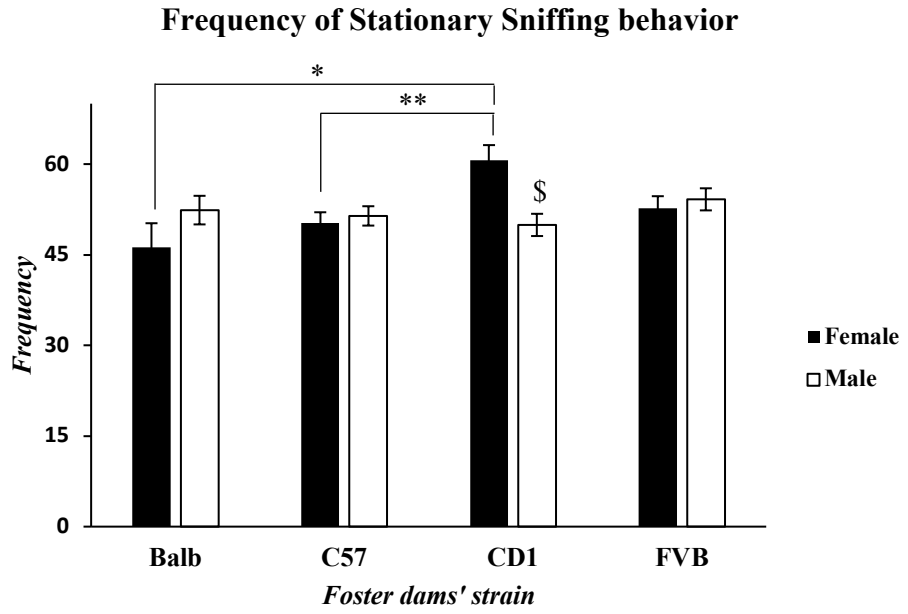
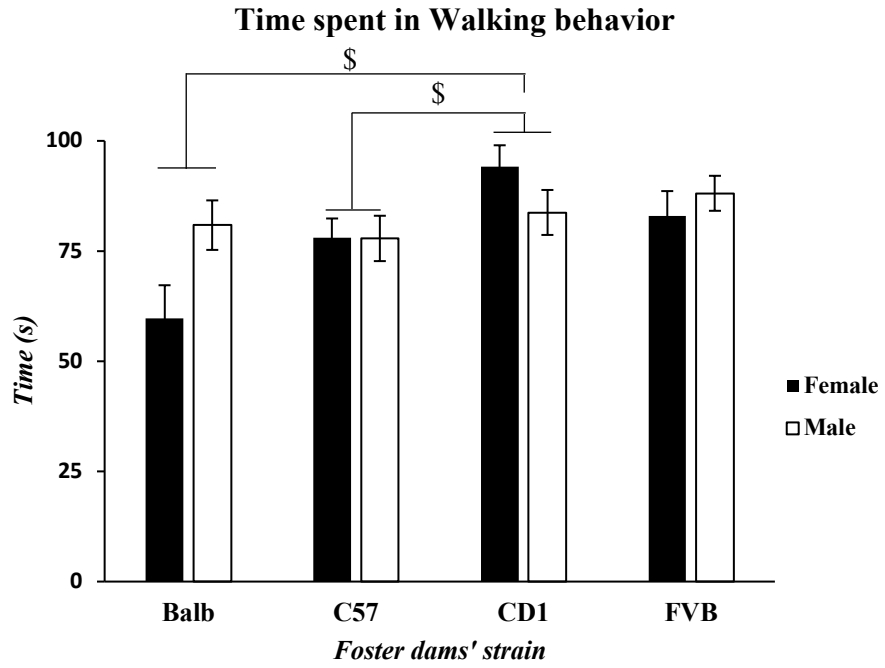


Figure 21. Time spent in and frequency of Stationary Sniffing behavior of male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. Mice reared by Balb dams tended to spend more time in stationary sniffing compared to mice reared by either FVB or CD1. Besides, mice reared by CD1 dams were observed more frequently in stationary sniffing behavior compared to mice reared by C57 (A). The observation of the frequency of stationary sniffing was higher in females reared by CD1 dams than their male counterparts and females reared by either Balb or C57 dams, whereas no difference was observed among males according to adoption (B). $ $p<0.1$; * $p<0.05$; ** $p<0.01$.

A



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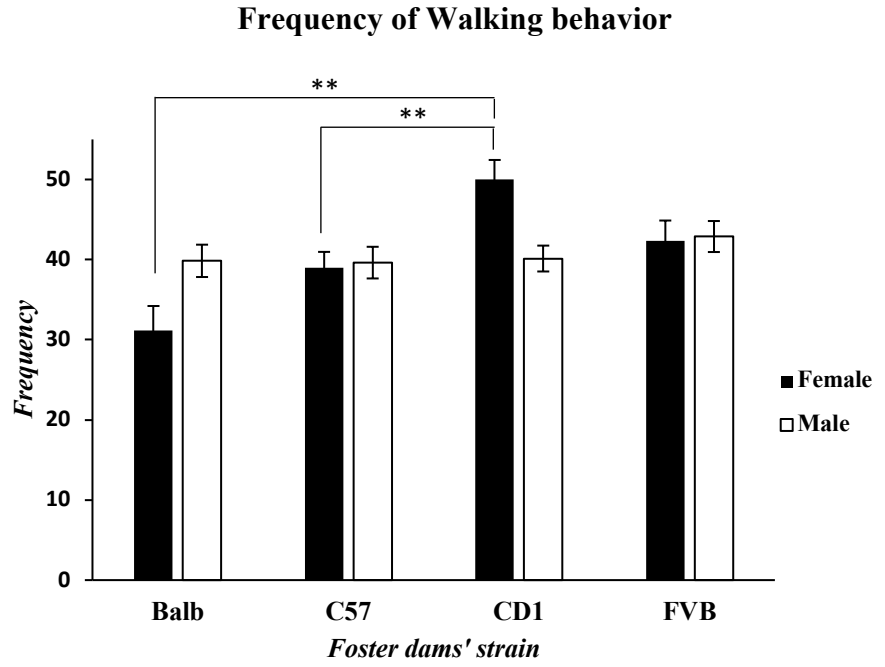
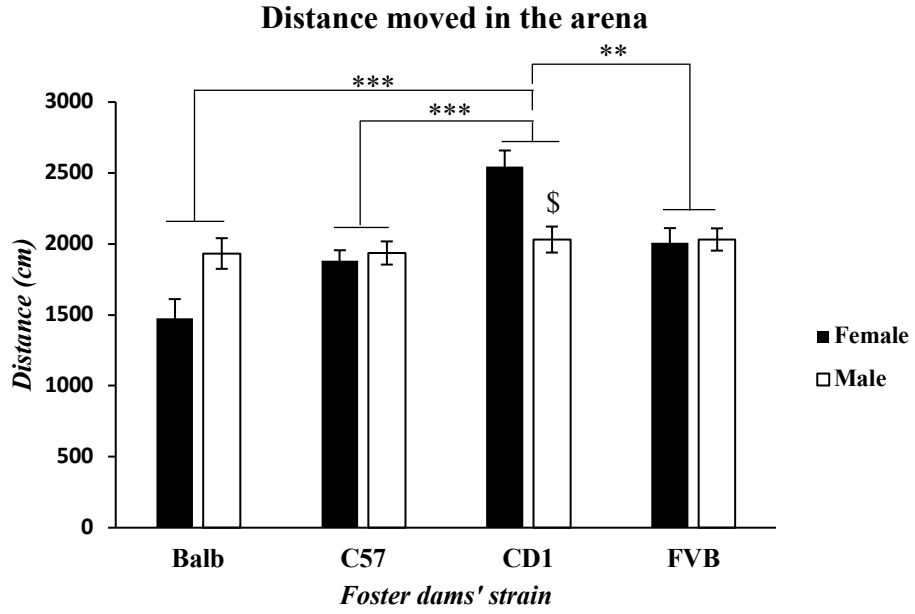


Figure 22. Time spent in and frequency of Walking behavior of male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. Both males and females reared by CD1 dams were more active compared to mice reared by either Balb or C57 (A). Females reared by CD1 were more active than females fostered by either Balb or C57 dams, whereas no difference was observed among males according to adoption (B).

A



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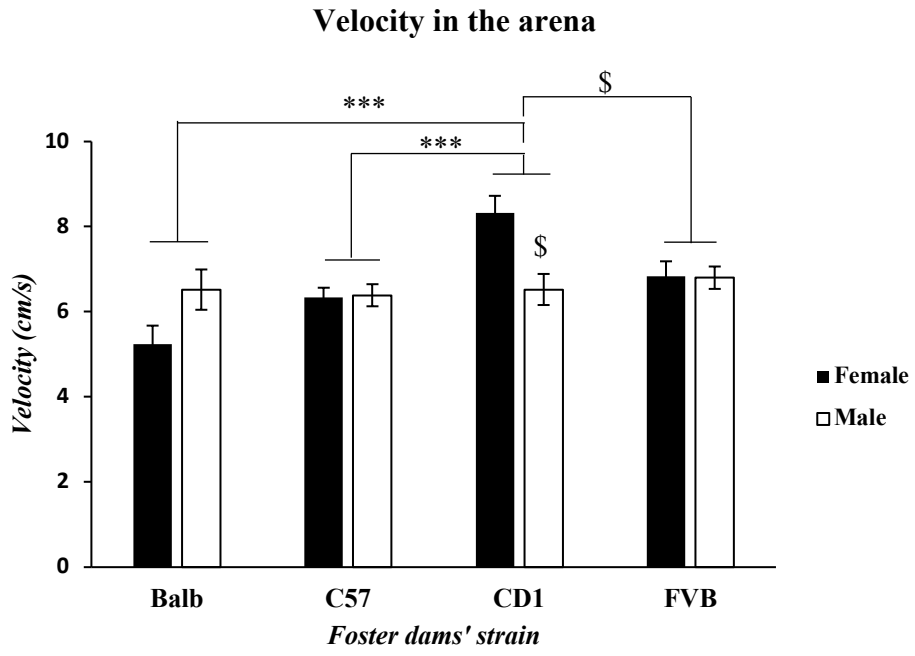
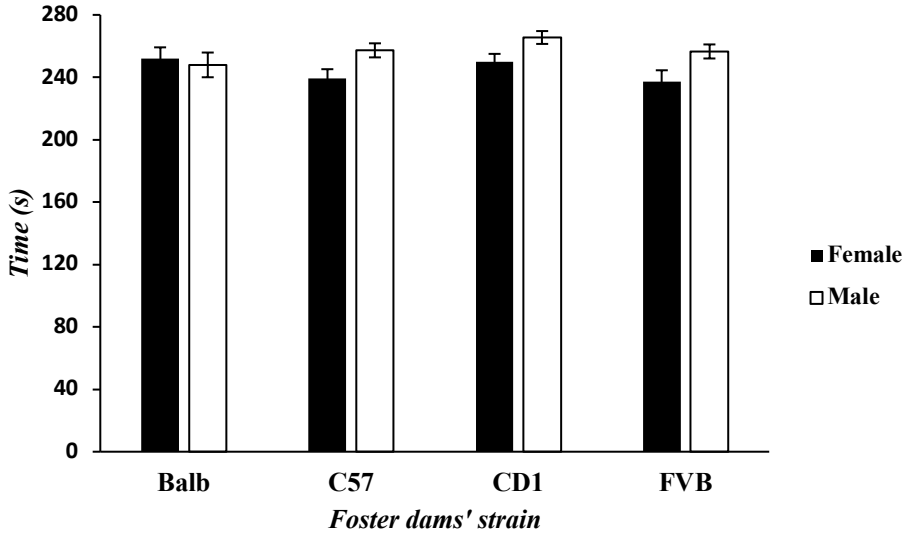


Figure 23. Distance moved and velocity in the arena of male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. Both males and females reared by CD1 dams were more active (A) and faster (B) in the arena compared to mice reared by either Balb or C57 dams and FVB. Females reared by CD1 moved more and faster than females fostered by either Balb or C57 dams and FVB, whereas no difference was observed among males according to adoption. Females reared by CD1 tended to move more and faster compared to their male counterparts, whereas this trend for a sex difference was not observed among other adoptions. $ $p<0.1$; ** $p<0.01$; *** $p<0.001$.

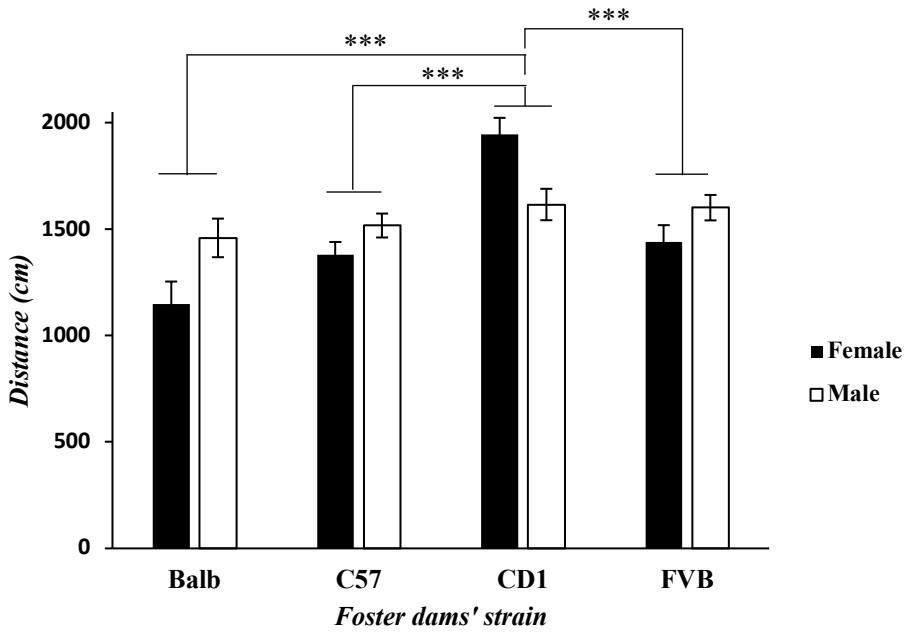
A

Time spent in the Edge



B

Distance moved in the Edge



C

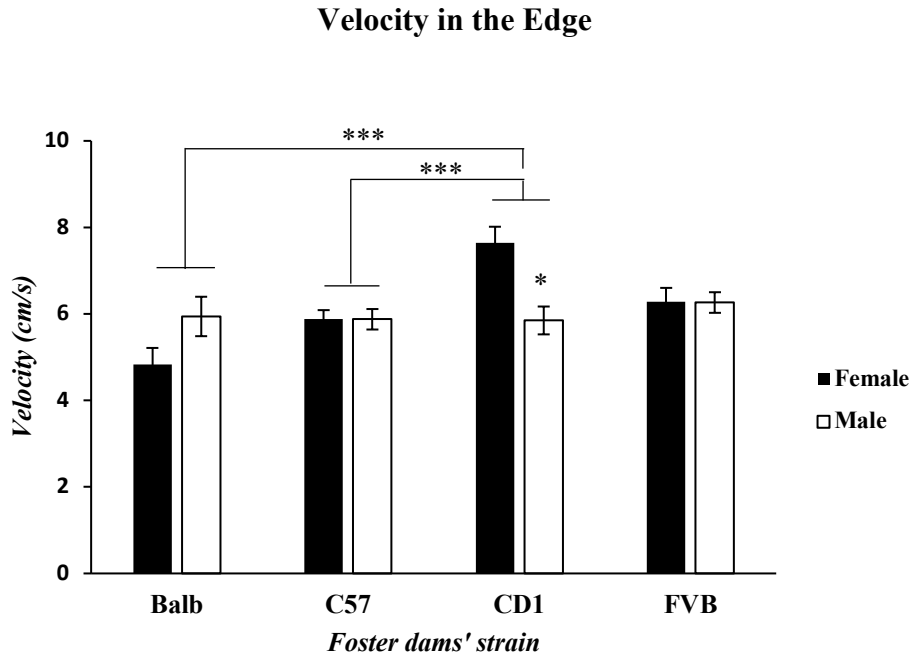
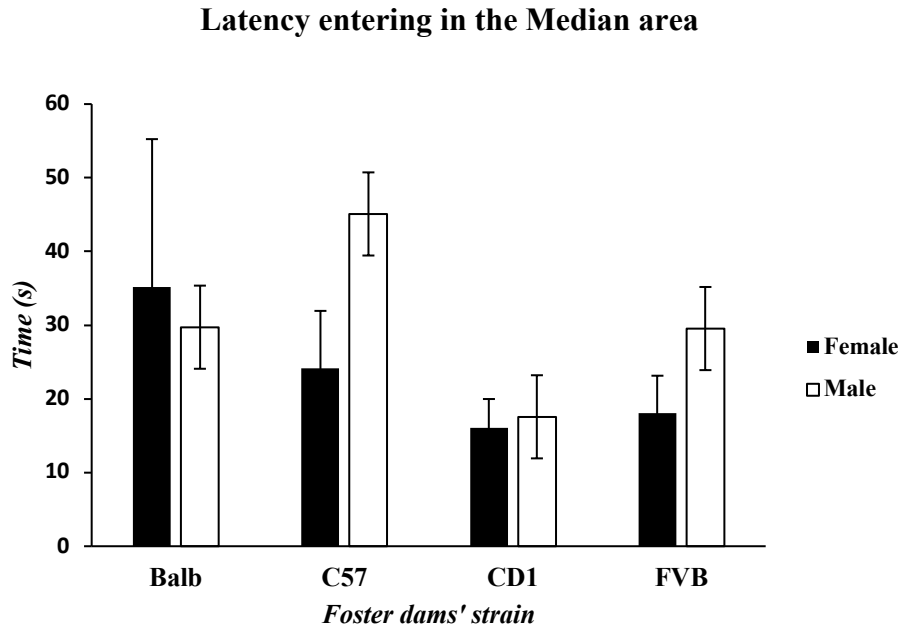
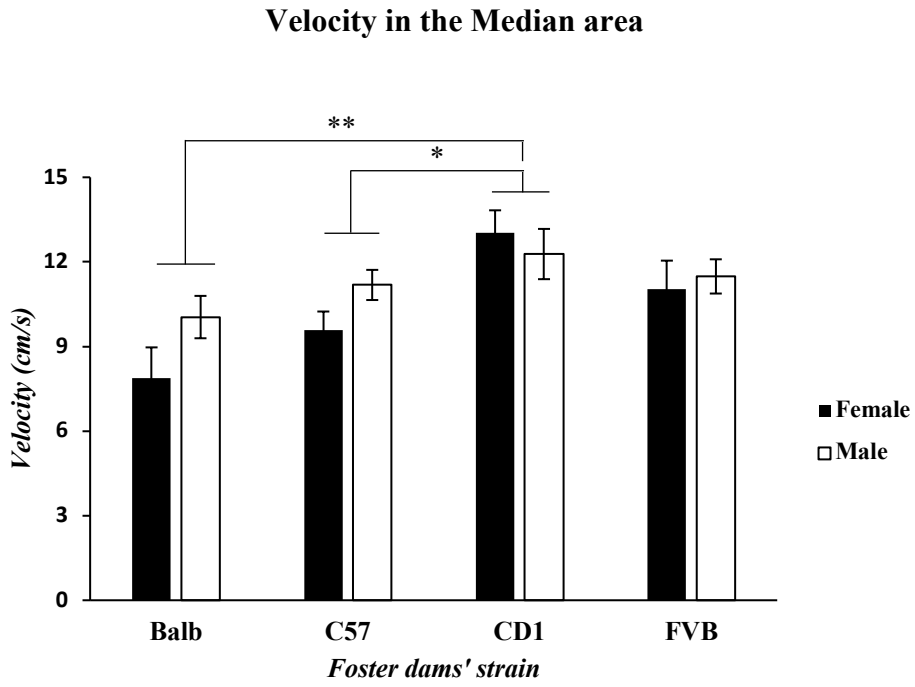


Figure 24. Distance moved and velocity in the arena of male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM**.** Male mice spent more time in the edge compared to females (A). Female mice reared by CD1 dams moved faster than their male counterparts, whereas no sex difference was observed among other adoptions. Besides, females reared by CD1 moved more and faster than females reared by either Balb or C57 dams and; whereas no difference was observed among males according to their adoption. Mice reared by CD1 dams moved significantly more in the edge compared to mice reared by Balb, C57 and FVB dams (B) and they were also faster compared to C57 and Balb (C). * $p<0.05$; *** $p<0.001$.

