REGULATION OF MOUSE BEHAVIOR BY EPHRIN-A5

By

MICHAL SHELEG

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Toxicology

written under the direction of

Dr. Renping Zhou

and approved by

_____

_____

_____

_____

_____

New Brunswick, New Jersey

January, 2015

ABSTRACT OF THE DISSERTATION

Regulation of Mouse Behavior by Ephrin-A5

By MICHAL SHELEG

Dissertation Director:

Renping Zhou, PhD

Social behaviors in mammals are regulated by core neural circuits that respond to stimuli from the environment. These circuits are formed by guidance molecules and create a "map" in the brain that corresponds to sensory surface and motor effectors. Among these guidance molecules are the Eph/ ephrin family of receptor tyrosine kinases. In this study we examined the role of ephrin-A5, a ligand for the Eph receptors, in animal behavior. We found that ephrin-A5 inactivation in the mouse caused delays in the maturation of sensorimotor skills during development and defective behaviors in adult life. Specifically, ephrin-A5$^{-/-}$ mothers are impaired in maternal behavior in the form of nest building and pup retrieval and male null mice show severe reduction in inter-male aggression. In addition, anxiety-like behavior is reduced in both male and female null mice. An examination of the general olfactory function revealed no apparent deficit; ephrin-A5$^{-/-}$ mice were able to use their sense of smell to locate a flavored cereal that was buried beneath the cage bedding, they showed increased investigatory sniffs in response to new odor, and they were able to discriminate between male and female mice, suggesting that neuronal circuits that control foraging and sex discrimination are intact. However stimuli that control maternal care, inter-male aggression and anxiety may not be directed to the appropriate

circuits in the null brain. We further examined molecules that have been shown to act in theses circuits such as testosterone, corticosterone, serotonin and arginine vasopressin (AVP) and found alteration in the expression of AVP in the null brain. Taken together, our studies revealed a novel role for ephrin-A5 in the development of appropriate behaviors.

ACKNOWLEDGEMENTS

I would like to first thank my advisor Dr. Renping Zhou, for his guidance, help and support both professionally and personally. I am incredibly fortunate to have such an amazing mentor who gave me the opportunity to grow and become the scientist I am today. Thank you for keeping your door open all the time and for having the patience to answer my questions.

I would like to also thank my committee members Dr. Gleb Shumyatsky, Dr. Jason Richardson, Dr. Suzie Chen and Dr. George Wagner for their supervision and expertise. Especially, I would like to thank Dr. Jason Richardson and Dr. George Wagner for being extremely supportive throughout these years.

Special thanks to Dr. Suzie Chen who was there for me every time when I needed help, advice and guidance in my research and personal life.

I want to thank all the people that helped me from the department of chemical biology, especially, Deborah Stalling, Bobbie Busch, Erica DiPaola and Annette Dionisio, I don’t know what I would have done without your help.

Finally, I would like to thank my family; my parents, my brother, my husband and my boys who are the reason for who I am.

DEDICATION

This thesis is dedicated to the men of my life: Assaf, Matan and Omri

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER 1: GENERAL INTRODUCTION

1.1 The Eph/ephrin Family of Receptor Tyrosine Kinase

Proper formation of neuronal circuits during development and synaptic plasticity in adults are essential for appropriate animal behavior. For example, sensory information from the basolateral amygdala to the bed nucleus of the stria terminalis (BNST) has been shown to be involved in fear, anxiety (Singewald, Salchner et al. 2003; Kim, Adhikari et al. 2013) and aggression (Nelson and Trainor 2007). In addition, circuits between motor neurons in the brain and their peripheral target, the muscles, are required for locomotor activities (Bonanomi and Pfaff 2010). These circuits are formed and maintained by molecular guidance cues found in the neuronal environment (Tessier-Lavigne and Goodman 1996), including the Eph/ephrin family.