A

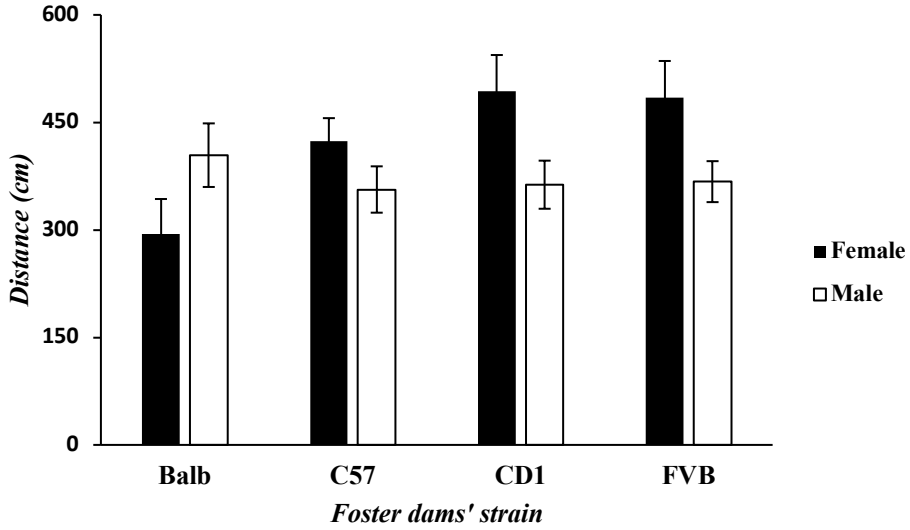

Latency entering in the Median area
Time (s)
60
50
40
30
20
10
0
Balb
C57
CD1
FVB
Foster dams' strain
Female
Male

B


Velocity in the Median area
**
*
Velocity (cm/s)
15
12
9
6
3
0
Balb
C57
CD1
FVB
Foster dams' strain
Female
Male

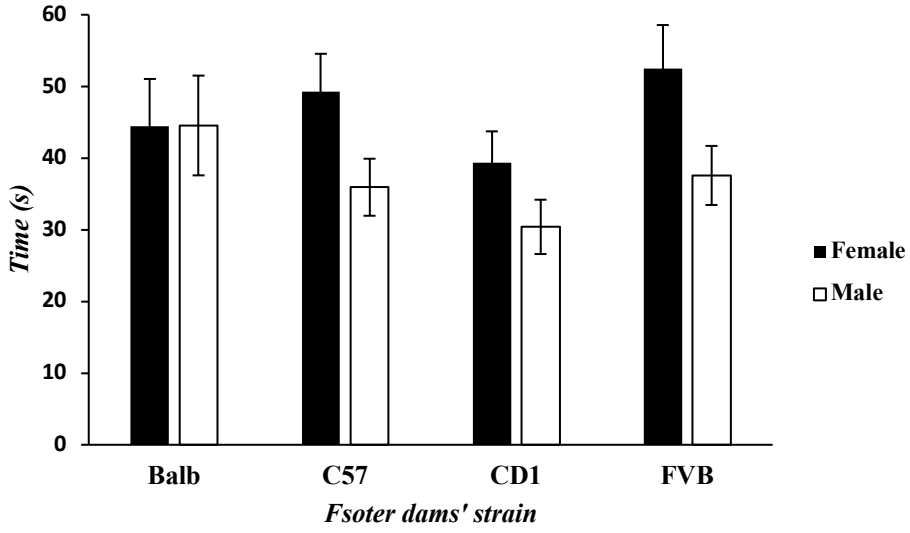
Distance moved in the Median area

C



Time spent in the Median area

D



E

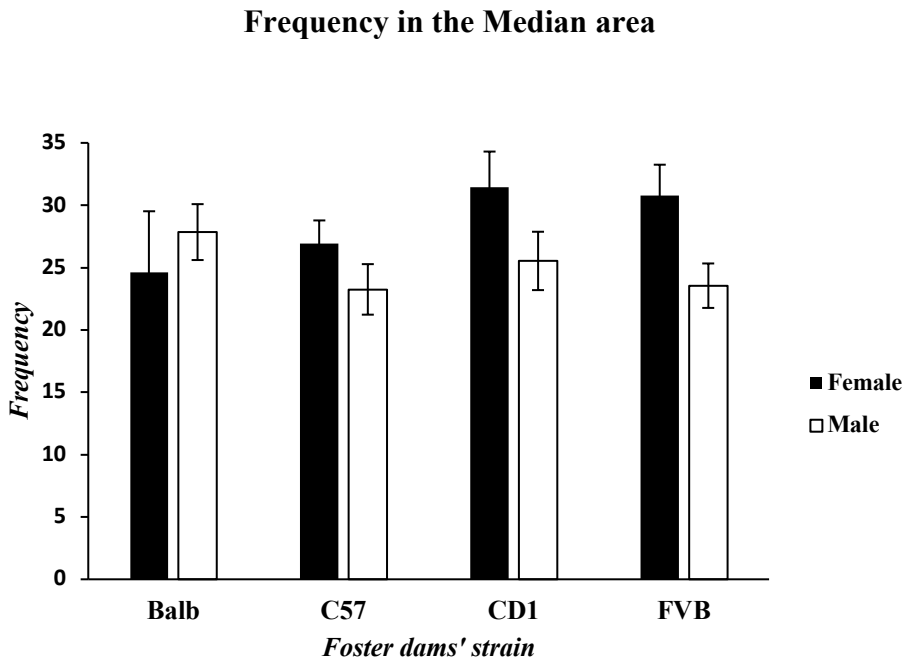
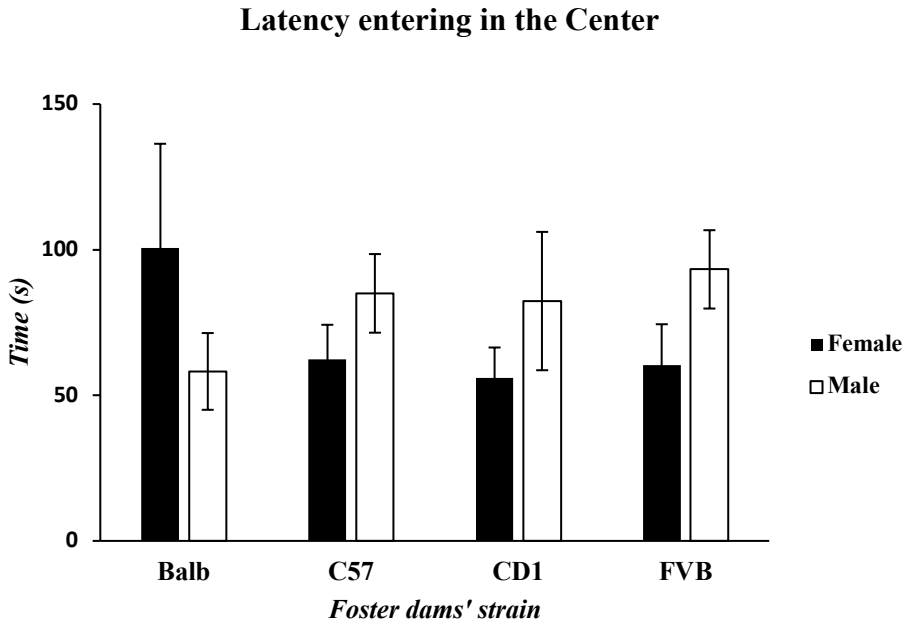
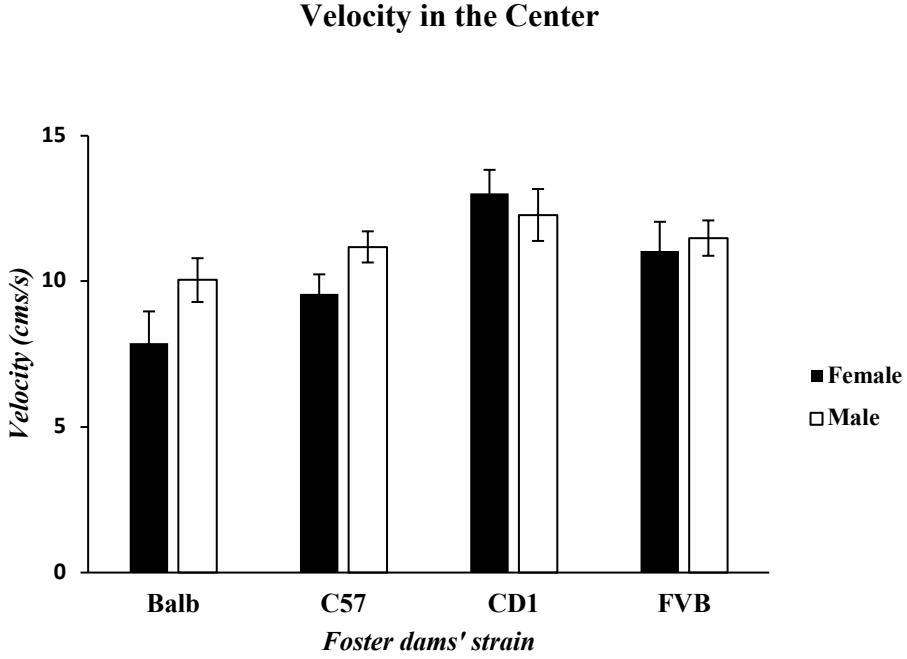


Figure 25. Latency to enter in, velocity, time spent in, distance moved and frequency in the Median area of male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. Female mice were quicker (smaller latencies) in entering (A), moved more (C), spent more time (D), and were observed more often (E) in the median area compared to male mice. Mice reared by CD1 dams were faster compared to mice reared either by C57 or Balb (B). * $p<0.05$; ** $p<0.01$.

A

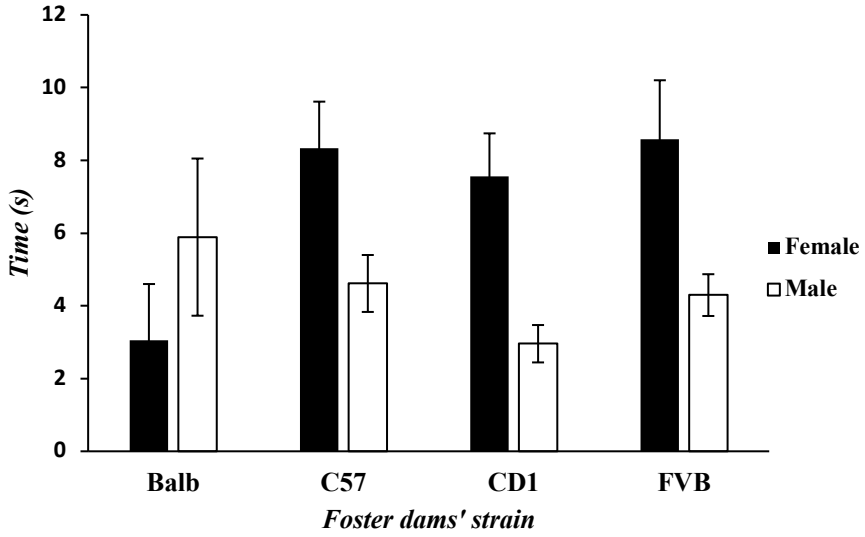

Latency entering in the Center
150
100
50
0
Time (s)
Balb
C57
CD1
FVB
Foster dams' strain
Female
Male

B


Velocity in the Center
15
10
5
0
Velocity (cms/s)
Balb
C57
CD1
FVB
Foster dams' strain
Female
Male

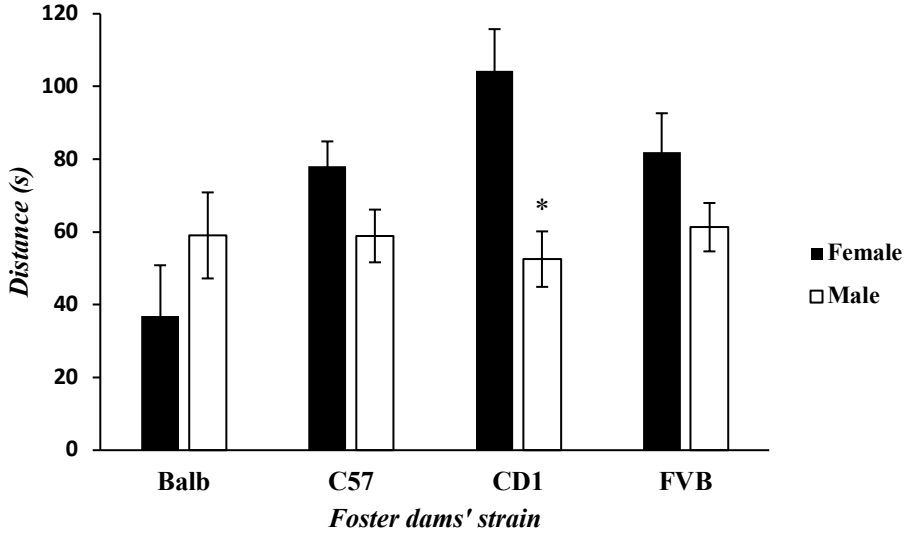
Time spent in the Center

C



Distance moved in the Center

D



E

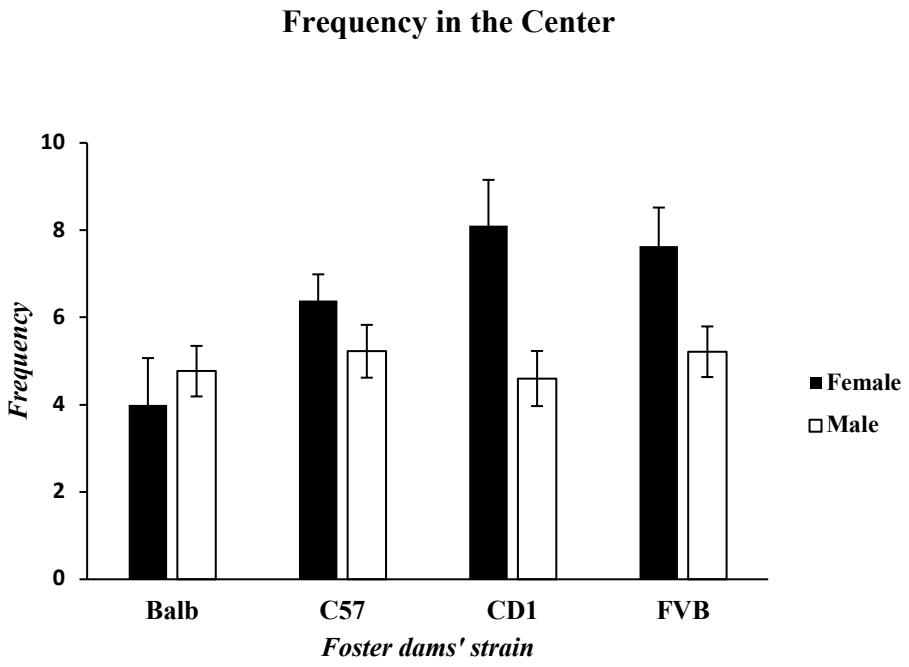


Figure 26. Latency to enter in, time spent in, distance moved and frequency in the Center of the arena of KO and WT, showed by male and female mice reared by Balb, C57, CD1 and FVB dams. Data are shown as mean ± SEM. Female mice had significantly smaller latencies in entering (A), were faster (B), spent more time (C), moved more (D), and were observed more often (E) in the center of the arena compared to male mice. * $p<0.01$.

4.2 Principal Component Analysis

367 experimental subjects were analyzed through Principal Component Analysis (PCA, Kayser's eigen-value-greater-than-one rule; Kaiser-Meyer-Olkin = .81; Bartlett's test: K-squared = 41641, df = 20, p <0.001). The purpose of this analysis was to group the different variables obtained from the analysis of the OF by means of both "Boris" and "Ethovision" softwares into a smaller number of factors, which were then found to be representative of a specific behavior. To assess the level of correlation between the dependent variables used in the PCA, I did a correlation matrix (table 2). I obtained five principal components and as can be seen from table 3, variables that have been grouped within a principal component are more correlated with each other than variables grouped into other principal components.

	Stationary sniffing (frequency)	Stationary sniffing (total time)	Stationary sniffing (mean time)	SAP (frequency)	SAP (total time)	SAP (mean time)	Walking (frequency)	Walking (total time)	Wall rearing (frequency)	Wall rearing (total time)	Wall rearing (mean time)	Median area (time spent)	Median area (frequency)	Center (time spent)	Center (frequency)	Edge (distance)	Center (distance)	Median area (distance)	Arena (distance)	Arena (mean velocity)	Edge (mean velocity)
Stationary sniffing (frequency)	1																				
Stationary sniffing (total time)	-0,42	1																			
Stationary sniffing (mean time)	-0,83	0,78	1																		
SAP (frequency)	-0,38	0,26	0,4	1																	
SAP (total time)	-0,4	0,19	0,36	0,91	1																
SAP (mean time)	-0,23	0,01	0,12	0,31	0,54	1															
Walking (frequency)	0,84	-0,68	-0,88	-0,49	-0,46	-0,19	1														
Walking (total time)	0,57	-0,87	-0,83	-0,44	-0,36	-0,07	0,84	1													
Wall rearing (frequency)	0,59	-0,67	-0,7	-0,36	-0,33	-0,19	0,64	0,58	1												
Wall rearing (total time)	0,43	-0,76	-0,66	-0,3	-0,25	-0,1	0,56	0,61	0,92	1											
Wall rearing (mean time)	-0,04	-0,49	-0,31	-0,1	-0,01	0,07	0,12	0,3	0,36	0,62	1										
Median area (time spent)	0,1	-0,1	-0,15	-0,19	-0,15	-0,04	0,26	0,27	-0,07	-0,05	-0,01	1									
Median area (frequency)	0,38	-0,47	-0,49	-0,33	-0,28	-0,09	0,58	0,63	0,28	0,27	0,04	0,74	1								
Center (time spent)	0,13	-0,07	-0,16	-0,14	-0,12	-0,07	0,2	0,17	-0,01	0	0,02	0,56	0,48	1							
Center (frequency)	0,35	-0,42	-0,44	-0,29	-0,25	-0,1	0,5	0,52	0,29	0,28	0,11	0,64	0,78	0,69	1						
Edge (distance)	0,69	-0,71	-0,79	-0,38	-0,34	-0,15	0,78	0,76	0,76	0,69	0,2	-0,19	0,27	-0,15	0,18	1					
Center (distance)	0,36	-0,4	-0,45	-0,28	-0,25	-0,08	0,5	0,51	0,3	0,27	0,09	0,57	0,73	0,78	0,88	0,21	1				
Median area (distance)	0,4	-0,51	-0,53	-0,28	-0,24	-0,08	0,59	0,64	0,33	0,31	0,12	0,7	0,82	0,5	0,73	0,3	0,74	1			
Arena (distance)	0,72	-0,79	-0,86	-0,44	-0,39	-0,16	0,88	0,89	0,74	0,67	0,2	0,2	0,62	0,15	0,52	0,9	0,54	0,65	1		
Arena (mean velocity)	0,65	-0,63	-0,72	-0,38	-0,34	-0,17	0,76	0,74	0,58	0,52	0,16	0,24	0,6	0,24	0,52	0,74	0,57	0,61	0,86	1	
Edge (mean velocity)	0,62	-0,6	-0,68	-0,37	-0,34	-0,18	0,75	0,72	0,55	0,48	0,14	0,32	0,59	0,27	0,51	0,71	0,55	0,59	0,83	0,98	1

Table 2. Correlation matrix between the different variables used in the PCA. Red shows positive correlations, while blue negative correlations.

	PC1	PC2	PC3	PC4	PC5	h2	u2	com
Mean velocity	**.88**					.82	.178	1.1
Total distance moved	**.86**					.95	.052	1.1
Edge velocity	**.84**					.78	.217	1.1
Edge distance moved	**.95**					.93	.071	1.2
Walking frequency	**.80**					.88	.117	1.2
Walking total time	**.61**	.41				.88	.123	2.2
Stationary sniffing frequency	**.91**					.81	.189	1.2
Stationary sniffing mean time	**-.79**					.81	.187	1.1
Stationary sniffing total time	**-.49**			-.52		.81	.187	2.6
Median duration		**.80**				.83	.170	1.3
Median distance		**.68**				.84	.161	1.5
Median frequency		**.67**				.88	.117	1.6
Stretch attend posture total time			**.99**			.96	.045	1.0
Stretch attend posture mean time		.35	**.67**			.59	.413	2.0
Stretch attend posture frequency			**.89**			.85	.155	1.1
Wall rearing frequency	.65			**.41**		.82	.176	2.1
Wall rearing total time	.47			**.67**		.91	.093	2.0
Wall rearing mean time				**.95**		.82	.177	1.1
Center duration					**.94**	.84	.161	1.0
Center frequency		.41			**.57**	.85	.148	2.1
Center distance					**.71**	.83	.169	1.4

Table 3. Loadings between different variables and the principal components extracted from those variables. *PC1, PC2, PC3, PC4, PC5*: different Principal Components extracted from the variables; PC1 is the first principal component, PC2 is the second principal component and so on. *h2*: proportion of variance in each variable that is explained by the principal components. *u2*: proportion of variance in each variable that is not explained by the principal components. *com*: communalities of the variables, it represents the amount of variance in each variable that is explained by all the principal components together. Not all loadings are represented as a *cut* was made to 0.3.

Principal component 1 included: distance moved and mean velocity both in the arena and in the edge, walking (total time and frequency), stationary sniffing (total time, mean time and frequency). For that reason, I named it "*exploration*".

Principal component 2 included: time spent in, distance moved and frequency in the median area. For that reason, I named it "*activity in the median area*".

Principal component 3 included: stretch attend posture (total time, mean time and frequency). For that reason, I named it "*risk assessment*".

Principal component 4 included: wall rearing (total time, mean time and frequency). For that reason, I named it "*vertical exploration*".

Principal component 5 included: time spent in, distance moved and frequency in the center of the arena. For that reason, I named it "*activity in the center*".

After obtaining the 5 principal components, I conducted the following two ANOVAs consistently with the preliminary analysis in chapter 4.1: a 3-way ANOVA (sex, genotype, and foster dams' strain) in the whole sample and a 2-way ANOVA on data from WT only.

4.2.1 3-way ANOVA on Principal Components in the whole sample

A main effect on genotype in *exploration* was found ($F_{(3,351)}$=9.748, $p<0.01$), in particular, regardless their sex and foster dams' strain, KO mice were less active than WT. 3-way ANOVA yielded a highly significant effect of sex by foster dams' strain interaction on *exploration* ($F_{(3,351)}$=4.562, $p<0.01$), specifically female mice reared by CD1 dams were more active than female mice reared by Balb, C57 (for both Balb and C57, $p<0.001$) and FVB ($p<0.01$), whereas no difference was observed among males according to adoption; female mice reared by CD1 dams were also more active than their male counterparts ($p<0.05$), whereas no difference was observed among other strains according to sex. Foster dams' strain was also highly significant

in exploration ($F_{(1,351)}$=7.166, p<0.001): in general, mice reared by CD1 dams were more active than mice reared by Balb, C57 and FVB (respectively: p<0.01; p<0.001; p<0.05).