The Eph receptors and their ephrin ligands are the largest family of receptor tyrosine kinases (RTKs), with 14 receptors and nine ligands which can be subdivided into two classes based on structural homology and binding affinities (Pasquale 2004). The A class consists of nine receptors (EphA1-A8, A10) and five ligands (ephrin-A1-A5), whereas the B class includes six receptors (EphB1-B4, B6) and three ligands (ephrin-B1-B3) (Klein 2009). Class A ephrin ligands are attached to the cell membrane by a glycerophosphatidyl-inositol (GPI) while class B has a transmembrane region and a cytoplasmic domain that includes three tyrosine residues and a c-terminal PDZ binding motif. In addition to these domains, ephrin-B3 has an ERK-binding domain (D-domain) in the juxtamembrane region which allows it to interact with Erk2 (McClelland, Hruska et al. 2010). Eph receptors have an extracellular region that contains the ligand binding domain, a cysteine-rich domain with an EGF-like motif and two fibronectin type III repeats. The

intracellular part includes a juxtamembrane region, a kinase domain, a sterile α-motif (SAM) and a PDZ-binding domain (Kullander and Klein 2002). In general A-class receptors will bind to all A ligands and B-class receptors will bind to all B ligands. However, some exceptions exist; EphB2 can bind to ephrin-A5 and EphA4 can bind to all the class B ligands (Himanen, Chumley et al. 2004; Klein 2009). In contrast to other RTKs, interaction between the Eph receptor and ephrin ligand generates signals that propagate bi-directionally into both receptor and ligand-expressing cells. These processes are known as forward and reverse signaling (Pasquale 2005; Daar 2011; Xu and Henkemeyer 2011). Forward signaling follows the RTK model, where signaling is initiated in the cytoplasmic side of the receptor upon binding to the ligand. Here, autophosphorylation of the juxtamembrane region of the receptor activates the kinase domain and allows adaptor molecules to be associate with the receptor and transmit signals into the Eph-expressing cell (Kullander and Klein 2002). Reverse signaling can occur through both the ephrin-B and the ephrin-A ligands. Activation of ephrin-B ligands can take one of two forms; phosphorylation-dependent or phosphorylation independent/PDZ-domain-dependent. Here signaling occurs through the tyrosine residues or the PDZ-binding motif respectively (Kullander and Klein 2002). In contrast, ephrin-A ligands lack an intracellular domain and have to be associated with adaptor proteins found within discrete plasma membrane microdomains (also known as lipid rafts or caveola like-domains) in order to reverse signal (Davy, Gale et al. 1999). Specifically, the neurotrophin receptors p75 and TrkB were found to act as signaling partners with ephrin-As on retinal axons. These interactions are required for the formation of proper retinocollicular mapping (Lim, McLaughlin et al. 2008; Marler,

Becker-Barroso et al. 2008). Interestingly, both forward and reverse signaling can occur simultaneously (Egea and Klein 2007).

In addition to Eph-ephrin *trans*-interaction, when the ligand and the receptor are expressed on opposite cells, many regions in the brain co-express Eph and ephrin in the same cell, resulting in *cis* interactions. These interactions will usually not lead to active signaling and can interfere by blocking EphA forward signaling (Carvalho, Beutler et al. 2006). However, in some neurons, the co-expressed Eph/ephrin are found in distinct membrane domains in which they can act independently from each other, resulting in active signals (Marquardt, Shirasaki et al. 2005). Finally, some studies suggest that receptor-ligand interaction is not always required for signaling. For example, overexpression (Bochenek, Dickinson et al. 2010) or deletion (Foo, Turner et al. 2006) of ephrin-B2 in a single, isolated cell is sufficient to cause changes in cell morphology, behavior and motility even in the absence of a ligand, suggesting a contact-independent, cell-autonomous mode of signaling.

Both receptor and ligand are highly expressed on pre- and post-synaptic neurons from early organogenesis to postnatal stages (Mori, Wanaka et al. 1995; Flenniken, Gale et al. 1996; Liebl, Morris et al. 2003). In the nervous system, the protein levels are dynamic and change during the lifetime. Yet, in areas that maintain high levels of synaptic plasticity such as the olfactory bulb, hippocampus and cerebellum, the levels remain high (Liebl, Morris et al. 2003; Attwood, Patel et al. 2012).

In general Eph-ephrin interactions trigger cell-cell repulsion however, since both receptor and ligand are anchored to the membrane, most signaling requires cell-cell contact. Therefore, in order to achieve repulsion, the Eph/ephrin complex needs to be

removed from the cell surface. Two mechanisms have been shown to convert adhesive into repulsive; protease cleavage of the extracellular domain and endocytosis (reviewed in (Egea and Klein 2007; Pitulescu and Adams 2010)). A member of the ADAM (A Disintegrin And Metalloproteinase) family, ADAM10/Kuzbanian, has been shown to cleave activated ephrin-A2 and ephrin-A5 from the membrane. ADAM10 is constitutively bound to ephrin-A2 in *cis* (on the same cell) and will trigger ligand shedding upon interaction with the EphA3 receptor (Hattori, Osterfield et al. 2000). Interestingly, a proteolysis-blocking mutation in ephrin-A2 inhibits axon withdrawal without effecting growth cone collapse *in vitro* (Hattori, Osterfield et al. 2000), suggesting that cleavage is require to execute ephrin-A2 signaling *in vivo*. ADAM10 is also associated with EphA3 on the cell surface. Here, binding of the receptor to ephrin-A5, on opposite cells repositions ADAM10 and activates its proteinase domain. This in turn causes *trans* cleavage of the ligand (Janes, Saha et al. 2005). In both cases, ADAM10 cleavage of the ligands breaks the contact between the Eph expressing cell to the ephrin expressing cell and results in contact-mediated repulsion.

An alternative mechanism to terminate the Eph/ephrin interaction is endocytosis. Here, the ligand-receptor complex internalized into the signaling Eph or ephrin-expressing cells. In the nervous system, Eph-endocytosis is clathrin-mediated and regulated by members of GTPase family such as Vav2, Rin1 and TIAM (Pitulescu and Adams 2010). Specifically, TIAM1, a Rac-specific guanine nucleotide exchange factor, has been shown to be associated with EphA8 and govern the endocytosis of the EphA8/ephrin-A5 complex (Yoo, Shin et al. 2010). Eph-forward signaling also regulates clathrin-mediated endocytosis of other proteins such as the AMPA-type glutamate receptors. For example,

ephrin-B2/EphB interaction causes the phosphorylation of synaptojanin 1 in its proline-rich domain. This domain is known to interact with endocytic proteins and sequentially control clathrin-mediated endocytosis (Irie, Okuno et al. 2005). Hence, EphB controls synaptojanin 1 activity and in turn, clathrin-mediated endocytosis of the AMPA receptor which plays a critical role in synaptic long-term depression (LTD).

1.2 Eph/ephrin Regulation of Synaptic Functions

In the central nervous system, Eph forward and ephrin reverse signaling impacts spine and synapse formation, synaptic transmission and long term changes in synaptic strength (reviewed in (Egea and Klein 2007; Klein 2009; Hruska and Dalva 2012)). Eph dependent forward signaling is required for normal spine morphogenesis and activity-dependent synaptic plasticity. For example, EphA4 activation caused shortening of dendritic spines in hippocampal slices, whereas its inhibition produced distorted spine shape both *in vitro* and *in vivo* (Murai, Nguyen et al. 2003). Here, cyclin- dependent kinase 5 (Cdk5) is recruited to the intracellular domain of the EphA4 receptor (Sahin, Greer et al. 2005). Cdk5 activates ephexin1 (Eph-interacting exchange factor) which activates RhoA, and in turn regulates actin reorganization in the spines (Sahin, Greer et al. 2005). Interestingly, at the CA3-CA1 synapse, EphA4 is required for long term potentiation (LTP) in a kinase-independent way, suggesting distinct mechanisms during plasticity and spine formation (Grunwald, Korte et al. 2004). EphBs are also important in spine formation, knockout mice lacking EphB1, B2, and B3 develop abnormal spines and had decreased spine density. In addition, EphB deletion in hippocampal neurons results in immature spines (Henkemeyer, Itkis et al. 2003). Here, signaling through the receptor modulates the

activation of Rho family GTPases, the key regulators of actin dynamics (Klein 2009). Furthermore, EphBs recruit and interact with NMDA receptors, which are important for synapse formation and plasticity (Dalva, Takasu et al. 2000). EphB2 knockout mice have reduced NMDA-dependent LTP at both CA1 and dentate granule synapses (Henderson, Georgiou et al. 2001) and the receptor expression controls the amount of NMDA-R at the synapses (Nolt, Lin et al. 2011).