Remarkably, conditional deletion of NPY1r significantly reduced *activity in the median area* (genotype: $F_{(1,351)}$=8.243, p<0.01). As expected, females were more active in the median area than males (sex: $F_{(1,351)}$=18.558, p<0.001).

A main effect on genotype in *risk assessment* was also found ($F_{(3,351)}$=4.508, p<0.05), in particular regardless their sex and foster dams' strain, KO mice displayed more SAP than WT. Foster dams' strain was also highly significant in *risk assessment* ($F_{(3,351)}$=12.586, p<0.001): mice reared by CD1 dams were less anxious-like than mice reared by either C57 or FVB (p<0.001) and Balb-reared mice displayed less risk assessment behaviors than C57 (p<0.1). The effect of sex did not reach significance in altering *risk assessment* ($F_{(1,351)}$=2.761, p<0.1): males tended to be more anxious-like (more risk assessment) than females.

3-way ANOVA revealed a significant main effect of sex in *vertical exploration* ($F_{(1,351)}$=9.517, p<0.01): males explored more than females. Foster dams' strain was found to be significant, too ($F_{(3,351)}$=5.154, p<0.01), in particular mice reared by CD1 and FVB dams were more exploratory than mice reared by C57 and Balb (p<0.05).

Sex by foster dams' strain interaction was significant in *activity in the center* ($F_{(3,351)}$=3.819, p<0.05), specifically females reared by CD1 dams were more active in the center than females reared by Balb, C57 and FVB dams (respectively: p<0.05; p<0.001; p<0.1), whereas no difference was observed among males according to adoption; female mice reared by C57 dams were more active in the center than their male counterparts (p<0.1), whereas no sex difference was observed in other strains.

4.2.2 2-way ANOVA on Principal Components on data from WT mice

2-way ANOVA yielded a significant effect of sex by foster dams' strain interaction in *exploration* ($F_{(3,218)}$=3.330, p<0.05), specifically female mice reared by CD1 dams were more active than female mice reared by Balb, C57 and FVB (p<0.01; p<0.001; p<0.05), whereas no difference was observed among males according to adoption. In general, foster dams' strain was highly significant in *exploration* ($F_{(3,218)}$=6.211, p<0.001): regardless their sex, mice reared by CD1 dams explored more than mice reared by Balb, C57 and FVB dams (p<0.01; p<0.001; p<0.1).

Female mice displayed significantly more *activity in the median area* than males ($F_{(1,218)}$=17.481, p<0.001).

Foster dams' strain was highly significant in *risk assessment* ($F_{(3,218)}$=7.168, p<0.001): mice reared by CD1 dams were less anxious-like than mice reared by either C57 or FVB (respectively: p<0.001; p<0.01).

2-way ANOVA revealed a significant main effect on sex in *vertical exploration* ($F_{(1,218)}$=6.911, p<0.01): males explored more than females. Foster dams' strain was found to be significant too ($F_{(3,218)}$=2.906, p<0.05), in particular mice reared by FVB dams were more exploratory than mice reared by C57 (p<0.05).

Foster dams' strain was significant in *activity in the center* ($F_{(3,218)}$=4.775, p<0.01), specifically mice reared by CD1 lactating females were more active in the center than mice reared by C57 and Balb dams (respectively: p<0.1; p<0.01).

4.3 Analysis on data from mice reared by FVB and C57 dams that underwent both sessions

4.3.1 4-way mixed ANOVA

I conducted a mixed 4-way ANOVA (sex, genotype, foster dams' strain and session) only on mice reared by C57 and FVB dams (that underwent both OF sessions), as the other two strains (Balb and CD1) had a strong sample imbalance. As shown in table 3, it is the session that reported the largest number of significant effects and also its interaction with other considered factors.

Sex	Frequency in stationary sniffing ($F_{(1,135)}$=5.03, p<0.05), mean velocity in the edge ($F_{(1,135)}$=12.18, p<0.001), median area (latency to enter: $F_{(1,135)}$=20.61, p<0.001; frequency: $F_{(1,135)}$=12.46, p<0.001), center (latency to enter: $F_{(1,135)}$=21.64, p<0.001; distance moved: $F_{(1,135)}$=14.04, p<0.001; time spent in: $F_{(1,135)}$=9.57, p<0.01; frequency: $F_{(1,135)}$=15.36, p<0.001)
Foster dams' strain	Frequency in grooming ($F_{(1,135)}$=7.66, p<0.01), frequency in walking ($F_{(1,135)}$=5.19, p<0.05), wall rearing (total time: $F_{(1,135)}$=4.58, p<0.05; frequency: $F_{(1,135)}$=5.18, p<0.05), SAP (total time: $F_{(1,135)}$=5.3, p<0.05; frequency: $F_{(1,135)}$=11.78, p<0.001), frequency in stationary sniffing ($F_{(1,135)}$=4.69, p<0.05)
Genotype	Grooming (total time: $F_{(1,135)}$=5.13, p<0.05; frequency: $F_{(1,135)}$=5.74, p<0.05)

Session	Grooming (total time: $F_{(1,135)}$=22.73, p<0.001; frequency: $F_{(1,135)}$=47.69, p<0.001), walking (total time: $F_{(1,135)}$=22.73, p<0.001; frequency: $F_{(1,135)}$=18.84, p<0.001), time spent in wall rearing ($F_{(1,135)}$=4.72, p<0.05), SAP (total time: $F_{(1,135)}$=115.12, p<0.001; frequency: $F_{(1,135)}$=103.73, p<0.001), stationary sniffing (total time: $F_{(1,135)}$=4.56, p<0.05; frequency: $F_{(1,135)}$=15.36, p<0.001), distance moved and mean velocity in the arena (respectively: $F_{(1,135)}$=29.19, p<0.001; $F_{(1,135)}$=18.53, p<0.001), edge (distance moved: $F_{(1,135)}$=25.17, p<0.001; frequency: $F_{(1,135)}$=20.06, p<0.001), frequency in the median area ($F_{(1,135)}$=33.14, p<0.001), center (latency to enter: $F_{(1,135)}$=8.37, p<0.01; velocity: $F_{(1,135)}$=4.57, p<0.05; distance moved: $F_{(1,135)}$=39.68, p<0.001; time spent in: $F_{(1,135)}$=7.62, p<0.01; frequency: $F_{(1,135)}$=35.67, p<0.001)
Sex × Foster dams' strain	Time spent in grooming ($F_{(1,135)}$=4.09, p<0.05)
Sex × Genotype	Velocity in the center ($F_{(1,135)}$=4.57, p<0.05)
Sex × Session	Walking (total time: $F_{(1,135)}$=6.9, p<0.01; frequency: $F_{(1,135)}$=9.54, p<0.01), wall rearing (total time: $F_{(1,135)}$=13.19, p<0.001; frequency: $F_{(1,135)}$=11.78, p<0.001), frequency in stationary sniffing ($F_{(1,135)}$=17.59, p<0.001), distance moved and mean velocity in the arena (respectively: $F_{(1,135)}$=11.26, p<0.01; $F_{(1,135)}$=7.13, p<0.01), edge (total time: $F_{(1,135)}$=22.22, p<0.001; velocity: $F_{(1,135)}$=24.01, p<0.001), median area (latency to enter: $F_{(1,135)}$=4.42, p<0.05; distance moved: $F_{(1,135)}$=28.31, p<0.001; time spent in: $F_{(1,135)}$=21.19, p<0.001), center (distance moved: $F_{(1,135)}$=5.26, p<0.05; frequency: $F_{(1,135)}$=4.12, p<0.05)
Foster dams' strain × Session	Time spent in SAP ($F_{(1,135)}$=4.75, p<0.05)
Genotype × Session	Wall rearing (total time: $F_{(1,135)}$=4.13, p<0.05; frequency: $F_{(1,135)}$=4.19, p<0.05)

Sex × Foster dams' strain × Genotype	Frequency in walking ($F_{(1,135)}$=5.76, p<0.05), frequency in stationary sniffing ($F_{(1,135)}$=6.54, p<0.05), defecation ($F_{(1,135)}$=4.39, p<0.05), frequency in the edge ($F_{(1,135)}$=6.96, p<0.01)
Foster dams' strain× Genotype × Session	Wall rearing (total time: $F_{(1,135)}$=7.39, p<0.01; frequency: $F_{(1,135)}$=6.29, p<0.05), center (velocity: $F_{(1,135)}$=4.83, p<0.05; time spent in: $F_{(1,135)}$=4.42, p<0.05)

Table 3. 4-way ANOVAs significant effects of sex, genotype, foster strain, session and their interactions on behavioral measures obtained from OF (F-statistic and p-value).

As reported in Table 3, the effect of the session was found significant in several behavioral measures: in general in the second experimental session the animals performed less active exploratory activity (walking, wall rearing, activity in the arena in the edge, median area and center) and more static activity (grooming and stationary sniffing). The animals displayed reduced anxiety-like behavior (SAP) and exploration levels as a result of habituation. Since the animals have already explored the environment previously, they would seem less interested in moving in the arena and less anxious than in the first session. Another consideration is that in the second experimental session the females were pregnant and therefore less active (*i.e.*, walking behavior, wall rearing behavior, activity in the arena, velocity in the edge, distance moved in the median are and distance moved and frequency in the center of the arena) than in the first session and compared to males.

Interestingly, in the first session, mice reared by C57 dams displayed more risk assessment than mice reared by FVB dams (p<0.01), whereas this difference was not observed in the second session (significant effect of the foster dams' strain by session interaction; Table 3).

There was a significant effect of genotype by session interaction (Table 3): KO mice showed no behavioral differences in wall rearing in the two sessions, while WT mice displayed less wall rearing in the second session than in the first session.

Given the significance of session and of its interactions, I chose to conduct two smaller 3-way ANOVAs separated for each session of OF. This choice was also legit because the sex by session interaction yielded several significant effects. These effects were attributed to the different experimental design that the two sexes underwent. Therefore, further examination was deemed unnecessary.

4.3.2 3-way ANOVA on OF first session

I performed a 3-way ANOVA on the OF first session to assess the effects of sex, genotype and foster dams' strain considering only mice reared by C57 and FVB. I analyzed the same dependent variables as in the preliminary analyses as follows.

Sex by foster dams' strain interaction missed significance on frequency of SAP ($F_{(1,135)}$=3.872, p=0.0512; Fig.27B): males reared by C57 dams were observed more frequently in SAP than males reared by FVB dams ($p<0.05$), whereas no difference was observed among females according to adoption. Foster dams' strain was significant on both time spent in and frequency of observation in SAP ($F_{(1,135)}$=5.316, $p<0.05$; $F_{(1,135)}$=4.856, $p<0.05$; Fig.27), in particular, mice reared by C57 dams were more anxious-like than mice reared by FVB dams.

3-way ANOVA yielded a significant effect for sex by genotype and foster dams' interaction on time spent in grooming behavior ($F_{(1,135)}$=7.578, $p<0.01$): specifically, KO females reared by FVB dams spent more time in grooming behavior than their WT counterparts ($p<0.1$), whereas no genotype difference was observed among males reared by FVB and mice reared by C57 dams regardless of their sex. Generally, genotype was significant on frequency of grooming and missed significance in time spent in grooming behavior (respectively:

$F_{(1,135)}$=8.09, p<0.01; $F_{(1,135)}$=3.896, p=0.0505; Fig.28): KO mice spent more time in and were observed more frequently in grooming behavior than WT. Sex by foster dams' interaction was found to be significant on time spent in grooming behavior, too ($F_{(1,135)}$=4.801, p<0.05): females reared by C57 dams groomed more than their males counterparts, whereas in mice reared by FVB dams this sex difference was reversed. However, foster dams' strain just missed significance on frequency in grooming behavior ($F_{(1,135)}$=3.773, p=0.054) in particular, mice reared by C57 dams groomed less frequently than mice reared by FVB dams.

3-way ANOVA revealed a missed significance of the genotype by foster strain interaction on frequency observed in wall rearing behavior ($F_{(1,135)}$=3.603, p=0.0598; 29B): KO mice reared by FVB dams were observed more frequently in wall rearing behavior than KO mice reared by C57 (p<0.05), whereas no foster strain difference was observed in WT mice. Foster dams' strain was significant on both time spent in and frequency of observation in wall rearing behavior (respectively: $F_{(1,135)}$=6.805, p<0.05; $F_{(1,135)}$=8.169, p<0.01; Fig.29), in particular, regardless their sex and genotype, mice reared by FVB dams explored more than mice reared by C57 dams.

Mice reared by FVB dams were observed more frequently in walking behavior than mice reared by C57 dams (foster dams' strain: $F_{(1,135)}$=5.478, p<0.05; Fig.30).

3-way ANOVA yielded a significant effect for sex by genotype and foster dams' strain interaction on frequency observed in the edge ($F_{(1,135)}$=6.941, p<0.01; Fig.31B): WT females reared by FVB dams were more often in the edge than their males counterparts, whereas there was no sex difference among WT mice reared by C57 dams and in general among KO mice. A significant effect was found for the genotype by foster dams' strain interaction on time spent in the edge ($F_{(1,135)}$=5.514, p<0.05; Fig.31A): specifically, KO mice reared by FVB mice spent more time in the edge than KO mice reared by C57 mice, whereas no difference was found

among WT mice according to adoption. In general, male mice spent significantly more time in the edge than females (sex: $F_{(1,135)}$=12.563, p<0.001).

3-way ANOVA yielded a significant effect for sex by genotype and foster dams' strain interaction on frequency observed in the median area ($F_{(1,135)}$=4.484, p<0.05): WT females reared by FVB dams were observed more frequently in the median area than their males counterparts (p<0.05), whereas there was no sex difference among WT mice reared by C57 dams; also they were observed more frequently in the median area than WT females reared by C57 dams; KO females mice reared by C57 dams had an higher frequency than their male counterparts, whereas no sex difference was found in KO reared by FVB strain. Genotype by foster dams' strain was significant on time spent in the median area ($F_{(1,135)}$=4.666, p<0.05; Fig.32C): KO mice reared by C57 dams spent more time in the median area than WT mice reared by C57 dams and KO mice reared by FVB dams, whereas no genotype difference was observed among mice reared by FVB dams. Sex was highly significant on time spent in, distance moved, latency to enter and frequency in the median area (respectively: $F_{(1,135)}$=13.975, p<0.001; $F_{(1,135)}$=13.464, p<0.001; $F_{(1,135)}$=25.470, p<0.001; $F_{(1,135)}$=14.446, p<0.001; Fig.32): in particular female mice spent more time in, moved more, were quicker to enter and were observed more frequently in the median area than male mice.

Sex was highly significant on time spent in, distance moved, latency to enter and frequency in the center (respectively: $F_{(1,135)}$=4.117, p<0.05; $F_{(1,135)}$=13.631, p<0.001; $F_{(1,135)}$=14.636, p<0.001; $F_{(1,135)}$=14.939, p<0.001; Fig.33): in particular female mice spent more time in, moved more, were quicker to enter and were observed more frequently in the center of the arena than male mice.

3-way ANOVA revealed a significant effect of sex by genotype interaction in defecation ($F_{(1,135)}$=5.450, p<0.05; Fig.34): in KO females the number of feces found in the arena at the end of the test was higher than in KO males, whereas in WT mice no sex difference was found.

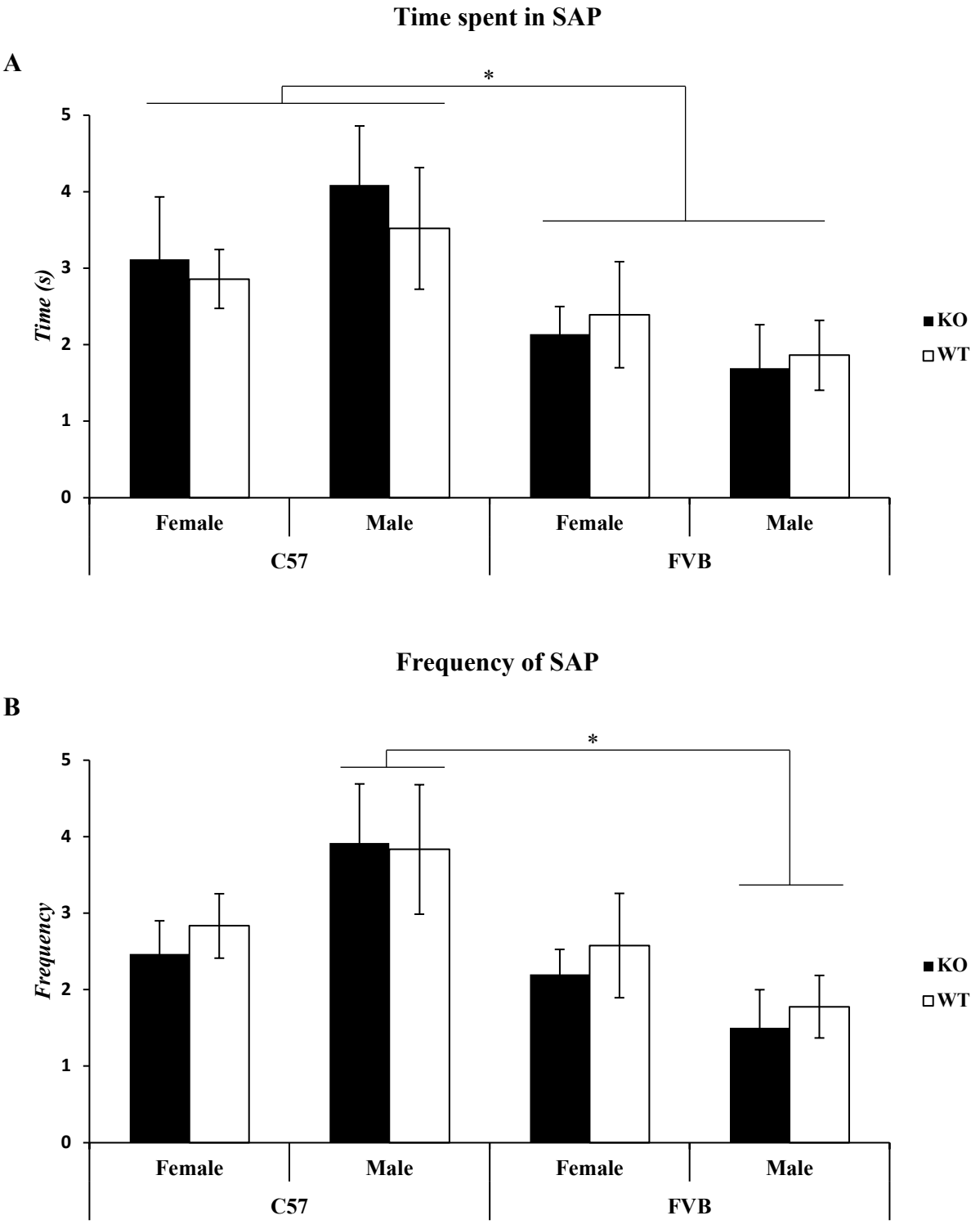


Figure 27. Time spent in and frequency of SAP of KO and WT showed by male and female mice reared by C57 and FVB dams (first experimental session). Data are shown as mean ± SEM. Males reared by C57 dams were observed more frequently in SAP than males reared by FVB dams, whereas no difference was observed among females according to adoption (B). In general, mice reared by C57 dams were more anxious-like than mice reared by FVB dams. * $p<0.05$

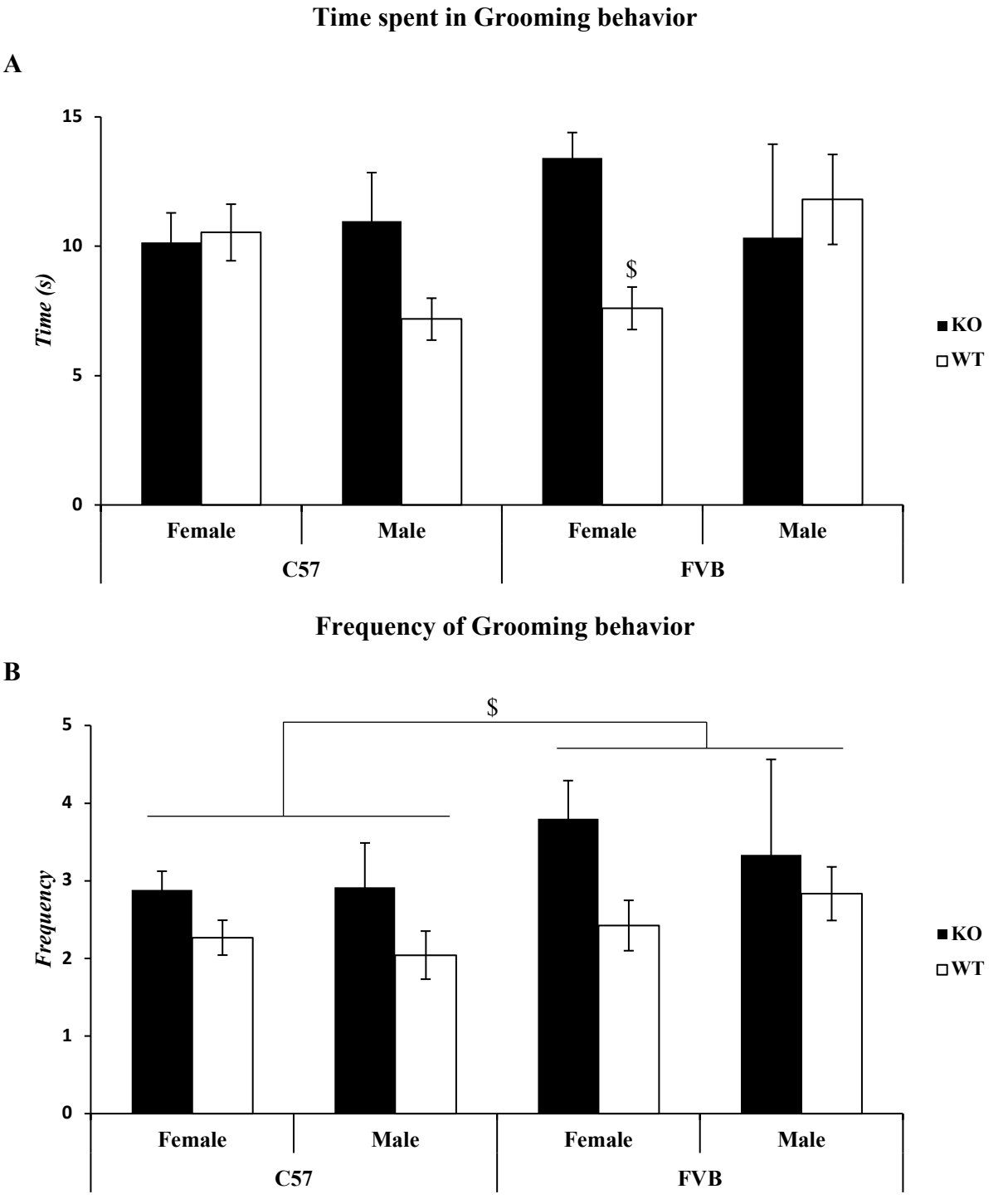


Figure 28. Time spent in and frequency of Grooming behavior of KO and WT showed by male and female mice reared by C57 and FVB dams (first experimental session). Data are shown as mean ± SEM. KO females reared by FVB dams spent more time in grooming behavior than their WT counterparts (A), whereas no genotype difference was observed among males reared by FVB and mice reared by C57 dams regardless of their sex. KO mice spent significantly more time in and were observed more frequently (B) in grooming behavior than WT. Females reared by C57 dams groomed more than their males' counterparts, whereas in mice reared by FVB dams this sex difference was reversed. However, mice reared by C57 dams groomed less frequently than mice reared by FVB dams. $ $p<0.1$.

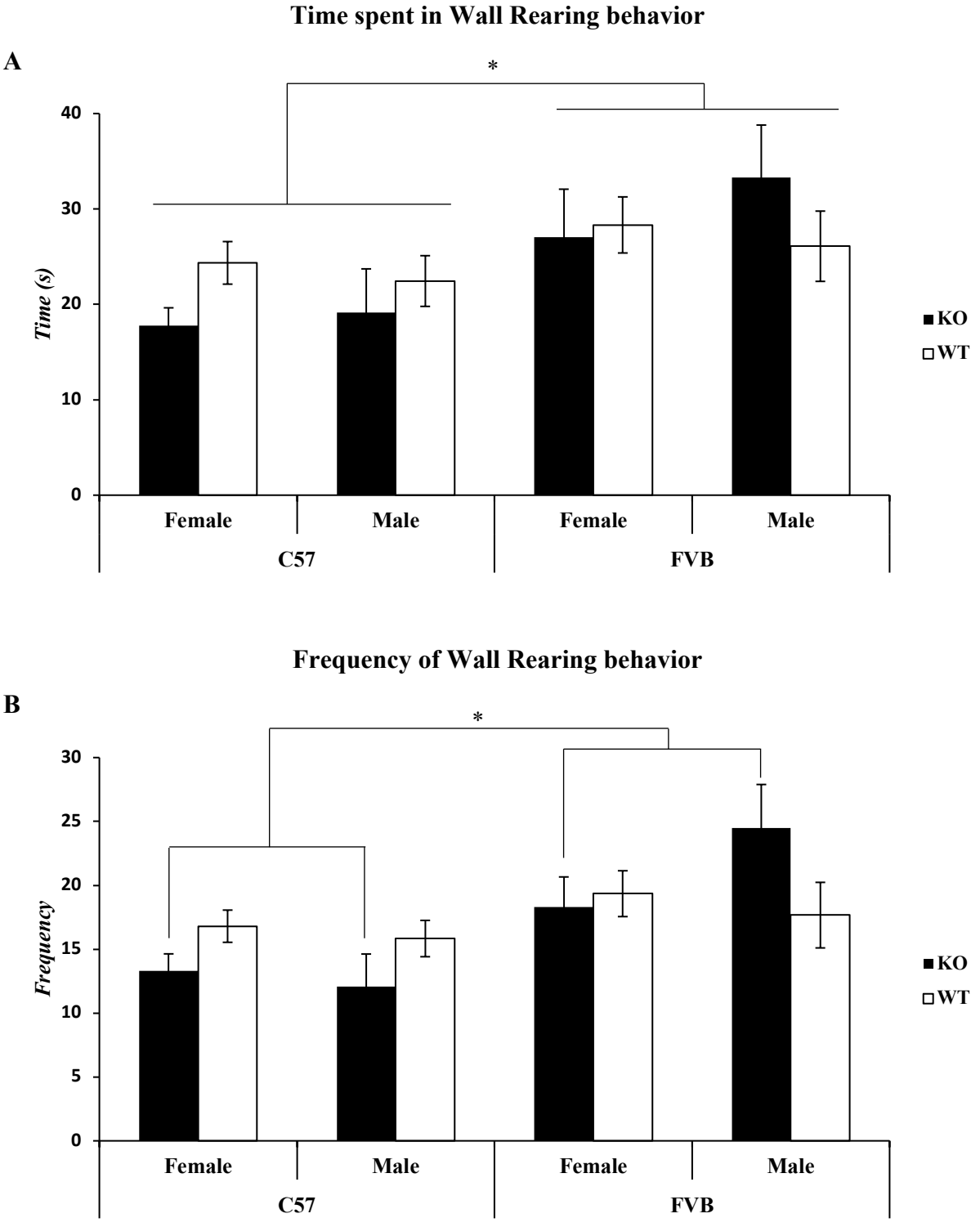


Figure 29. Time spent in and frequency of Wall Rearing behavior of KO and WT showed by male and female mice reared by C57 and FVB dams (first experimental session). Data are shown as mean ± SEM. KO mice reared by FVB dams were observed more frequently in wall rearing behavior than KO mice reared by C57 whereas no foster strain difference was observed in WT mice (B). Regardless their sex and genotype, mice reared by FVB dams explored more than mice reared by C57 dams (A and B). * $p<0.05$.

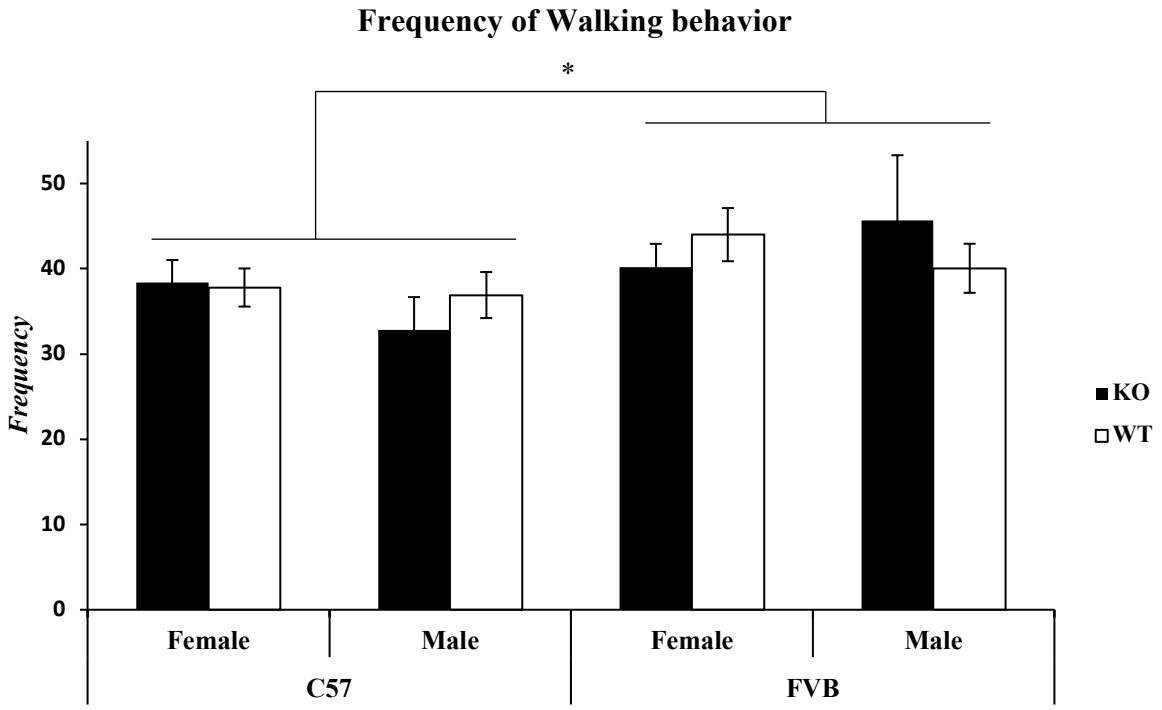


Figure 30. Frequency of Walking behavior of KO and WT showed by male and female mice reared by C57 and FVB dams (first experimental session). Data are shown as mean ± SEM. Mice reared by FVB dams were observed more frequently in walking behavior than mice reared by C57 dams. * $p<0.05$.

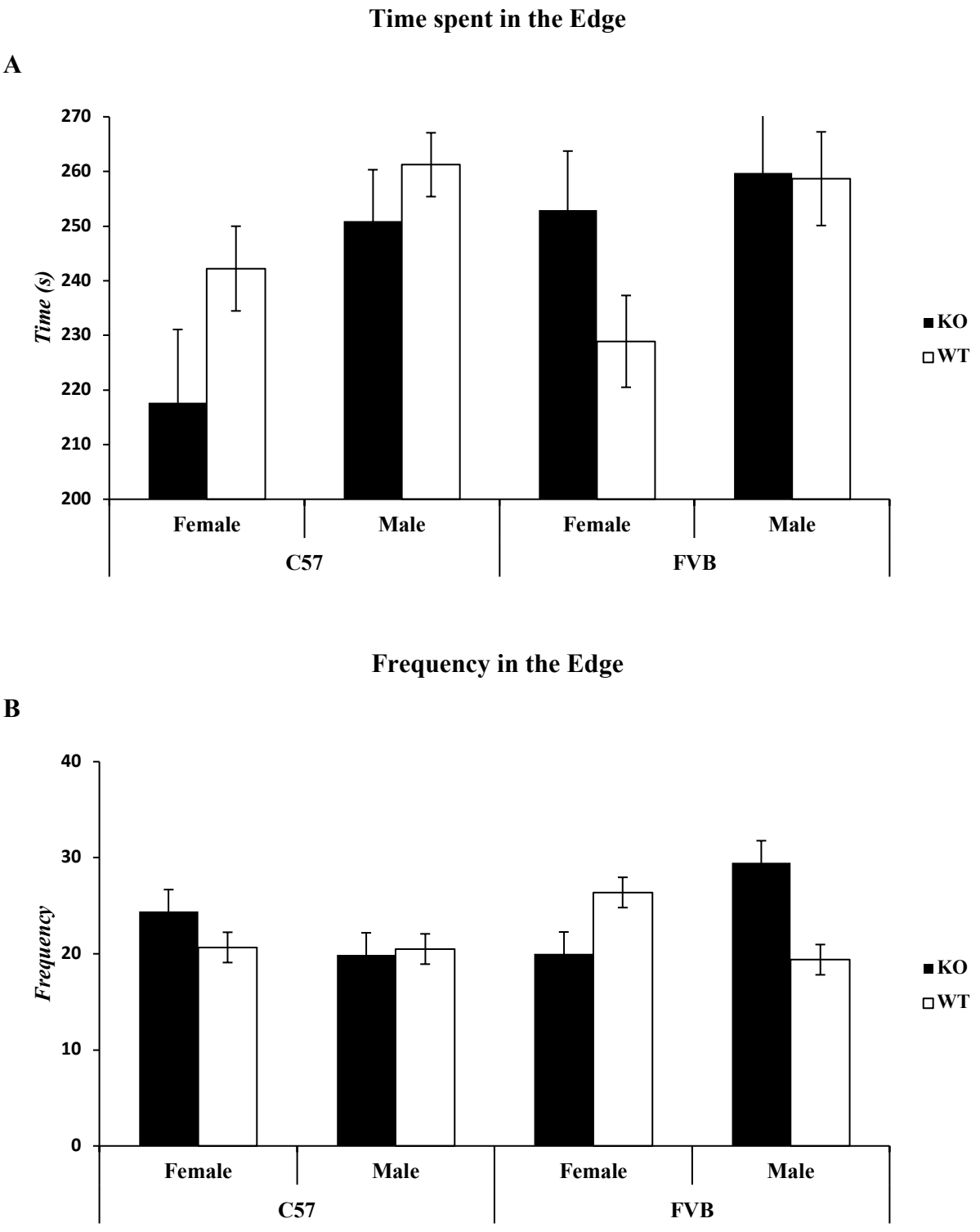
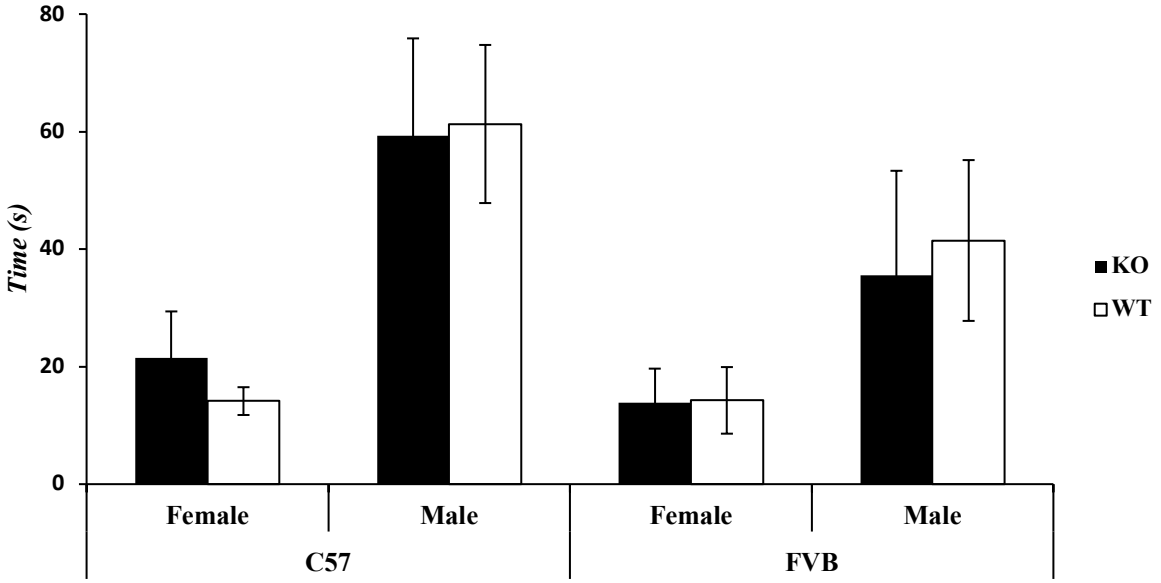


Figure 31. Time spent in and frequency in the Edge of the arena of KO and WT showed by male and female mice reared by C57 and FVB dams (first experimental session). Data are shown as mean ± SEM. WT females reared by FVB dams were more often in the edge than their males' counterparts, whereas there was no sex difference among WT mice reared by C57 dams and in general among KO mice (B). KO mice reared by FVB mice spent more time in the edge than KO mice reared by C57 mice, whereas no difference was found among WT mice according to adoption. In general, male mice spent significantly more time in the edge than females (A).

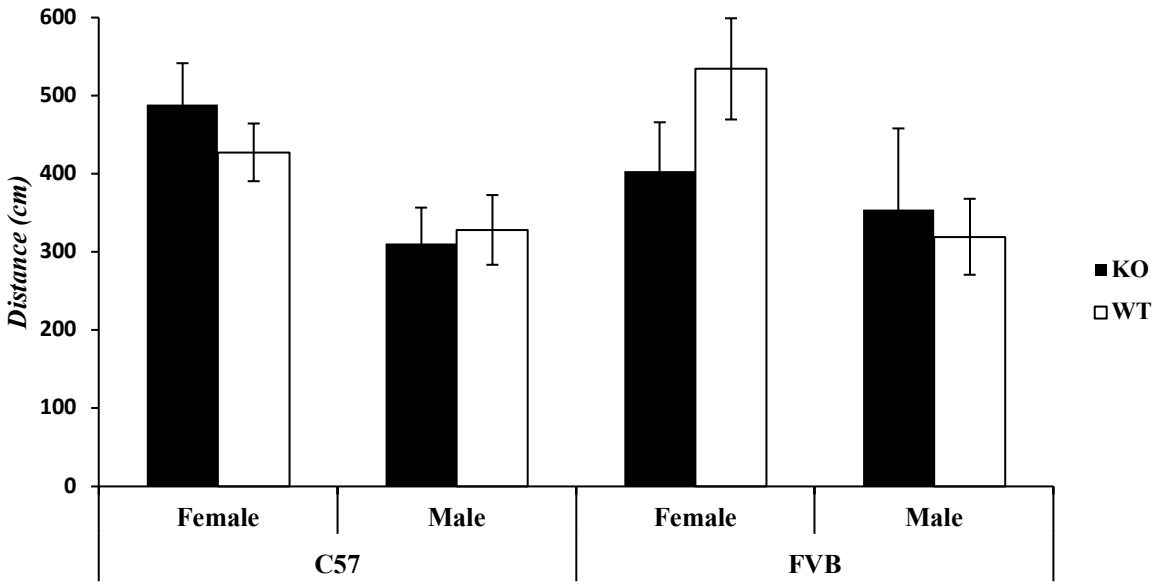
Latency entering in the Median area

A



Distance moved in the Median area

B



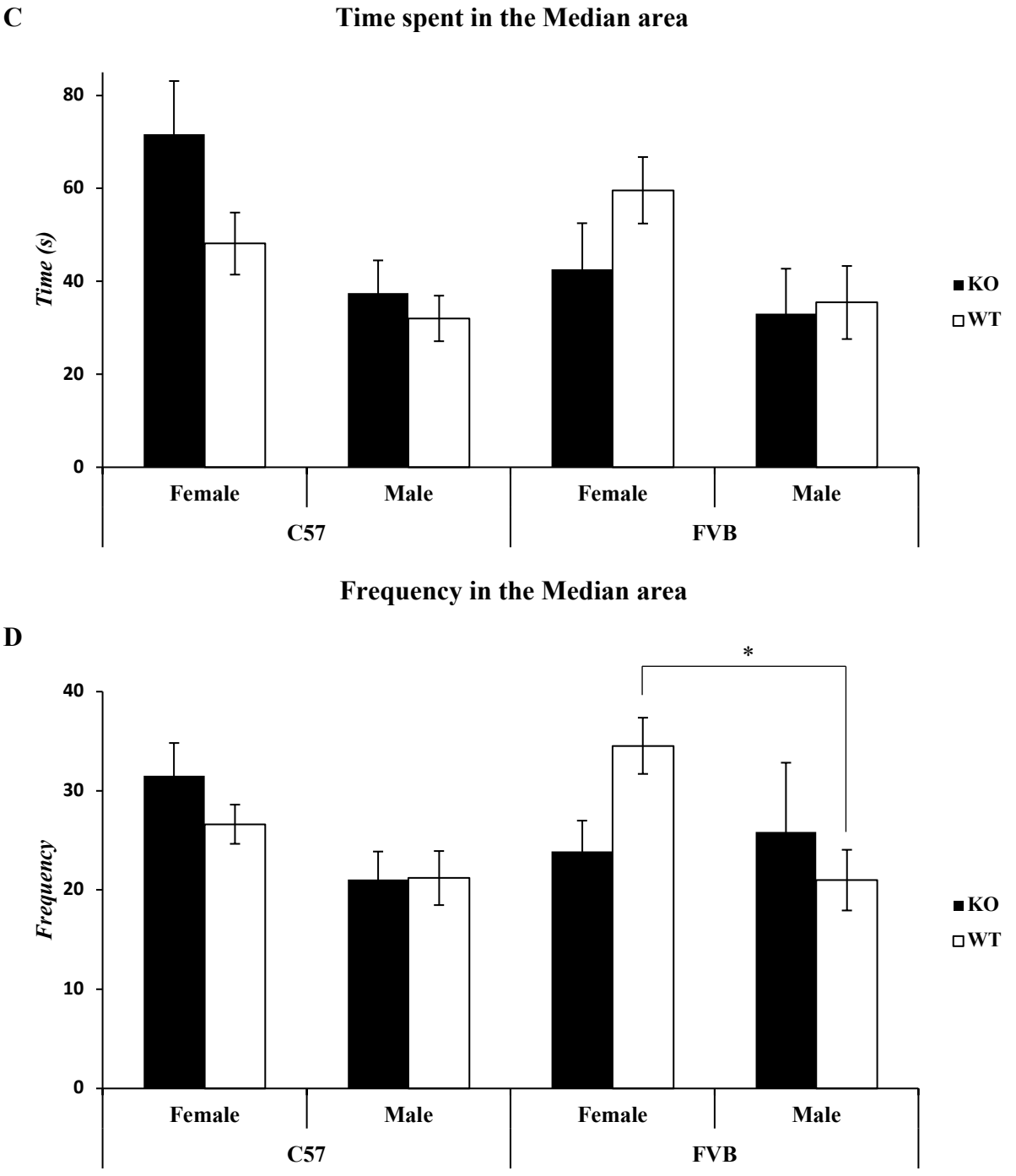
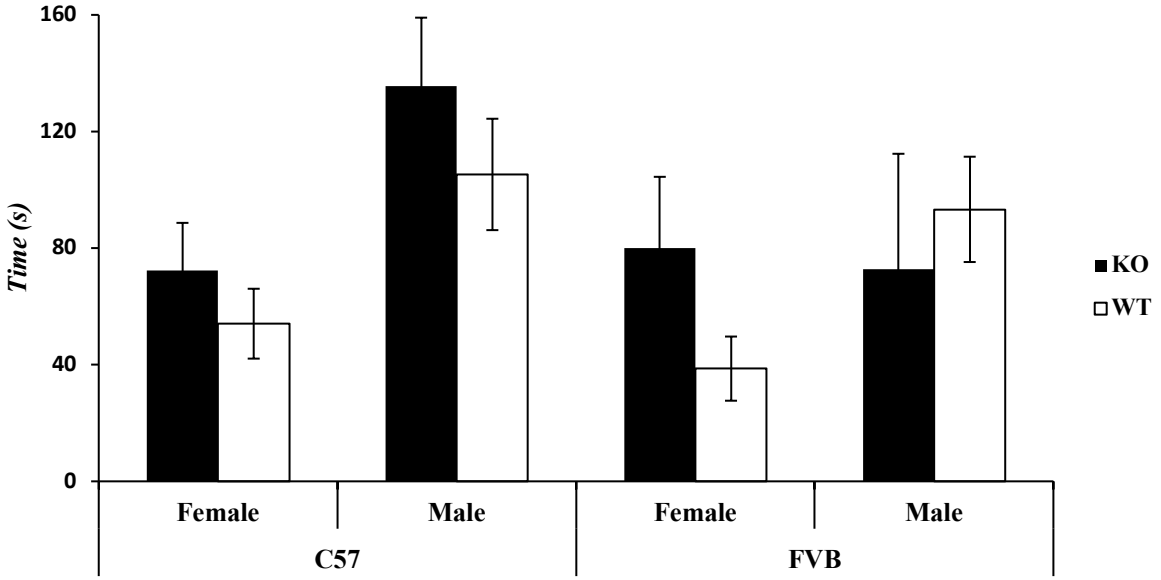