Ephrin ligands also function as post- and pre-synaptic receptors to control synapse formation, spine morphogenesis and synaptic plasticity. Recently, using an ephrin-B3 mutant mice ($Efnb3^{-/-}$), Xu *et al*. (Xu, Sun et al. 2011) reported that postsynaptic hippocampal neurons lacking ephrin-B3 have increased dendrites and reduced number of spines. To verify that reverse signaling (and not EphB forward signaling) was responsible for these changes, the authors studied mutant mice that express a truncated ephrin-B3-β-gal fusion protein ($Efnb3^{lacZ/lacZ}$). These mice lack the cytoplasmic segment of ephrin-B3 and therefore cannot transduce reverse signals. Since dendritic morphologies in $Efnb3^{lacZ/lacZ}$ mice were similar to the ones in $Efnb3^{-/-}$, the authors concluded that reverse signaling through the ligand, as opposed to forward signaling through the receptor, regulates the number of dendrites and spines on these neurons. In the cortex, ephrin-B3 controls synapse density through its unique Erk-binding/D-domain. The D-domain allows the interaction of the ligand with Erk2, which regulates synapse density and the formation of dendritic spines through inhibition of MAPK signaling (McClelland, Hruska et al. 2010). Similar to ephrin-B3, ephrin-B2 is also expressed on postsynaptic neurons in the hippocampus and affects LTP and long term depression (LTD) (Bouzioukh, Wilkinson et al. 2007). Here, signaling through the C-terminal PDZ- binding domain is necessary for

synaptic function, since mice lacking this domain are unable to mediate LTP and LTD (Bouzioukh, Wilkinson et al. 2007). Furthermore, members of the ephrin-A family, ephrin-A2, A3 and A5, are also involved in synapse function. Ephrin-A2 regulates experience-dependent synaptic pruning as well as synaptic glutamate transmission in the cortex. In addition, levels of glial glutamate transporters are significantly lower in the cortex of ephrin-A2-null mice compared to wild-type mice (Yu, Wang et al. 2013). In contrast, in the hippocampus of ephrin-A3-null mice, level of glial glutamate transporters is up-regulated and the spines are longer (Carmona, Murai et al. 2009).

Finally, ephrin-A5 has been shown to regulate synapse development and function (Marler, Becker-Barroso et al. 2008; Guellmar, Rudolph et al. 2009). Ephrin-A5-null mice have longer stellate cell dendrites and increased number of filopodia during early development. However, after synapse formation when spines are formed, null mice have decreased spine density, suggesting a reduced number of excitatory synapses (Guellmar, Rudolph et al. 2009). Furthermore, in the retino-tectal system, *cis* interaction of ephrin-A5 with the TrkB receptor suppressed axon branching (Drescher, Kremoser et al. 1995; Frisen, Yates et al. 1998; Marler, Becker-Barroso et al. 2008). Here, activation of TrkB by the neurotrophin BDNF led to increase axon branching possibly via activation of the PI-3 kinase signaling pathway (Marler, Becker-Barroso et al. 2008).

1.3 Regulation of Animal Behavior by the Eph/ephrin Family

Eph receptors have been shown to regulate the proper development of motor and social behavior in mice. Specifically, genetic inactivation of EphA6 (Savelieva, Rajan et al. 2008) and EphB2 (Grunwald, Korte et al. 2001), as well as intra-hippocampal infusion

of an EphA5 antagonist (Gerlai, Shinsky et al. 1999), produced behavioral deficits in tests for learning and memory. In these studies hippocampal-dependent performance in the Morris water maze (MWM) task and fear conditioning training was evaluated. Both EphB2$^{-/-}$ and EphA6$^{-/-}$ had specific impairment in the MWM task. In the fear conditioning test, a significant deficit in the context-dependent task was found in EphA6$^{-/-}$ mice, as well as in mice that were treated with an EphA5 antagonist, suggesting a deficit in learning. EphA4 disruption has also been shown to have significant effects on normal behavior in adult mice. EphA4$^{-/-}$ mice have impaired locomotor habituation in the open field test, decreased spatial recognition in the Y-maze and deficits in motor coordination and balance on the accelerating rotarod (Willi, Winter et al. 2012). Here, heterozygous null mice did not have the same behavioral deficit as homozygous mice, suggesting that complete deletion of the gene is required to produce these behavioral changes. In addition, expression of EphA4 and one of its ligands, ephrin-A3, is dysregulated in the hippocampus of stress susceptible rats; EphA4 expression is higher while ephrin-A3 expression are lower. Interestingly, treatment with the anti-depression drug fluoxetine restored the levels back to normal, suggesting that EphA4 and ephrin-A3 are involved in stress regulation and antidepressant response (Li, Wang et al. 2014). Furthermore, Rodenas- Ruano *et al*. (Rodenas-Ruano, Perez-Pinzon et al. 2006) found that ephrin-B3-dependant signaling is required for proper performance in the MWM learning task independent of its cytoplasmic domain. In the spatial test, in which the platform is hidden below the water surface, ephrin-B3$^{-/-}$ mice took significantly longer to locate the hidden platform than wild-type controls, implying reduced learning. However, the cytoplasmic deletion mutants, ephrin-B3^{lacz}, did not differ then wild-type mice, suggesting that reverse signaling was not required for this