Figure 32. Latency to enter in, distance moved, time spent in, and frequency in the Median area of KO and WT, showed by male and female mice reared by C57 and FVB dams (first experimental session). Data are shown as mean ± SEM. WT females reared by FVB dams were observed more frequently in the median area than their males counterparts, whereas there was no sex difference among WT mice reared by C57 dams; also they were observed more frequently in the median area than WT females reared by C57 dams; KO females mice reared by C57 dams had an higher frequency than their male counterparts, whereas no sex difference was found in KO reared by FVB strain (D). KO mice reared by C57 dams spent more time in the median area than WT mice reared by C57 dams and KO mice reared by FVB dams, whereas no genotype difference was observed among mice reared by FVB dams (C). Female mice were significantly quicker to enter in (A), moved more (B), spent more time in and were observed more frequently in the median area than male mice. * $p<0.05$.

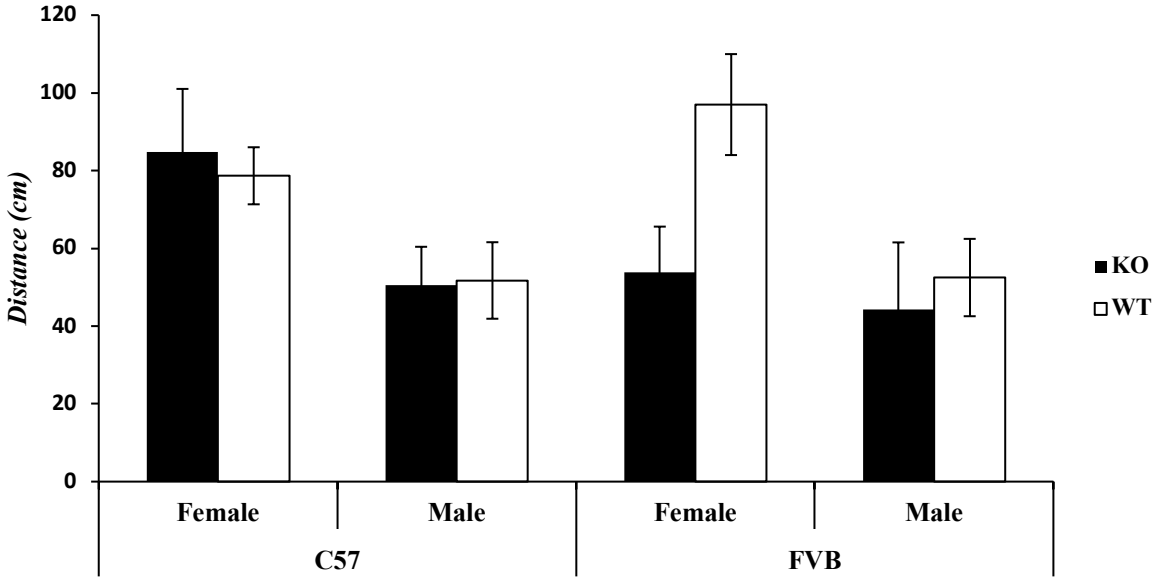
Latency entering in the Center

A



Distance moved in the Center

B



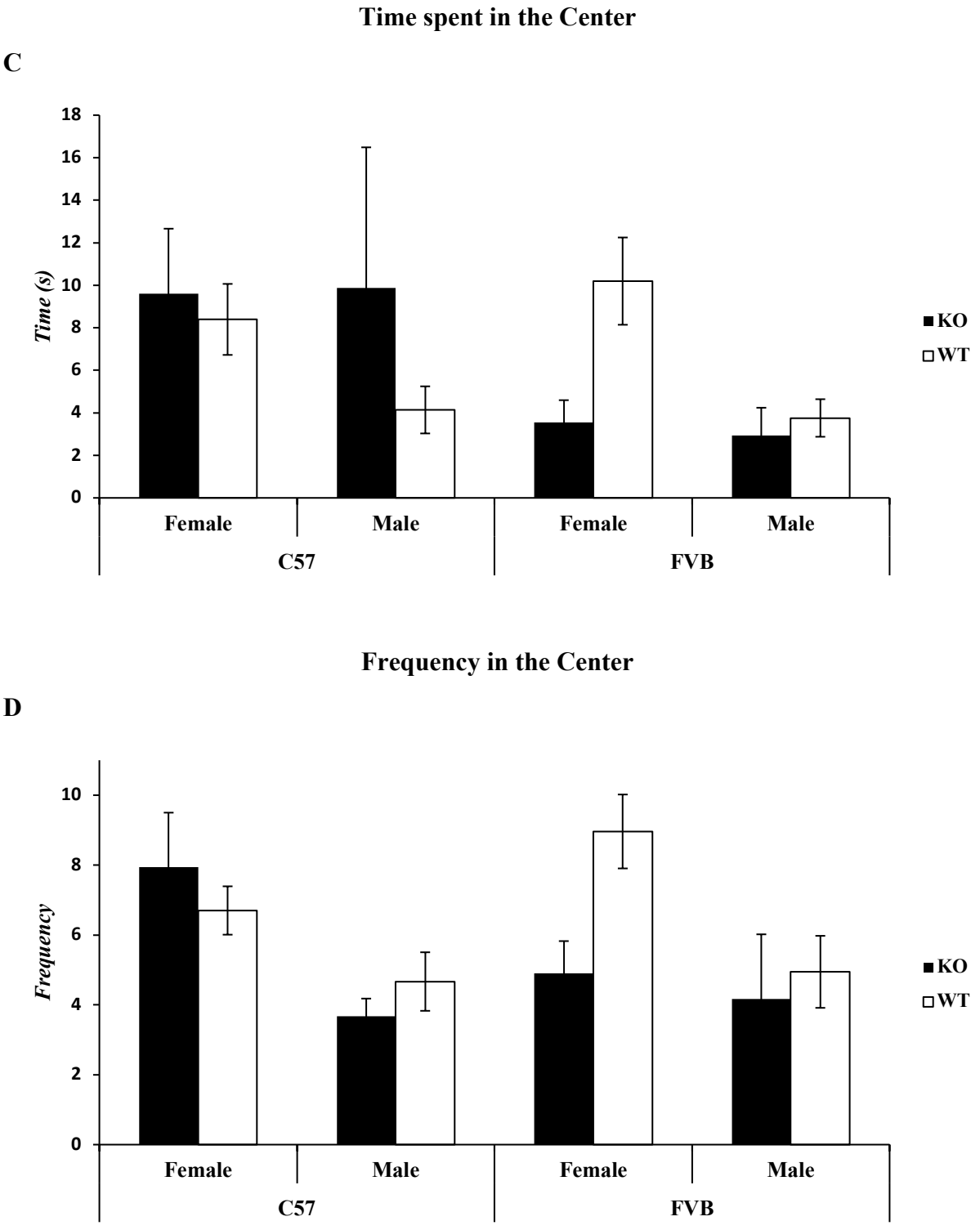


Figure 33. Latency to enter in, distance moved, time spent in, and frequency in the Median area of KO and WT, showed by male and female mice reared by C57 and FVB dams (first experimental session). Data are shown as mean ± SEM. Female mice were significantly quicker to enter in (A), moved more (B), spent more time in (C), and were observed more frequently (D) in the center of the arena than male mice.

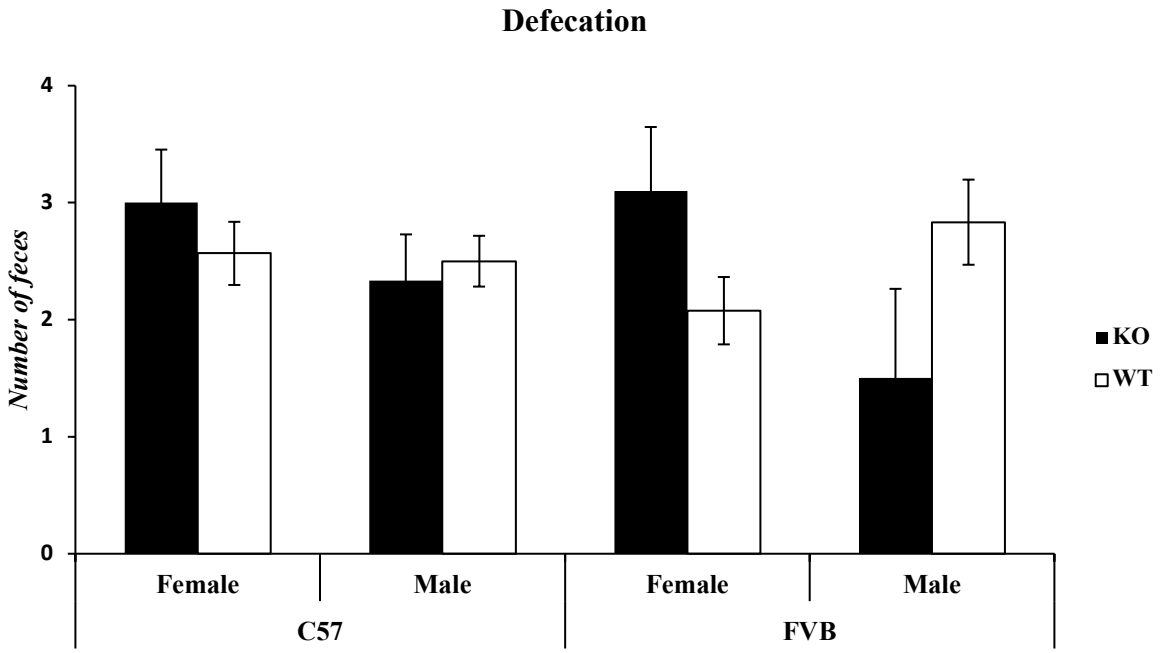


Figure 34. Number of feces in the arena after the five-minute OF test of KO and WT, showed by male and female mice reared by C57 and FVB dams (first experimental session). Data are shown as mean ± SEM. In KO females the number of feces found in the arena at the end of the test was higher than in KO males, whereas in WT mice no sex difference was found.

4.3.3 3-way ANOVA on OF second session

I performed a 3-way ANOVA on the OF second session to assess the effects of sex, genotype and foster dams' strain considering only mice reared by C57 and FVB. I analyzed the same dependent variables as in the 3-way ANOVA on the first session.

Similarly as in the first OF session, foster dams' strain was significant on the frequency observed in grooming behavior ($F_{(1,135)}$=4.613, $p<0.05$; Fig.35): mice reared by FVB were observed more frequently in grooming behavior than mice reared by C57.

3-way ANOVA yielded a highly significant main effect for sex on both time spent in and frequency observed in wall rearing behavior (respectively: $F_{(1,135)}$=12.140, $p<0.001$; $F_{(1,135)}$=8.661, $p<0.01$; Fig.36): specifically male mice spent more time in and were observed more frequently in wall rearing behavior than female mice.

Remarkably, the sex by genotype and foster dams' strain was significant on frequency observed in stationary sniffing ($F_{(1,135)}$=7.445, $p<0.01$; Fig.37): KO males reared by FVB dams were observed more frequently in stationary sniffing than KO females reared by FVB dams ($p<0.01$) and KO males reared by C57, whereas no difference was found in KO mice reared by C57 dams according to sex and among WT mice according to sex and strains. Generally, male mice were observed more frequently in stationary sniffing than females (sex: $F_{(1,135)}$=17.254, $p<0.001$). The analysis also revealed a significant main effect on foster dams' strain on frequency observed in stationary sniffing ($F_{(1,135)}$=4.304, $p<0.05$), mice reared by FVB were observed more frequently in stationary sniffing than mice reared by C57 dams.

3-way ANOVA revealed a highly significant effect for sex by genotype and foster dams' strain on both time spent in and frequency observed in walking behavior (respectively: $F_{(1,135)}$=7.635, $p<0.01$; $F_{(1,135)}$=7.987, $p<0.01$; Fig.38): in particular KO males reared by FVB dams spent more time in and were observed more frequently in walking behavior than KO females reared by FVB dams ($p<0.05$ for frequency), whereas no sex difference was observed

among KO mice reared by C57 and among WT mice according to sex and foster dams' strain. Sex was found to be significant on both time spent in and frequency observed (like in the first session) in walking behavior (respectively: $F_{(1,135)}$=7.408, p<0.01; $F_{(1,135)}$=6.185, p<0.05): in particular males spent more time in and were observed more frequently in the arena than females.

Remarkably, males moved more and faster in the arena than females (sex: $F_{(1,135)}$=11.553, p<0.001 for distance moved; $F_{(1,135)}$=6.851, p<0.01 for mean velocity; Fig.39).

Sex by foster dams' strain interaction was significant on time spent in the edge ($F_{(1,135)}$=4.901, p<0.05; Fig.40A): in particular males reared by C57 dams spent significantly less time in the edge than females reared by C57 dams and males reared by FVB dams (respectively: p<0.01; p<0.05), whereas no sex difference was observed among mice reared by FVB dams. Sex was also significant on both time spent in and mean velocity in the edge ($F_{(1,135)}$=8.147, p<0.01; $F_{(1,135)}$=9.583, p<0.01; Fig.40): in particular male mice spent more time in and were faster in the edge than female mice.

3-way ANOVA showed a significant effect for sex by foster dams' strain interaction on time spent in the median area ($F_{(1,135)}$=4.546, p<0.05; Fig.41B): specifically males reared by C57 dams spent significantly less time in the median area than females reared by C57 dams and males reared by FVB dams (respectively: p<0.01; p<0.05), whereas no sex difference was observed among mice reared by FVB dams. Sex was significant on time spent in, distance moved and frequency observed in the median area (respectively: $F_{(1,135)}$=8.498, p<0.01; $F_{(1,135)}$=12.298, p<0.001; $F_{(1,135)}$=3.897, p<0.0504; Fig.41): in particular male mice spent more time in, moved more and were observed less frequently in the median area than female mice.

Conditional deletion for NPY1r significantly increased time spent in the center (genotype: $F_{(1,135)}$=4.144, p<0.05; Fig.42B). 3-way ANOVA yielded a significant main effect for sex on time spent in, latency to enter and frequency observed in the center ($F_{(1,135)}$=6.939,

p<0.01; $F_{(1,135)}$=7.175, p<0.01; $F_{(1,135)}$=4.195, p<0.05; Fig.42): female mice spent more time in, were observe more frequently and were slower to enter in the center than male mice.

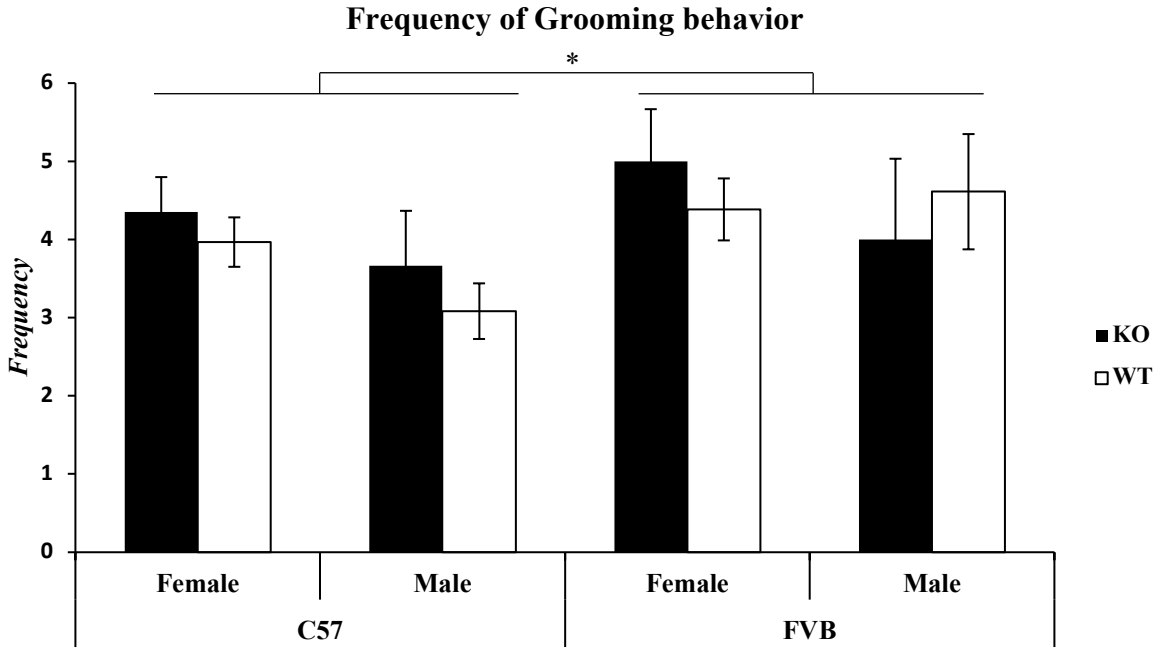


Figure 35. Frequency of Grooming behavior of KO and WT showed by male and female mice reared by C57 and FVB dams (second experimental session). Data are shown as mean ± SEM. Mice reared by FVB were significantly observed more frequently in grooming behavior than mice reared by C57 dams. * p<0.05**.**

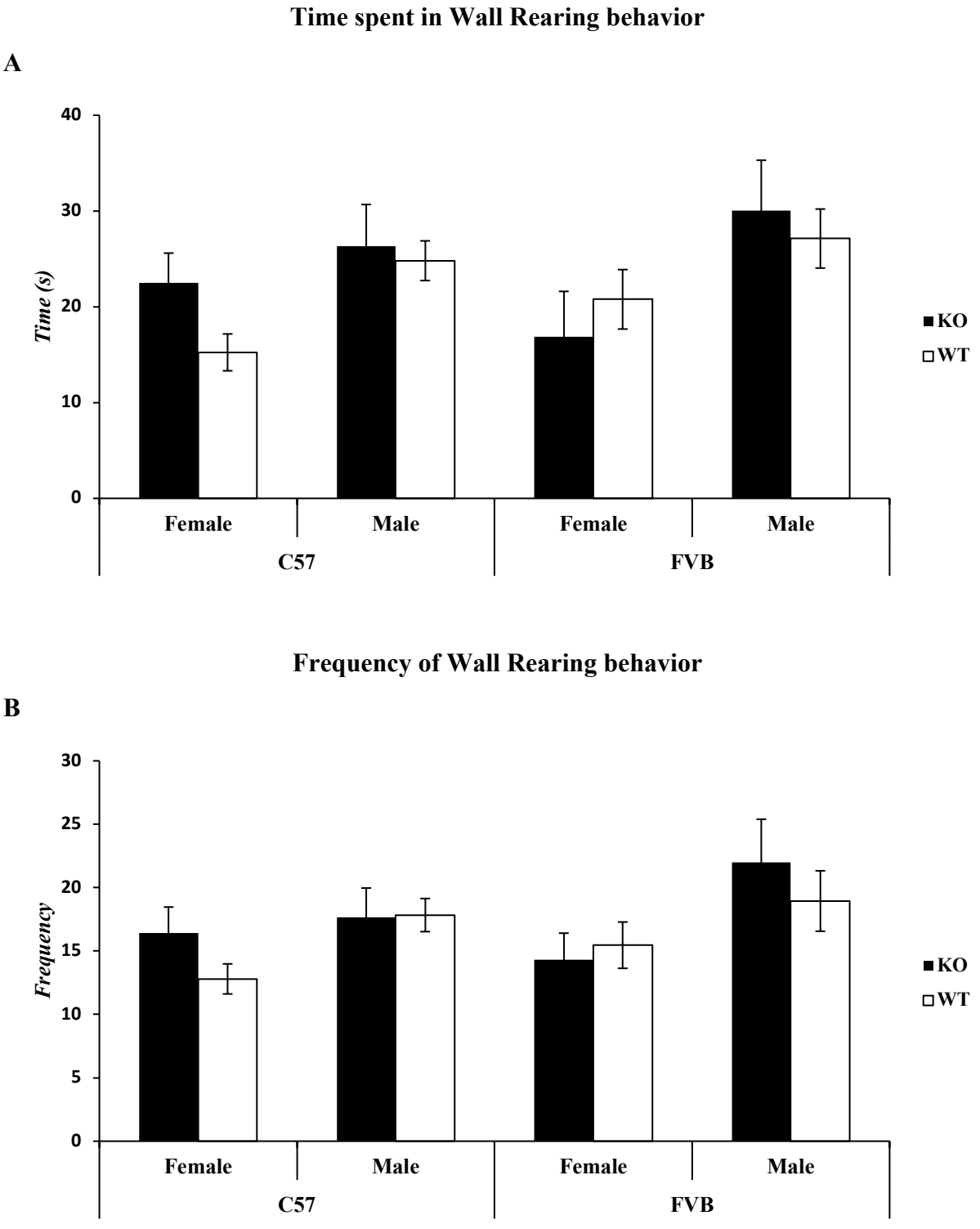


Figure 36. Time spent in and frequency of Wall Rearing behavior of KO and WT showed by male and female mice reared by C57 and FVB dams (second experimental session). Data are shown as mean ± SEM. Male mice significantly spent more time in (A) and were observed more frequently in (B) wall rearing behavior than female mice.

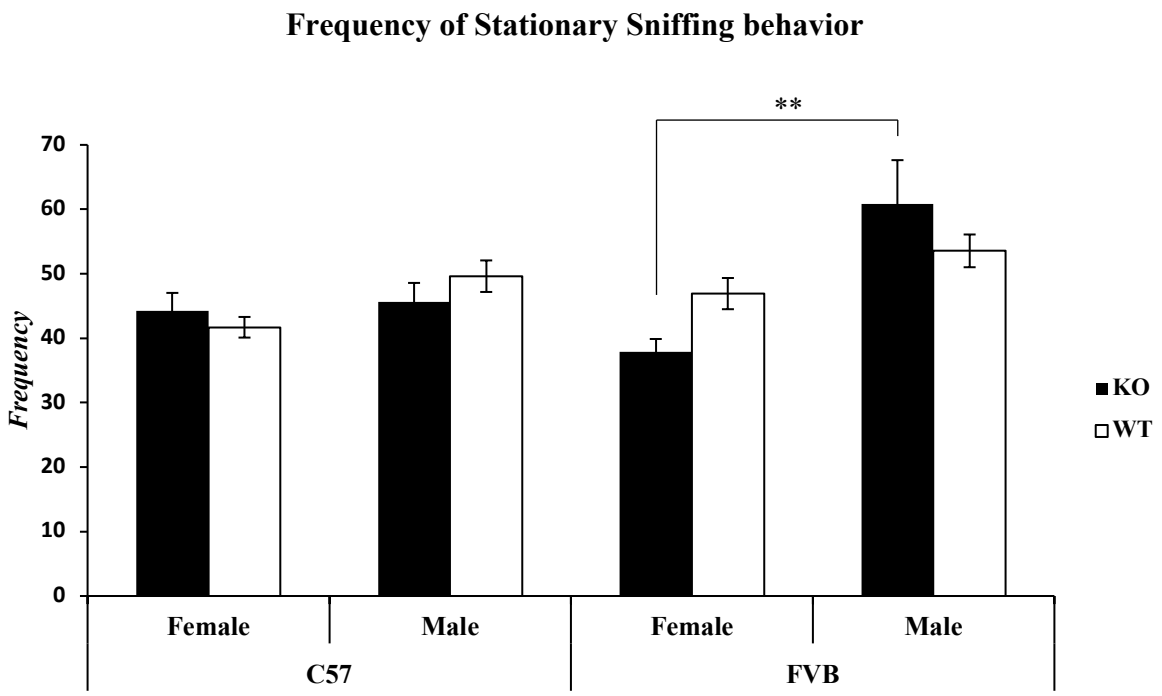


Figure 37. Frequency of Stationary Sniffing behavior of KO and WT showed by male and female mice reared by C57 and FVB dams (second experimental session). Data are shown as mean ± SEM. KO males reared by FVB dams were significantly observed more frequently in stationary sniffing than KO females reared by FVB dams and KO males reared by C57, whereas no difference was found in KO mice reared by C57 dams according to sex and among WT mice according to sex and strains. Generally, male mice were observed more frequently in stationary sniffing than females. Besides, mice reared by FVB were observed more frequently in stationary sniffing than mice reared by C57 dams. ** $p<0.01$.

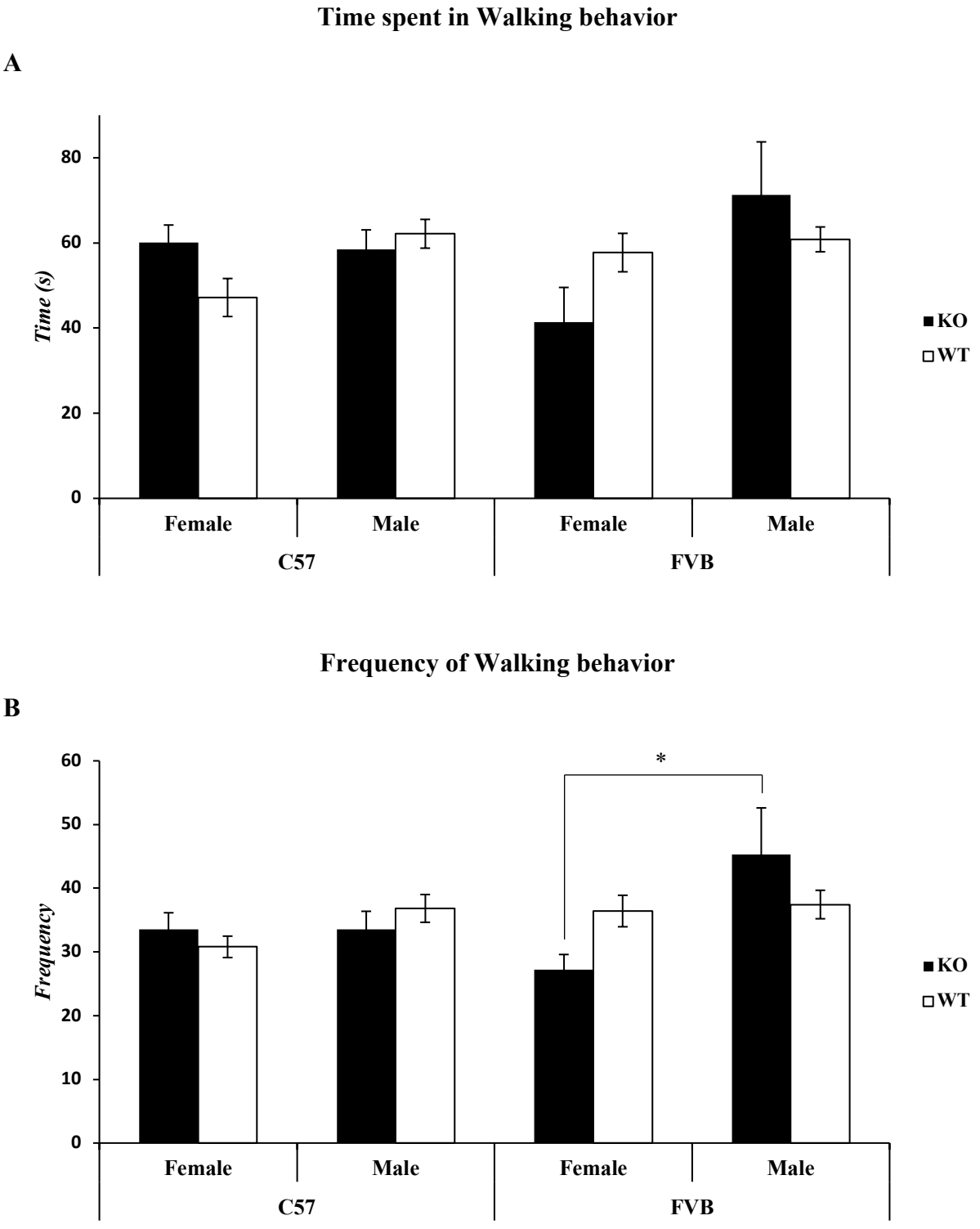


Figure 38. Time spent in and frequency of Walking behavior of KO and WT showed by male and female mice reared by C57 and FVB dams (second experimental session). Data are shown as mean ± SEM. KO males reared by FVB dams spent more time in (A) and were observed more frequently (B) in walking behavior than KO females reared by FVB dams, whereas no sex difference was observed among KO mice reared by C57 and among WT mice according to sex and foster dams' strain. Furthermore, males significantly spent more time in and were observed more frequently in the arena than females. * $p<0.05$.

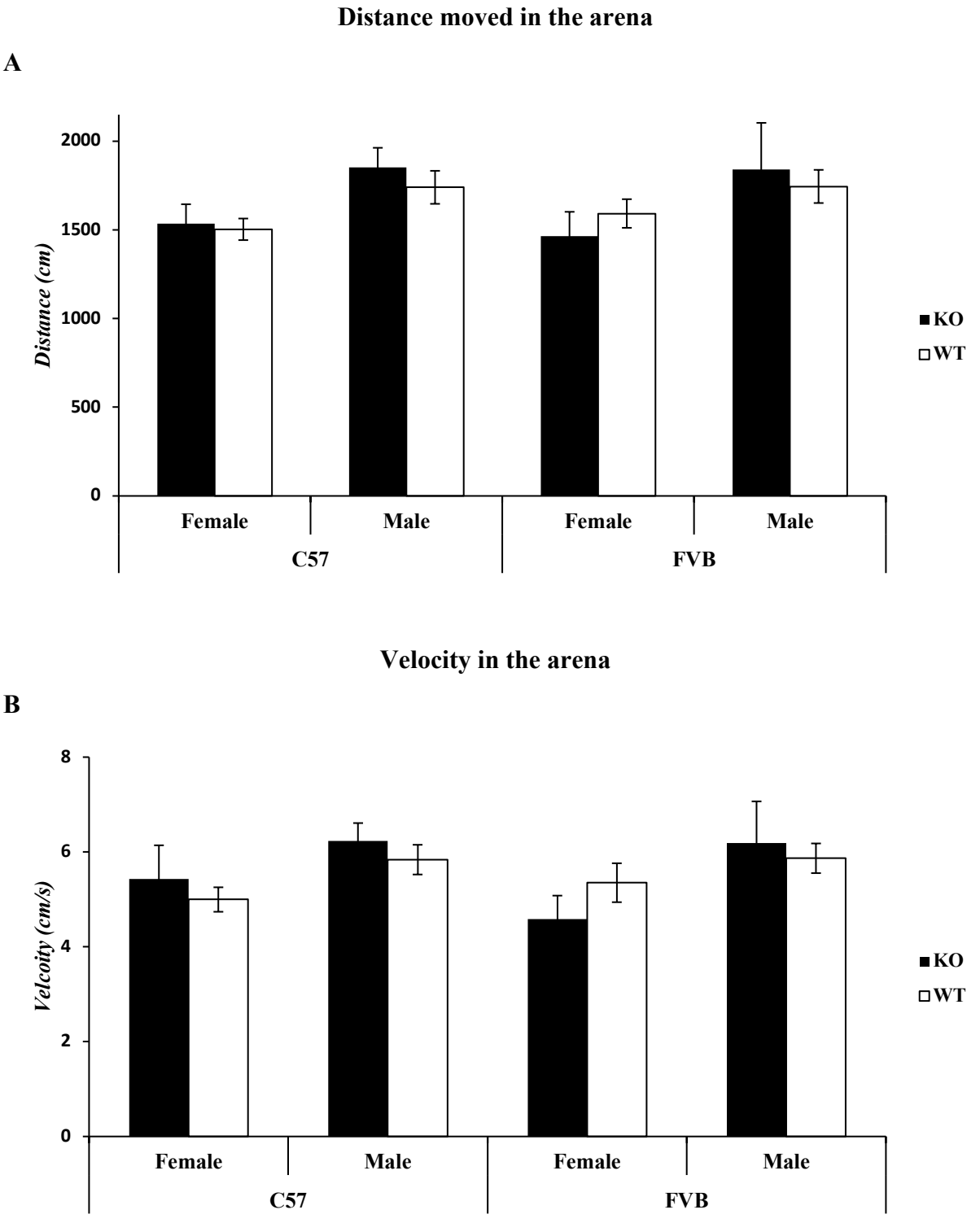


Figure 39. Distance moved and velocity in the arena of KO and WT showed by male and female mice reared by C57 and FVB dams (second experimental session). Data are shown as mean ± SEM. Males moved more (A) and faster (B) in the arena than females.

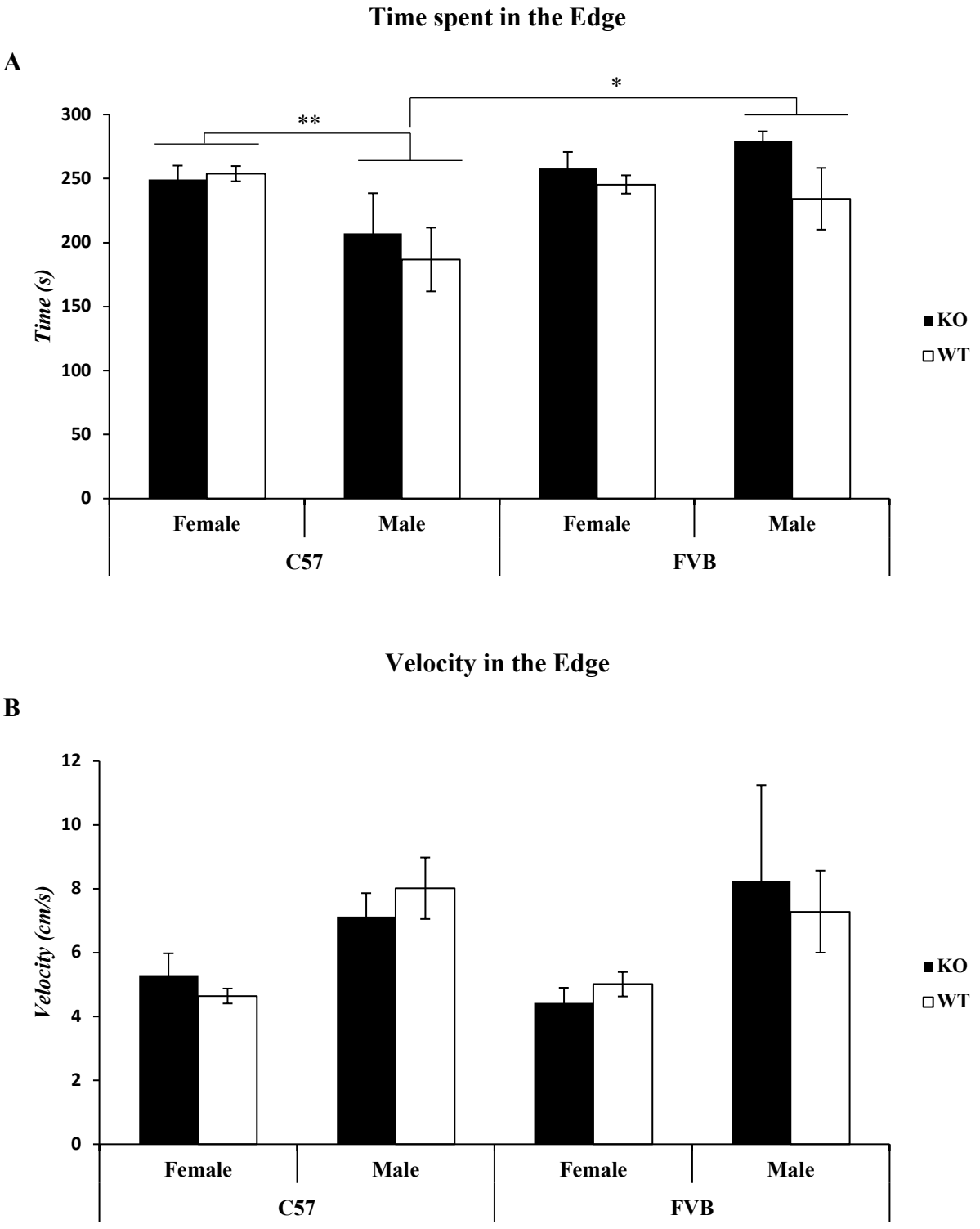


Figure 40. Time spent in and velocity in the Edge of the arena of KO and WT showed by male and female mice reared by C57 and FVB dams (second experimental session). Data are shown as mean ± SEM. Males reared by C57 dams spent significantly less time in the edge than females reared by C57 dams and males reared by FVB dams whereas no sex difference was observed among mice reared by FVB dams (A). In general, male mice spent more time in and were faster (B) in the edge than female mice. * $p<0.05$; ** $p<0.01$.

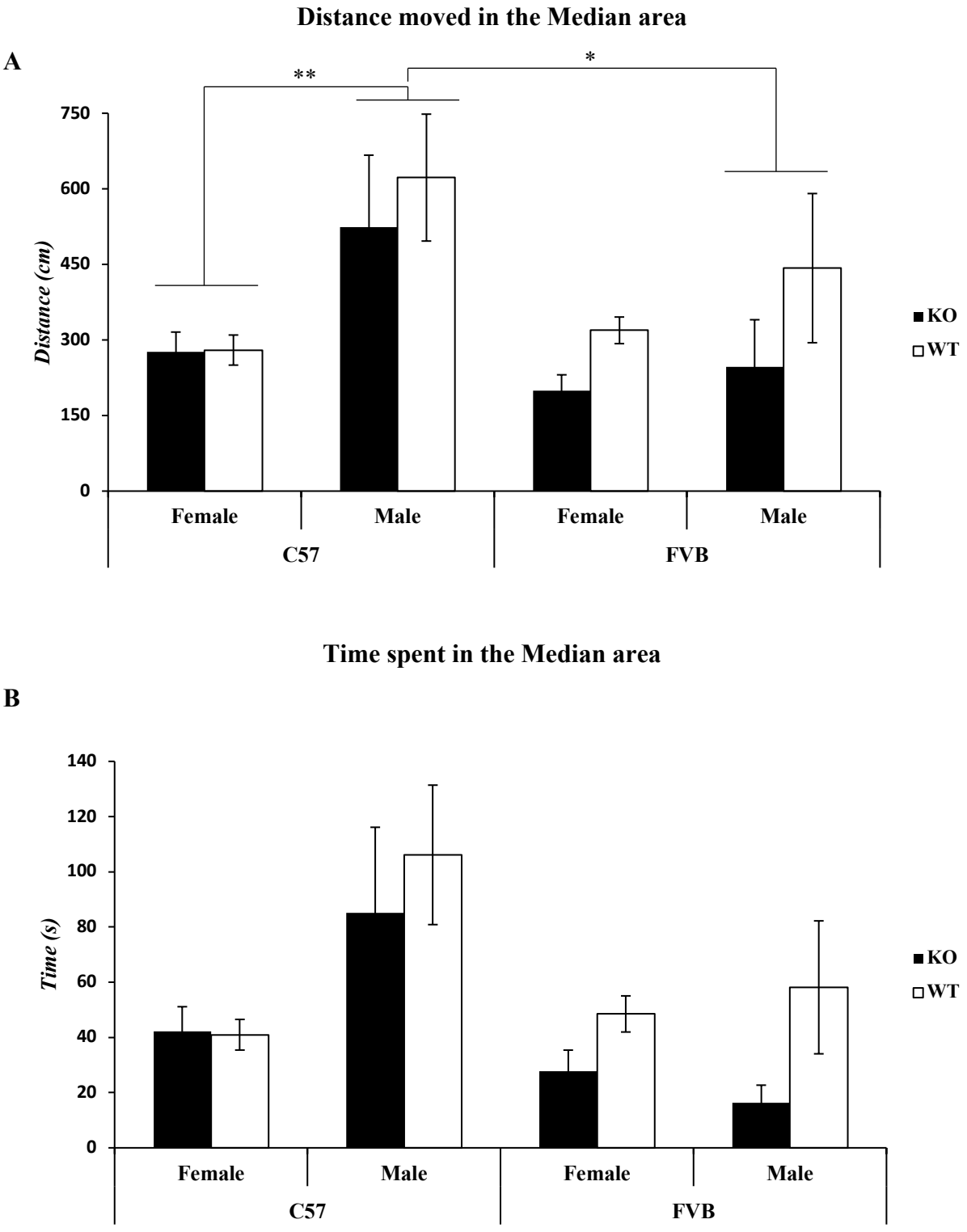
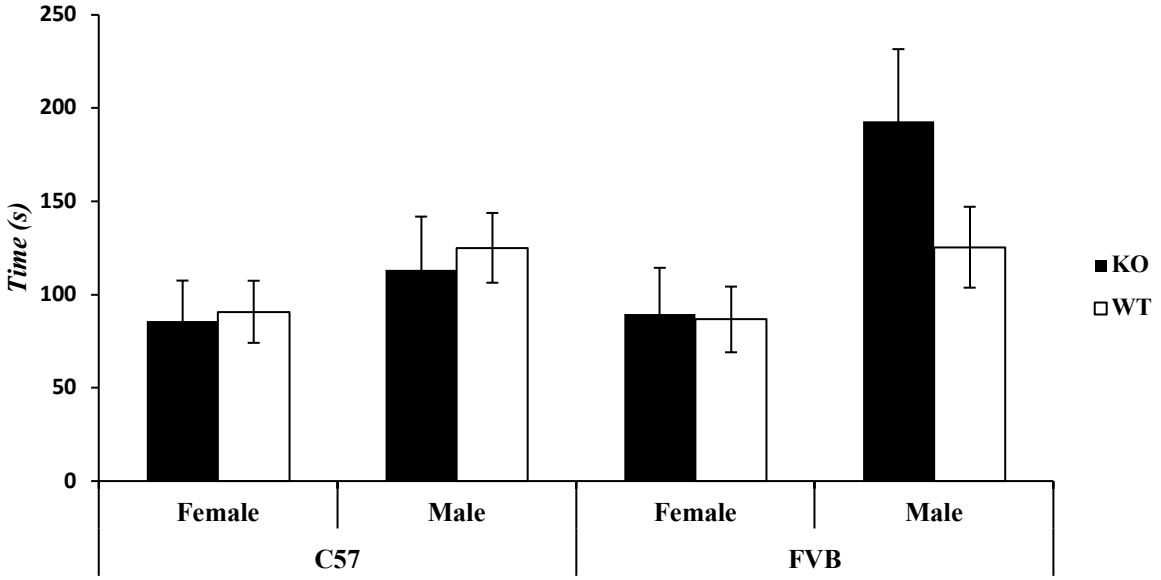


Figure 41. Distance moved and time spent in the Median area of KO and WT showed by male and female mice reared by C57 and FVB dams (second experimental session). Data are shown as mean ± SEM. Males reared by C57 dams spent significantly less time in the median area than females reared by C57 dams and males reared by FVB dams, whereas no sex difference was observed among mice reared by FVB dams (A). In general, male mice spent more time in and moved more in the median area than female mice. * $p<0.05$ ** $p<0.01$.