task. Finally, our laboratory has shown that EphA5 inactivation caused decrease aggressive behavior in the mice; EphA5$^{-/-}$ mice took longer to attack an intruder male and had fewer attacks during the resident-intruder offensive test (Mamiya, Hennesy et al. 2008).

1.4 Ephrin-A5

The ligand ephrin-A5 (also known as AL-1/LERK-7) is a 25 kDa GPI-anchored protein that was first isolated in 1995 from a human breast carcinoma cell line (Winslow, Moran et al. 1995). Later that year, a second group purified and cloned the chick homolog of ephrin-A5 and named it RAGS (for repulsive axon guidance signal) (Drescher, Kremoser et al. 1995). Finally, in 1997, a third group reported the isolation of ephrin-A5 from a human fetal brain cDNA library (Kozlosky, VandenBos et al. 1997). Although expressed predominantly in the central nervous system, ephrin-A5 is also found in non-neuronal tissues such as the heart, placenta, lung, kidney, spleen, prostate, testes, ovary, small intestine and colon (Winslow, Moran et al. 1995; Kozlosky, VandenBos et al. 1997). In the developing brain, ephrin-A5 is highly expressed from early organogenesis through postnatal life (Zhang, Cerretti et al. 1996; Deschamps, Morel et al. 2010) in areas such as the sensory and motor regions of the cortex (Gao, Shinsky et al. 1998), the pituitary, the hypothalamus (Zarbalis and Wurst 2000), the olfactory system (Cutforth, Moring et al. 2003) and both the dorsolateral and ventromedial striatum (Passante, Gaspard et al. 2008; Cooper, Kobayashi et al. 2009). It has been shown to function as an axonal guidance molecule in patterning the development of several axon pathways including the retino-tectal (Drescher, Kremoser et al. 1995; Frisen, Yates et al. 1998) and the thalamacortical

(Gao, Yue et al. 1998; Bolz, Uziel et al. 2004) projections, as well as regulating synaptic plasticity in the mature brain (Gao, Shinsky et al. 1998).

Ephrin-A5 can act as both repellent and attractive cue. During development of the visual system ephrin-A5 and its receptors, EphA3 (in the chick) and EphA5 (in the mouse), are expressed in complementary manner within the retino-tectal system (or its mammalian equivalent, the superior colliculus). Ephrin-A5 is expressed in a low-high gradient across the anterio-posterior axis of the tectum, while its receptor is expressed in a high-low temporal-nasal gradient across the retinal axons (Drescher, Kremoser et al. 1995; Marcus, Gale et al. 1996; Feldheim, Vanderhaeghen et al. 1998; Frisen, Yates et al. 1998). This complementary expression are implicated in the guidance of retinal axons to their target in the tectum. Thus, high receptor expressing axons from the temporal retinal are repelled by high ligand expressing cells in the posterior tectum and vice versa. Furthermore, ephrin-A5 was found to inhibit neurite outgrowth of spinal cord neurons (Yue, Su et al. 1999) as well as limbic axon invasion into the somatosensory cortex *in vitro* (Gao, Yue et al. 1998). In addition to its inhibitory effects, ephrin-A5 can function as a positive cue. One example can be found in the mammalian vomeronasal organ (VNO), where ephrin-A5 is expressed in a high-low gradient along the apical-basal region of the vomeronasal epithelium and its receptor, EphA6, is expressed at a high-low concentration along the anterior-posterior part of the accessory olfactory bulb (AOB) (Knoll, Zarbalis et al. 2001). In contrast to the retino-tectal system, the apical epithelium axons, where ephrin-A5 is highly expressed, are projected into the anterior part of the AOB where the receptors are found in high concentration. Another example for its positive effect was reported in the striatum where ephrin-A5 and its receptor EphA5 elicit a positive effect on midbrain

dopaminergic axon termination by promoting survival and neurite growth (Cooper, Kobayashi et al. 2009).