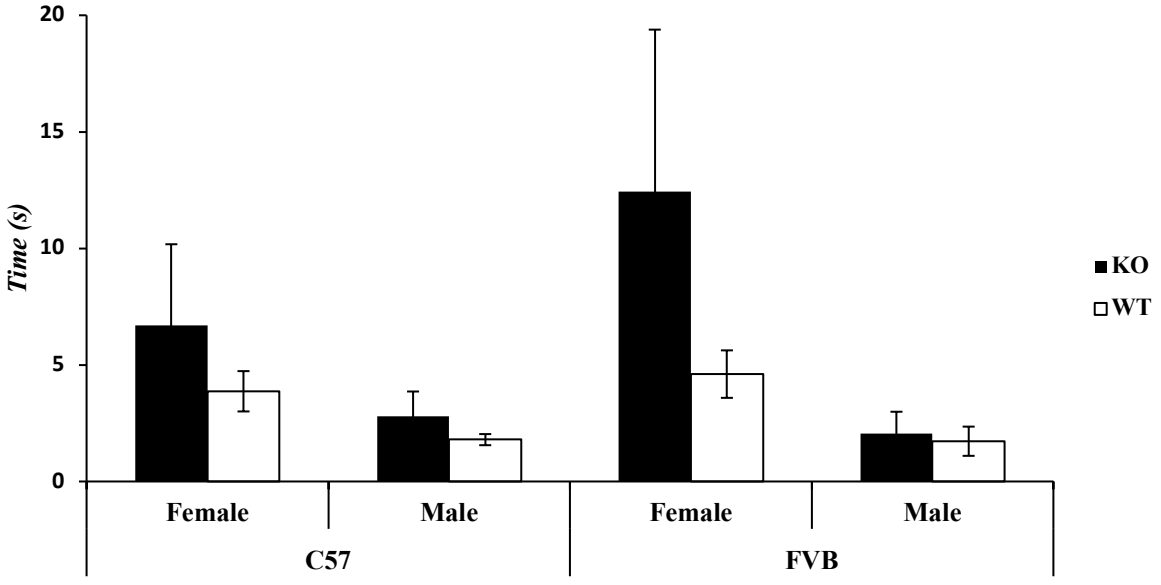
Latency in entering the Center

A



Time spent in the Center

B



C

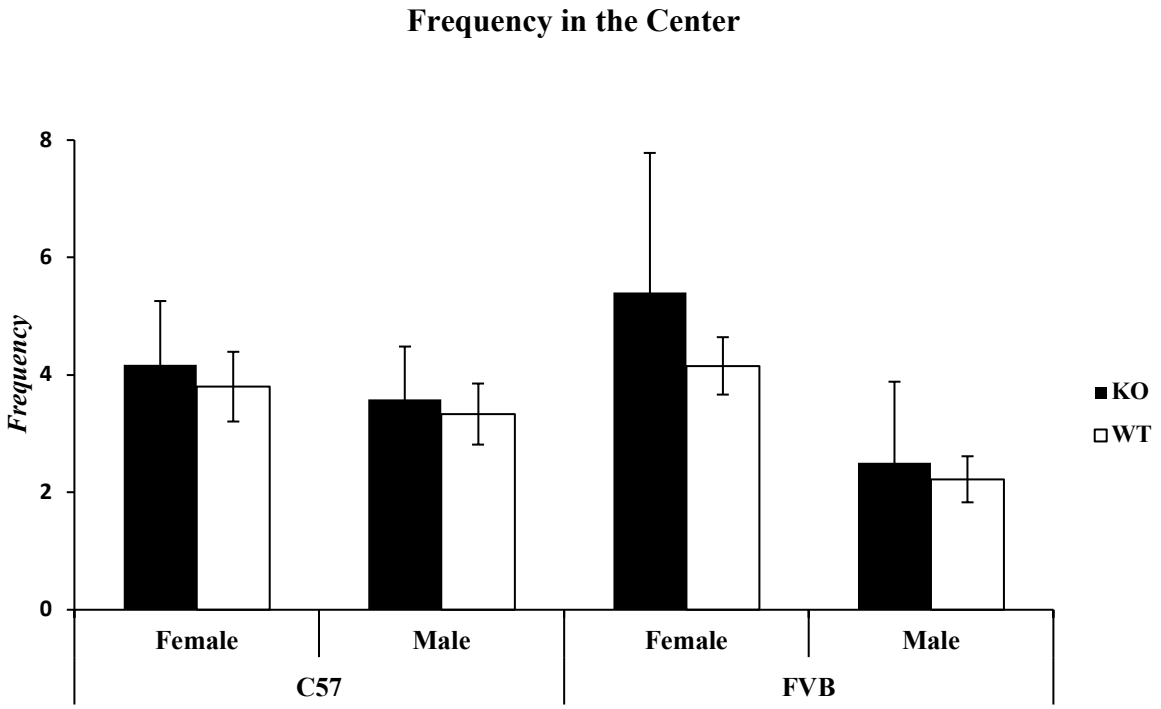


Figure 42. Latency to enter in, time spent and frequency in the Center of the arena of KO and WT showed by male and female mice reared by C57 and FVB dams (second experimental session). Data are shown as mean ± SEM. Conditional deletion for NPY1r significantly increased time spent in the center (B). Female mice were significantly slower to enter in (A), spent more time in (B) and were observe more frequently (C) in the center of the arena than male mice.

5 DISCUSSION

My project focused on the analysis of exploratory and emotional behavior in conditional KO mice for the NPY1r gene in relation to sex and early maternal environment (i.e., maternal care received during the first week of postnatal life). Under present conditions, the experimental design requires the experimental subjects to be reared by foster dams belonging to different strains. In order to assess the early maternal environment, the lactating foster dams were observed during the first week of lactation to measure their spontaneous maternal behavior, which was analyzed in other thesis (data not published). In any case, the behavioral analysis showed that females of CD1 and FVB females were found to show a higher level of maternal care, while C57 and Balb females displayed a lower level of maternal care, as assessed on the basis of lactation postures (e.g., Arched Back Posture and Nursing Behavior).

In the conditional NPY1r-KO mouse model, a complete inactivation of NPY1r gene occurs after postnatal day 40 and only in specific brain areas such as: hippocampus, amygdala and prefrontal cortex (Bertocchi et al., 2011). Therefore, as adults, experimental subjects underwent a battery of tests, including traditional Open Field test (OF), a test widely used in behavioral neuroscience to assess exploratory and anxiety-like behaviors in rodents (Hall and Ballachey, 1932; Crawley, 1985). My thesis research focused on a detailed, ethological analysis of the behaviors expressed by mice during this particular test.

I performed a comprehensive analysis on all mice examined during research conducted over several years in the Palanza laboratory. Some cohorts performed the test a second time following a challenging event, (i.e., pregnancy for females and Stress procedure for males); this allowed also to assess mouse behavior following a challenging event in a previously explored environment. Once I had scored all video footage of the OF tests related to the NPY1r KO and Wild Type (WT) mice from the different cohorts, I conducted a preliminary statistical analysis

on the whole sample on the first experimental session to assess the impact of sex, genotype, and foster dams' strain and their possible interactions on exploratory and anxious-like behavior. Next, I conducted a two-factor analysis only on the control group to assess the influence of sex and early maternal environment on exploratory behavior in the overall population. Given the high sample size (367) and many dependent variables, I conducted a Principal Component Analysis only in the first experimental session to group the ethological measures into a smaller number of factors representative of the main behavioral categories shown by the experimental subjects. I obtained 5 principal components, indicative of: exploratory activity, activity in the median area, risk assessment, vertical exploration and activity in the center of the arena. I conducted two preliminary analyses on these 5 factors: the former on the data from all animals, and the latter only on the control group. Finally, I conducted analyses to assess the effect of a previous challenge on mice behavior.

5.1 Principal Component Analysis

The size of the sample analyzed and the absence of possible variation due to differences in the observer allowed principal component analysis to be applied to the data produced. Complexity reduction is achieved by simply analyzing the principal components among the new variables. From the principal component analysis, I obtained 5 factors as follows:

- Principal component 1 included: distance moved and mean velocity both in the arena and in the edge, walking (total time and frequency), stationary sniffing (total time, mean time and frequency). Since it includes parameters of exploration of the arena base, I named it "**Exploration**".
- Principal component 2 included: time spent in, distance moved and frequency in the median area. For that reason, I named it "**Activity in the Median Area**".

- Principal component 3 included: stretch attend posture (total time, mean time and frequency). Because it includes parameters of active risk assessment in the environment, I named it "**Risk Assessment**".
- Principal component 4 included: wall rearing (total time, mean time and frequency). For that reason, I named it "**Vertical Exploration**".
- Principal component 5 included: time spent in, distance moved and frequency in the center of the arena. For that reason, I named it "**Activity in the Center**".

By means of this analysis, KO mice resulted to be less active and more anxious than WT mice. A consistent difference related to conditional gene deletion was also found in preliminary analysis: KO mice displayed more grooming behavior than WT. However, regardless of their genotype and sex, all the animals reared by high maternal care dams (specifically CD1) were more active and less anxious than those reared by low maternal care dams (C57 and Balb). In particular, females reared by CD1 dams were more exploratory than their male counterparts, while among mice reared by Balb dams it was the opposite: males were more active than the females. In the remaining two strains (both FVB and C57), a sex difference was not found. In summary, inactivation of the gene produced a behavioral profile more indicative of anxiety-like state, interacting neither with sex nor with the foster dams' strain. These results are consistent with previous results obtained by Bertocchi et al. (2011); NPY1r^{rfb} mice (i.e., KO mice) display reduced exploration of the OF. Furthermore, foster dams' strain exploratory and anxiety-like behavior in their adult offspring, in that mice receiving a higher level of maternal care were less anxious and more exploratory than mice exposed to lower levels of maternal behavior. This finding confirms that in rodents the levels of maternal care experienced affect later behavioral responses (Meaney, 2001; Champagne, 2008).

When considering only control mice (WT), mice reared by CD1 lactating females, particularly females, were more exploratory and less anxious in the edge of the arena than

animals reared by the other strains, specifically C57 and Balb. On the other hand, males performed more wall rearing than females, and mice reared by CD1 and FVB showed more vertical exploration than both C57 and Balb. Mice reared by C57 dams appeared to be more anxious (more SAP) than other strains. When looking at the behavioral sex differences, females reared by CD1 were more active and less anxious than their male counterparts; while in the other strains this difference is either reversed (Balb) or null (FVB and C57), because the females from these strains became more to the males, i.e. less exploratory and more anxious-like. Consistently with the preliminary analysis, female mice reared by Balb dams spent more time in grooming behavior compared to their male counterparts, while in the other strains this sex difference was either reversed (CD1) or null (C57 and FVB). This result is in line with literature, with grooming being commonly linked to emotionality, that is considered to be a reaction to a stressful situation (e.g., Hoover-Plow et al., 2001), a response often provoked by novelty (Dunn et al., 1981). From this analysis, we inferred how early maternal environment influenced exploratory activity and anxiety in response to a new, potentially dangerous, environment represented by the OF. In addition, early maternal environment had a different effect in the two sexes: higher maternal care seemed to affect female mice, but not male mice (Caviola, 2017).

5.2 Focus on mice reared by C57 and FVB dams during first and second session of open field test

As adults, experimental mice were tested twice during the open field test. In the first session, the animals faced the apparatus for the first time; while before being tested for the second session they've been subjected to a specific challenge. Females underwent the test while pregnant (a couple of days before delivery); whereas males after a month of the Social Stress procedure. Briefly, during this procedure, which lasted 30 days, an intruder was placed in the experimental animal's cage daily likewise in the resident/intruder paradigm.

As for these analyses only subjects who underwent both sessions were included. As mentioned before, only a subset of the animals underwent two sessions of the OF test. This led to strong imbalances in the sample, as for instance there was only one KO male reared by CD1 dams. I had thus to exclude data from CD1 adoption. On the other hand, many animals had been reared by the C57 and FVB strains. Besides, these two strains differ on the level of maternal care (high for FVB and low for C57). Therefore, I decided to consider these two groups for the present analysis. Unfortunately, such further sample reduction did not allow me to conduct a PCA.

Overall and in line with our expectations, when comparing the second experimental session with the first one, mice performed less exploratory activity. Animals displayed more frequently behaviors in which they remain in place, such as grooming behavior and stationary sniffing. This result could be due either to the effect of habituation to the apparatus or to the challenge experienced. Remarkably, behavioral differences in the two experimental sessions are also influenced by the sex of the animal. In the second session females were less exploratory than in the first session, while in males there are no such differences between the two sessions. Besides, during the second session females explored less than males. This was to be expected given that females in the second experimental session were pregnant and therefore the energy expenditure to perform some exploratory activities were greater. Precisely this difference is more noticeable in walking behavior, average animal speed and wall rearing behavior. Since females did more exploratory activity in the central areas of the arena, they were found to show less anxious-like behaviors than males.

To further investigate the possible relationship between sex, genotype and early maternal environment, I analyzed separately data from first and second session of open field. In the first experimental session, regardless of their sex and early maternal environment, KO mice displayed more grooming than WT mice. KO mice seemed to be more emotional (Hoover-

Plow et al., 2001) in a novelty environment (Dunn et al., 1981). There were no differences in exploratory activity due to genotype in general, though. In particular, KO mice reared by FVB dams appeared to be more anxious since they spent more time in the edge than WT mice reared by FVB and KO mice reared by C57 dams. As opposed to mice reared by C57 dams, KO mice are less anxious than WT counterparts. Consistently, KO mice reared by FVB dams spent less time in the median area and center of the arena than FVB-reared WTs and C57-reared KOs who spent more time in the central areas than C57-reared WTs. As a whole, different maternal care seemed to influence exploratory behavior in KO mice in opposite ways, those reared by FVB are more anxious while those reared from C57 are less so. Present results are slightly different from what Bertocchi and colleagues (2011) found; in their study the effect of the genotype becomes evident only in males reared by FVB foster mothers but not by C57 foster mothers. Furthermore, when considering only the adoption, mice reared by FVB dams displayed more exploratory behaviors and were less anxious-like behaviors than mice reared by C57. This is consistent with the effect of high level of maternal care, since FVB dams, exhibiting a higher level of maternal care, raise less anxious mice than those raised by C57 dam s.Finally, as also shown in previous analyses, females performed more exploratory activity and were more likely to explore the central areas of the arena while males spent more time in the peripheral area.

In the second experimental session, all animals reduced the time spent in the center due to habituation. However, habituation seemed to have a greater impact on WT, who showed a greater reduction in time spent in the center as opposed to KOs. Indeed, in the second experimental session, KO mice spent more time in the center of the arena than WT mice. The overall reduction in locomotor and exploratory activity in the second session, especially showed by WT mice, is in line with that found by Leppänen and colleagues (2005), who used high and low thigmotaxis lines of mice. Although they found no difference between the two lines of mice in exploratory activity during the second session of the OF, they observed a reduction in activity

in subsequent exposure to the apparatus. Under present conditions, KOs were probably less affected by habituation, and this led them to explore more in the central area of the arena. Unfortunately, in the second session the SAP had a high percentage of zeros so it was not possible to conduct an analysis on that behavior that would have been useful for understanding the anxiety state of KOs. Interestingly, KO males reared by FVB dams were observed more frequently in walking and stationary sniffing compared to KO females reared by FVB dams and KO males reared by C57. No sex difference was observed among KO mice reared by C57. Besides, among WT mice I did not observe neither sex nor strain differences. Given the reduction in exploratory activity, the effect of habituation was greater in WTs compared to the first session; whereas in male KOs habituation effect is reduced especially in those reared by FVB. This result seemed appear to be consistent with the results found by Bertocchi and colleagues (2011) whereby KO males reared by FVB dams were more anxious than FVB-reared KO females, even though they had been exposed only once to the open field. Present results are consistent with the activity performed in the center of the arena: KOs seemed to be less affected by habituation. Since anxiety state and habituation ability are related (van der Goot et al., 2021), I could hypothesize that higher anxiety state caused by gene deletion led KO mice to be less prone to habituate. When considering the behavioral sex differences, as expected, in the second session female mice were generally less active than male mice, this is because the females were pregnant and heavier. Therefore, their energy expenditure for exploratory activity was greater. As a consequence, inferences according to sex differences in the second session cannot be made because of the different experimental design applied to the two sexes. Under present conditions, another consistent result was on strain of foster mothers; mice reared by FVB dams were observed more frequently in grooming behavior and stationary sniffing.

A major result of the present study is that, consistently with our previous studies (Bertocchi et al., 2011), overall KO mice displayed a more anxious-like behavior and lower

exploration than control mice. Actually, as suggested by the second experimental session, the effect of habituation was smaller in KO mice than in WT mice. A possible question is whether we observed an impairment on the habituation effect, or rather on the ability of KO mice to cope with the challenge they experienced. Indeed, an anxious-like behavioral profile in the second session is not supported by the results on SAP. Consequently, a possible hypothesis is that the lack of habituation in KO mice depends mainly upon the conditional deletion of NPY1r itself rather than on the challenge they underwent.

In line with the doctoral thesis study by Caviola (2020), mice reared by CD1 dams were less anxious than mice reared by either C57 or Balb dams. Consistently with present results suggest that such a difference is shown in females, whereby CD1-fostered females were less anxious and more exploratory than those reared by strains with low maternal care. While in males such strain difference did not occur.

Previous studies examining laboratory rodents' sex differences in emotionality have shown varying results (see Ramos et al., 2003), although, in line with our results, females have often been suggested to be less fearful than males (e.g., Gray, 1971).

6 CONCLUSIONS

Overall, the results of present analysis carried out on 367 animals belonging to 9 cohorts showed that conditional inactivation of NPY1r in limbic brain areas induced increased anxiety-like levels, reduced exploratory activity and habituation ability of conditional KO mice during the OF test. The genotype effect is more pronounced in FVB-fostered males (more frequency of walking and stationary sniffing behaviors), but it is generally not influenced by either sex or early maternal environment. Despite the high sample size, the number of animals per foster dams' strain was not homogeneous and this may have weakened the statistical analysis. The

strain of the adoptive mother, characterized by different levels of maternal care, appeared to influence exploratory activity in a sex-dependent manner in the control population; specifically, females reared by CD1 dams appear to be the most exploratory and least anxious.

Several studies have investigated whether NPY1r is a target of epigenetic modifications in response to maternal care or sex. For instance, one study found that in rats higher maternal care can lead to an increase in DNA methylation of the NPY1r gene in the hippocampus (Weaver et al., 2004). Another study found that sex-specific DNA methylation patterns and transcription of the NPY1r gene are associated with sex-dimorphic anxiety-like behaviors in mice exposed to perinatal low protein diet (LPD), with males being more anxious-like than females (Nätt et al., 2017). It is possible that maternal care and sex can influence the expression of NPY1r and related physiological and behavioral processes due epigenetic modifications. Moreover, Bertocchi and colleagues (2020) found that conditional inactivation of NPY1r gene differentially affects the phenotype of male and female $Npy1r^{rfb}$ mice with females being more resilient than males to the conditional deletion of NPY1r gene in the limbic system. This can suggest the presence of an estrogen-dependent relay necessary to ensure the maintenance of the homeostasis also in case of Y1R malfunctioning.

Future perspectives involve expanding the research toward studies including the use of strains with different levels of maternal care in order to assess how this variable might affect the phenotype of the animal model in adulthood, and how this factor influences NPY1r gene expression. Furthermore, present study also highlighted how the habituation ability of the animal could be affected by the conditional deletion of the gene; therefore, this phenomenon should also be further investigated. Further studies may help to better understand the etiopathogenesis of anxiety-related psychopathological disorders and help to develop future therapies to reduce these psychopathological disorders.

7 REFERENCE LIST

Adrian, T. E., Allen, J. M., Bloom, S. R., Ghatei, M. A., Rossor, M. N., Roberts, G. W., ... & Polak, J. M. (1983). Neuropeptide Y distribution in human brain. *Nature*, *306*(5943), 584-586.

Akerlund, J. E., & Björkhem, I. (1990). Studies on the regulation of cholesterol 7 alpha-hydroxylase and HMG-CoA reductase in rat liver: effects of lymphatic drainage and ligation of the lymph duct. *Journal of Lipid Research*, *31*(12), 2159-2166.

Bailey, K. R., & Crawley, J. N. (2009). Anxiety-related behaviors in mice.

Baraban, S. C., Hollopeter, G., Erickson, J. C., Schwartzkroin, P. A., & Palmiter, R. D. (1997). Knock-out mice reveal a critical antiepileptic role for neuropeptide Y. *Journal of Neuroscience*, *17*(23), 8927-8936.

Bard, J. A., Walker, M. W., Branchek, T. A., & Weinshank, R. L. (1995). Cloning and functional expression of a human Y4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. *Journal of Biological Chemistry*, *270*(45), 26762-26765.

Batterham, R. L., & Bloom, S. R. (2003). The gut hormone peptide YY regulates appetite. *Annals of the New York Academy of Sciences*, *994*(1), 162-168.

Batterham, R. L., Cowley, M. A., Small, C. J., Herzog, H., Cohen, M. A., Dakin, C. L., ... & Bloom, S. R. (2004). Does gut hormone PYY3–36 decrease food intake in rodents? (reply). *Nature*, *430*(6996), 3-4.

Berglund, M. M., Holmberg, S. K., Eriksson, H., Gedda, K., Maffrand, J. P., Serradeil–Le Gal, C., ... & Larhammar, D. (1999). The cloned guinea pig neuropeptide Y receptor Y1 conforms to other mammalian Y1 receptors. *Peptides*, *20*(9), 1043-1053.

Berglund, M. M., Lundell, I., Eriksson, H., Söll, R., Beck-Sickinger, A. G., & Larhammar, D. (2001). Studies of the human, rat, and guinea pig Y4 receptors using neuropeptide Y analogues and two distinct radioligands. *Peptides*, *22*(3), 351-356.

Bertocchi, I., Oberto, A., Longo, A., Mele, P., Sabetta, M., Bartolomucci, A., ... & Eva, C. (2011). Regulatory functions of limbic Y1 receptors in body weight and anxiety uncovered by conditional knockout and maternal care. *Proceedings of the National Academy of Sciences*, *108*(48), 19395-19400.

Bertocchi, I., Oberto, A., Longo, A., Palanza, P., & Eva, C. (2020). Conditional inactivation of Npy1r gene in mice induces sex-related differences of metabolic and behavioral functions. *Hormones and behavior*, *125*, 104824.

Borowsky, B., Walker, M. W., Bard, J., Weinshank, R. L., Laz, T. M., Vaysse, P., ... & Gerald, C. (1998). Molecular biology and pharmacology of multiple NPY Y5 receptor species homologs. *Regulatory peptides*, *75*, 45-53.

Bowlby, J. (1958). The nature of the child's tie to his mother. *The International Journal of Psychoanalysis,* 39, 350-373.

Broqua, P., Wettstein, J. G., Rocher, M. N., Gauthier-Martin, B., & Junien, J. L. (1995). Behavioral effects of neuropeptide Y receptor agonists in the elevated plus-maze and fear-potentiated startle procedures. *Behavioural pharmacology*.

Caberlotto, L., & Hurd, Y. L. (1999). Reduced neuropeptide Y mRNA expression in the prefrontal cortex of subjects with bipolar disorder. *Neuroreport*, *10*(8), 1747-1750.

Campbell, R. E., Smith, M. S., Allen, S. E., Grayson, B. E., & Grove, K. L. (2003). Orexin neurons express a functional pancreatic polypeptide Y4 receptor. *Journal of Neuroscience*, *23*(4), 1487-1497.

Carvajal, C., Dumont, Y., & Quirion, R. (2006). Neuropeptide y: role in emotion and alcohol dependence. *CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders)*, *5*(2), 181-195.

Caviola, G. (2017). Impact of early maternal environment and sex on behaviour and metabolism in two KO mouse models (PhD thesis). University of Modena & Reggio-Emilia, Italy.

Champagne, F. A. (2008). Epigenetic mechanisms and the transgenerational effects of maternal care. *Frontiers in neuroendocrinology*, *29*(3), 386-397.

Champagne, F., Diorio, J., Sharma, S., & Meaney, M. J. (2001). Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *Proceedings of the National Academy of Sciences*, *98*(22), 12736-12741.

Choleris, E., Thomas, A. W., Kavaliers, M., & Prato, F. S. (2001). A detailed ethological analysis of the mouse open field test: effects of diazepam, chlordiazepoxide and an extremely low frequency pulsed magnetic field. *Neuroscience & Biobehavioral Reviews*, *25*(3), 235-260.

Colmers, W. F., & Bleakman, D. (1994). Effects of neuropeptide Y on the electrical properties of neurons. *Trends in neurosciences*, *17*(9), 373-379.

Crawley, J. N. (1985). Exploratory behavior models of anxiety in mice. *Neuroscience & Biobehavioral Reviews*, *9*(1), 37-44.

Dumont, Y., Jacques, D., Bouchard, P., & Quirion, R. (1998). Species differences in the expression and distribution of the neuropeptide Y Y1, Y2, Y4, and Y5 receptors in rodents, guinea pig, and primates brains. *Journal of Comparative Neurology*, *402*(3), 372-384.

Dunn, A. J., Guild, A. L., Kramarcy, N. R., & Ware, M. D. (1981). Benzodiazepines decrease grooming in response to novelty but not ACTH or β-endorphin. *Pharmacology Biochemistry and Behavior*, *15*(4), 605-608.

Eva, C., Keinänen, K., Monyer, H., Seeburg, P., & Sprengel, R. (1990). Molecular cloning of a novel G protein-coupled receptor that may belong to the neuropeptide receptor family. *FEBS letters*, *271*(1-2), 81-84.

Eva, C., Serra, M., Mele, P., Panzica, G., & Oberto, A. (2006). Physiology and gene regulation of the brain NPY Y1 receptor. *Frontiers in neuroendocrinology*, *27*(3), 308-339.

Fabrigar, L. R., Wegener, D. T., MacCallum, R. C., & Strahan, E. J. (1999). Evaluating the use of exploratory factor analysis in psychological research. *Psychological methods*, *4*(3), 272.

Gerald, C., Walker, M. W., Criscione, L., Gustafson, E. L., Batzl-Hartmann, C., Smith, K. E., ... & Weinshank, R. L. (1996). A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature*, *382*(6587), 168-171.

Gerald, C., Walker, M. W., Vaysse, P. J. J., He, C., Branchek, T. A., & Weinshank, R. L. (1995). Expression cloning and pharmacological characterization of a human hippocampal neuropeptide Y/peptide YY Y2 receptor subtype. *Journal of Biological Chemistry*, *270*(45), 26758-26761.

Geuther, B. Q., Peer, A., He, H., Sabnis, G., Philip, V. M., & Kumar, V. (2021). Action detection using a neural network elucidates the genetics of mouse grooming behavior. *Elife*, *10*, e63207.

Gould, T. D., Dao, D. T., & Kovacsics, C. E. (2009). The open field test. *Mood and anxiety related phenotypes in mice*, 1-20.

Gray, J. A. (1971). Sex differences in emotional behaviour in mammals including man: endocrine bases. *Acta psychologica*, *35*(1), 29-46.

Gregor, P., Feng, Y., DeCarr, L. B., Cornfield, L. J., & McCaleb, M. L. (1996). Molecular characterization of a second mouse pancreatic polypeptide receptor and its inactivated human homologue. *Journal of Biological Chemistry*, *271*(44), 27776-27781.

Gregor, P., Millham, M. L., Feng, Y., DeCarr, L. B., McCaleb, M. L., & Cornfield, L. J. (1996). Cloning and characterization of a novel receptor to pancreatic polypeptide, a member of the neuropeptide Y receptor family. *FEBS letters*, *381*(1-2), 58-62.

Hall, C., & Ballachey, E. L. (1932). A study of the rat's behavior in a field. A contribution to method in comparative psychology. *University of California Publications in Psychology*.

Hamassaki, D. E., & Britto, L. R. (1990). Thalamic origin of neuropeptide Y innervation of the accessory optic nucleus of the pigeon (Columba livia). *Visual Neuroscience*, *5*(3), 249-259.

Hane, A. A., & Fox, N. A. (2006). Ordinary variations in maternal caregiving influence human infants' stress reactivity. *Psychological science*, *17*(6), 550-556.

Harlow, H. F. (1958). The nature of love. *American psychologist*, *13*(12), 673.

Heilig, M. (1995). Antisense inhibition of neuropeptide Y (NPY)-Y1 receptor expression blocks the anxiolytic-like action of NPY in amygdala and paradoxically increases feeding. *Regulatory peptides*, *59*(2), 201-205.

Heilig, M. (2004). The NPY system in stress, anxiety and depression. *Neuropeptides*, *38*(4), 213-224.

Henry, M., Ghibaudi, L., Gao, J., & Hwa, J. J. (2005). Energy metabolic profile of mice after chronic activation of central NPY Y1, Y2, or Y5 receptors. *Obesity research*, *13*(1), 36-47.

Herzog, H., Darby, K., Ball, H., Hort, Y., Beck-Sickinger, A., & Shine, J. (1997). Overlapping gene structure of the human neuropeptide Y receptor subtypes Y1 and Y5 suggests coordinate transcriptional regulation. *Genomics*, *41*(3), 315-319.

Herzog, H., Hort, Y. J., Ball, H. J., Hayes, G., Shine, J., & Selbie, L. A. (1992). Cloned human neuropeptide Y receptor couples to two different second messenger systems. *Proceedings of the National Academy of Sciences*, *89*(13), 5794-5798.

Hoover-Plow, J., Skomorovska-Prokvolit, O., & Welsh, S. (2001). Selective behaviors altered in plasminogen-deficient mice are reconstituted with intracerebroventricular injection of plasminogen. *Brain research*, *898*(2), 256-264.

Kalra, S. P., & Crowley, W. R. (1992). Neuropeptide Y: a novel neuroendocrine peptide in the control of pituitary hormone secretion, and its relation to luteinizing hormone. *Frontiers in neuroendocrinology*, *13*(1), 1-46.

Kanatani, A., Mashiko, S., Murai, N., Sugimoto, N., Ito, J., Fukuroda, T., ... & Ihara, M. (2000). Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice. *Endocrinology*, *141*(3), 1011-1016.

Karl, T., & Herzog, H. (2007). Behavioral profiling of NPY in aggression and neuropsychiatric diseases. *Peptides*, *28*(2), 326-333.

Kask, A., Rägo, L., & Harro, J. (1998). Evidence for involvement of neuropeptide Y receptors in the regulation of food intake: studies with Y1-selective antagonist BIBP3226. *British journal of pharmacology*, *124*(7), 1507-1515.

Katsuura, G., Asakawa, A., & Inui, A. (2002). Roles of pancreatic polypeptide in regulation of food intake. *Peptides*, *23*(2), 323-329.

Kraeuter, A. K., Guest, P. C., & Sarnyai, Z. (2019). The open field test for measuring locomotor activity and anxiety-like behavior. In *Pre-clinical models* (pp. 99-103). Humana Press, New York, NY.

Larhammar, D. (1996). Structural diversity of receptors for neuropeptide Y, peptide YY and pancreatic polypeptide. *Regulatory peptides*, *65*(3), 165-174.

Larhammar, D., & Salaneck, E. (2004). Molecular evolution of NPY receptor subtypes. *Neuropeptides*, *38*(4), 141-151.

Larhammar, D., Blomqvist, A. G., Yee, F., Jazin, E., Yoo, H., & Wahlested, C. (1992). Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. *Journal of Biological Chemistry*, *267*(16), 10935-10938.

Larhammar, D., Wraith, A., Berglund, M. M., Holmberg, S. K., & Lundell, I. (2001). Origins of the many NPY-family receptors in mammals. *Peptides*, *22*(3), 295-307.

Leppänen, P. K., Ravaja, N., & Ewalds-Kvist, S. B. M. (2006). Twenty-three generations of mice bidirectionally selected for open-field thigmotaxis: Selection response and repeated exposure to the open field. *Behavioural processes*, *72*(1), 23-31.

Lin, S., Boey, D., & Herzog, H. (2004). NPY and Y receptors: lessons from transgenic and knockout models. *Neuropeptides*, *38*(4), 189-200.

Lopez-Valpuesta, F. J., Nyce, J. W., & Myers, R. D. (1996). NPY-Y1 receptor antisense injected centrally in rats causes hyperthermia and feeding. *Neuroreport*, *7*(15-17), 2781-2784.

Lundell, I., Blomqvist, A. G., Berglund, M. M., Schober, D. A., Johnson, D., Statnick, M. A., ... & Larhammar, D. (1995). Cloning of a human receptor of the NPY receptor family with high affinity for pancreatic polypeptide and peptide YY. *Journal of Biological Chemistry*, *270*(49), 29123-29128.

Mannon, P. J., & Mele, J. M. (2000). Peptide YY (PYY) transactivates the EGF receptor (EGFR) via the Y1 receptor (Y1R) to stimulate MAPK in gut epithelial cells in a pathway requiring BΓ-subunits and PKCE. *Gastroenterology*, *4*(118), A437.

Marsh, D. J., Hollopeter, G., Kafer, K. E., & Palmiter, R. D. (1998). Role of the Y5 neuropeptide Y receptor in feeding and obesity. *Nature medicine*, *4*(6), 718-721.

Meaney, M. J. (2001). Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. *Annual review of neuroscience*, *24*(1), 1161-1192.

Michel, M. C., Beck-Sickinger, A., Cox, H., Doods, H. N., Herzog, H., Larhammar, D., ... & Westfall, T. (1998). XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacological reviews*, *50*(1), 143-150.

Nätt, D., Barchiesi, R., Murad, J., Feng, J., Nestler, E. J., Champagne, F. A., & Thorsell, A. (2017). Perinatal malnutrition leads to sexually dimorphic behavioral responses with associated epigenetic changes in the mouse brain. *Scientific reports*, *7*(1), 11082.

Naveilhan, P., Canals, J. M., Valjakka, A., Vartiainen, J., Arenas, E., & Ernfors, P. (2001). Neuropeptide Y alters sedation through a hypothalamic Y1-mediated mechanism. *European Journal of Neuroscience*, *13*(12), 2241-2246.

Nie, M., & Selbie, L. A. (1998). Neuropeptide Y Y1 and Y2 receptor-mediated stimulation of mitogen-activated protein kinase activity. *Regulatory peptides*, *75*, 207-213.

Palanza, P., Morellini, F., Parmigiani, S., & Vom Saal, F. S. (2002). Ethological methods to study the effects of maternal exposure to estrogenic endocrine disrupters: a study with methoxychlor. *Neurotoxicology and Teratology*, *24*(1), 55-69.

Parker, R. M. C., & Herzog, H. (1999). Regional distribution of Y-receptor subtype mRNAs in rat brain. *European Journal of Neuroscience*, *11*(4), 1431-1448.

Plotsky, P. M., & Meaney, M. J. (1993). Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Molecular brain research*, *18*(3), 195-200.

Pronchuk, N., Beck-Sickinger, A. G., & Colmers, W. F. (2002). Multiple NPY receptors inhibit GABAA synaptic responses of rat medial parvocellular effector neurons in the hypothalamic paraventricular nucleus. *Endocrinology*, *143*(2), 535-543.

Ramos, A., Correia, E. C., Izídio, G. S., & Brüske, G. R. (2003). Genetic selection of two new rat lines displaying different levels of anxiety-related behaviors. *Behavior Genetics*, *33*, 657-668.

Rasmusson, A. M., Hauger, R. L., Morgan III, C. A., Bremner, J. D., Charney, D. S., & Southwick, S. M. (2000). Low baseline and yohimbine-stimulated plasma neuropeptide Y (NPY) levels in combat-related PTSD. *Biological psychiatry*, *47*(6), 526-539.

Roseboom, P. H., Nanda, S. A., Fox, A. S., Oler, J. A., Shackman, A. J., Shelton, S. E., ... & Kalin, N. H. (2014). Neuropeptide Y receptor gene expression in the primate amygdala predicts anxious temperament and brain metabolism. *Biological psychiatry*, *76*(11), 850-857.

Sah, R., & Geracioti, T. D. (2013). Neuropeptide Y and posttraumatic stress disorder. *Molecular psychiatry*, *18*(6), 646-655.

Sah, R., Ekhator, N. N., Strawn, J. R., Sallee, F. R., Baker, D. G., Horn, P. S., & Geracioti Jr, T. D. (2009). Low cerebrospinal fluid neuropeptide Y concentrations in posttraumatic stress disorder. *Biological psychiatry*, *66*(7), 705-707.

Salaneck, E., Ardell, D. H., Larson, E. T., & Larhammar, D. (2003). Three neuropeptide Y receptor genes in the spiny dogfish, Squalus acanthias, support en bloc duplications in early vertebrate evolution. *Molecular biology and evolution*, *20*(8), 1271-1280.

Stanley, B. G., Magdalin, W., Seirafi, A., Thomas, W. J., & Leibowitz, S. F. (1993). The perifornical area: the major focus of (a) patchily distributed hypothalamic neuropeptide Y-sensitive feeding system (s). *Brain research*, *604*(1-2), 304-317.

Stern, J. M., Yu, Y. L., & Crockett, D. P. (2002). Dorsolateral columns of the spinal cord are necessary for both suckling-induced neuroendocrine reflexes and the kyphotic nursing posture in lactating rats. *Brain research*, *947*(1), 110-121.

Sundström, G., Larsson, T. A., Xu, B., Heldin, J., & Larhammar, D. (2013). Interactions of zebrafish peptide YYb with the neuropeptide Y-family receptors Y4, Y7, Y8a, and Y8b. *Frontiers in Neuroscience*, *7*, 29.

Tatemoto, K., Carlquist, M., & Mutt, V. (1982). Neuropeptide Y—a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature*, *296*(5858), 659-660.

Ueno, N., Inui, A., Iwamoto, M., Kaga, T., Asakawa, A., Okita, M., ... & Kasuga, M. (1999). Decreased food intake and body weight in pancreatic polypeptide-overexpressing mice. *Gastroenterology*, *117*(6), 1427-1432.

van der Goot, M. H., Keijsper, M., Baars, A., Drost, L., Hendriks, J., Kirchhoff, S., ... & Arndt, S. S. (2021). Inter-individual variability in habituation of anxiety-related responses within three mouse inbred strains. *Physiology & behavior*, *239*, 113503.

Wahlestedt, C., & Reis, D. J. (1993). Neuropeptide Y-related peptides and their receptors--are the receptors potential therapeutic drug targets?. *Annual Review of Pharmacology and Toxicology*, *33*(1), 309-352.

Wahlestedt, C., Yanaihara, N., & Håkanson, R. (1986). Evidence for different pre-and post-junctional receptors for neuropeptide Y and related peptides. *Regulatory peptides*, *13*(3-4), 307-318.

Weaver, I. C. (2014). Integrating early life experience, gene expression, brain development, and emergent phenotypes: unraveling the thread of nature via nurture. *Advances in Genetics*, *86*, 277-307.

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., ... & Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature neuroscience*, *7*(8), 847-854.

Westrin, Å., Ekman, R., & Träskman-Bendz, L. (1999). Alterations of corticotropin releasing hormone (CRH) and neuropeptide Y (NPY) plasma levels in mood disorder patients with a recent suicide attempt. *European Neuropsychopharmacology*, *9*(3), 205-211.

Widerlöv, E., Lindström, L. H., Wahlestedt, C., & Ekman, R. (1988). Neuropeptide Y and peptide YY as possible cerebrospinal fluid markers for major depression and schizophrenia, respectively. *Journal of psychiatric research*, *22*(1), 69-79.

Wisialowski, T., Parker, R., Preston, E., Sainsbury, A., Kraegen, E., Herzog, H., & Cooney, G. (2000). Adrenalectomy reduces neuropeptide Y–induced insulin release and NPY receptor expression in the rat ventromedial hypothalamus. *The Journal of Clinical Investigation*, *105*(9), 1253-1259.

Wraith, A., Törnsten, A., Chardon, P., Harbitz, I., Chowdhary, B. P., Andersson, L., ... & Larhammar, D. (2000). Evolution of the neuropeptide Y receptor family: gene and chromosome duplications deduced from the cloning and mapping of the five receptor subtype genes in pig. *Genome research*, *10*(3), 302-310.

Wu, Q., Zhao, Z., & Shen, P. (2005). Regulation of aversion to noxious food by Drosophila neuropeptide Y–and insulin-like systems. *Nature neuroscience*, *8*(10), 1350-1355.

Zhang, X., Wiesenfeld-Hallin, Z., & Hökfelt, T. (1994). Effect of peripheral axotomy on expression of neuropeptide Y receptor mRNA in rat lumbar dorsal root ganglia. *European Journal of Neuroscience*, *6*(1), 43-57.