In addition to its effects during development, ephrin-A5 has been shown to play a role in different types of cancer such as colon, ovarian, and pancreatic cancer (Herath, Spanevello et al. 2006; Giaginis, Tsourouflis et al. 2010; Wang, Chang et al. 2012). Here, ephrin-A5 is either overexpressed and associated with poor prognosis or downregulated and identified as a tumor suppressor depends on the cancer type. Finally, our laboratory has recently identified a novel function of ephrin-A5 in eye development, loss of ephrin-A5 in the mouse resulted in the development of cataracts in almost 90 percent of the mutant mice with varies degrees of severity including posterior lens rupture and tissue degeneration (Cooper, Son et al. 2008). In addition, mutant animals developed a hyperplastic mass posterior to the lens similar to the human disease, persistent hyperplastic primary vitreous (PHPV) (Son, Sheleg et al. 2014), a disorder in which tissue originating from the primary vitreous remain in the postnatal eye and leads to blurred vision.

Here, we revealed a novel function for ephrin-A5 in animal behavior both during development and in adult life.

CHAPTER 2: DEVELOPMENTAL DELAYS AND HYPERACTIVITY IN EPHRIN-A5$^{-/-}$ MICE

2.1 Introduction

Proper development of the central nervous system is dependent on cell to cell communication mediated by cell surface molecules including receptor tyrosine kinases (RTKs) and their ligands (Palmer and Klein 2003). Members of the largest RTK family, the Eph receptors and their ephrin ligands, have been shown to play critical roles in neural development, particularly in the guidance of migrating cells, topographic organization of axon connection and neurogenesis (reviewed in Wilkinson 2001; Reber, Hindges et al. 2007; Klein 2009). Abnormal axon growth and guidance during development can lead to behavior deficit or even cause severe motor disorders (Engle 2010). Specifically, genetic mutations in number of the Eph receptors have been shown to regulate proper behavior in mice (Gerlai, Shinsky et al. 1999; Grunwald, Korte et al. 2001; Savelieva, Rajan et al. 2008) However, how different ephrins affect behavior, remains largely unexplored.

Ephrin-A5 is a GPI-linked ligand for the Eph receptors. It is highly expressed in the developing nervous system from early organogenesis to postnatal life (Zhang, Cerretti et al. 1996; Deschamps, Morel et al. 2010). During embryogenesis, ephrin-A5 transcripts are highly expressed in sensory and motor regions of the cortex (Gao, Shinsky et al. 1998), in the pituitary, the hypothalamus, (Zarbalis and Wurst 2000) the olfactory system (Cutforth, Moring et al. 2003) and in both the dorsolateral and ventromedial striatum (Passante, Gaspard et al. 2008; Cooper, Kobayashi et al. 2009). Its expression in the striatum was found to regulate the formation of the ascending midbrain dopaminergic pathways (Passante, Gaspard et al. 2008; Cooper, Kobayashi et al. 2009) which are

important for the integration of sensorimotor, reward, and social interactions (Robinson, Zitzman et al. 2011).

Because of its high expression during early development and its role in axon guidance, we hypothesized that loss of ephrin-A5 may result in impaired behavioral development. To test this hypothesis, we compared developmental milestones and behaviors as well as brain neurochemistry in wild-type and ephrin-A5$^{-/-}$ mice. Specifically, we used a battery of behavioral tests to monitor motor development throughout the early postnatal life of mice of both genotypes. In addition, brain neurochemistry was examined at postnatal days (P) 15 and 30. We found that loss of ephrin-A5 in the mouse caused delays in motor development and increased locomotor activity. These behavioral deficits were associated with decreased monoamine levels in several brain regions that have been associated with motor functions. Taken together, these data demonstrate that ephrin-A5 expression is important for proper neurobehavioral development and the development of central monoaminergic pathways.

2.2 Materials and Methods

Animals: Ephrin-A5 animals were originally generated on a mixed background (C57BL/6 and 129/SV) as described earlier (Frisen, Yates et al. 1998). This line was maintained in our colony in the same background. Animals used in this study were generated using the following breeding scheme: heterozygous ephrin-A5 mice were bred with each other to generate wild-type, heterozygous, and homozygous knockout animals. Due to the large number of animals needed for the assays, additional animals were generated using wild-type x wild-type and homozygous knockout x knockout mating using siblings from the initial heterozygous x heterozygous mating. Male and female wild-type (n=22 and 14

respectively from 6 litters) and ephrin-A5$^{-/-}$ mice (n=16 and 16 respectively from 6 litters) were separately housed in plastic cages with standard beta chip bedding and free access to food and water. Lights were set on a 12 h on 12 h off reverse cycle (lights off from 07:00 to 19:00 h), and temperature was maintained at 25° C. All behavioral experiments were performed during the first phase of the dark cycle. Pregnant females were housed separately until time of birth. Day of birth was recorded as P0 and pup body weights were recorded. All pups were labeled for individual identification by marking their tails. A maximum of 4 male and 4 female offspring were used from each litter. Following P5, body weight was measured every 4 days.

Developmental and behavioral assessment: Testing ranged from P7 to P30 and consisted of eye opening, negative geotaxis test, hanging wire grip strength, mid-air righting, social interaction and motor activity. Pups were randomly picked and tests were performed in the order stated above (Figure 2-1). For tests that were done on the same day, tests were performed with one hour breaks in between.

1. Eye opening: Starting at P7 pups were examined daily to determine the first day that both eyes were open.

2. Negative geotaxis test: General coordination and strength were evaluated using the negative geotaxis test on P9-19 by placing the pup on a grid wire surface (30 cm x 18 cm divided into 1.2 cm grid squares) facing downward along a 45° incline. Latency to turn 180° such that the head was facing upward along the incline was recorded with a maximum of 60 s each trial. The test was repeated daily until the pup reached the test criteria, performing the behavior correctly in less than 30 seconds for two consecutive days.

3. Hanging wire grip strength: Wire grip strength is commonly used as a test for neuromuscular strength and stamina (Crawley 2000). Pups were placed on a wire 30 cm above a padded surface on P13-19 and the latency to fall was recorded with a maximum of 60 s each trial.

4. Mid-air righting: Ability to right in mid-air was tested on P13-19 to examine motor coordination and reflex. Pups were held 30 cm above a padded surface by the scruff of the neck ventral side up with all four paws extended upward. The ability to right was scored positive if the mouse landed on all four paws. There were three trials each day and a score of 0-3 was recorded. A score of 0 means that the animal was not able to complete the test successfully (landing on its 4 paws) in all 3 trails. A score of 1 means that the animal was able to complete the test successfully 1 out of the 3 trails. A score of 2 means that the animal was able to complete the test successfully 2 out of the 3 trails and a score of 3 means that the animal was able to complete the test successfully 3 out of the 3 trails.

5. Social Interaction and play behavior: The behavior chamber was a standard large cage with fresh bedding. Locomotor behavior was recorded via photocell beams located around the cage (Opto-Varimex, Columbus, OH). Naïve mice were individually housed for one week prior to the test session. Pairs of non-sibling, 30 day old, ephrin-A5$^{-/-}$ and wild-type male mice were observed for social interactions for one 30 min session and scored by two trained observers for the number of times that a member of the pair engaged in a behavior. All observers were blind to the genotype, and were trained on mouse pairs not used in this study until there was 98% agreement in the observation scores. Testing was conducted at the start of the dark cycle and the testing room was illuminated by red light. During testing, the behaviors observed were: face sniffs, crawl-under/over behaviors (defined as one

mouse crawling under or over another mouse) self-grooming, and allogrooming (defined as one mouse rising up on its hind legs to touch paws and snout to the other mouse to perform grooming motions).

6. Motor activity: Horizontal motor activity was tested under both social and non-social conditions. In the non-social test, young (P21) and adult (P60) mice were placed in an empty plastic cage which was then placed into a black plexiglass box. Interuptions of horizontal-projecting photocell sensors (placed approximately 7 cm apart and 2.5 cm above the floor) were recorded for 30 minutes with six, five minute bouts per session. The social interaction test was done as described above.

HPLC analysis: A separate group of naïve**,** wild-type and ephrin-A5$^{-/-}$ mice were sacrificed at P15 and 30 and the brains removed. Selected brain regions were dissected on ice, snap frozen, and stored at -80 ^{o}C until analysis, as described previously (Schuh, Richardson et al. 2009). Briefly, frozen samples were sonicated in 500 µL of 0.1 N perchloric acid and centrifuged at 15,000 x g for 20 min at 4 ^{o}C. The pellets were kept for protein assay and the supernatants were re-centrifuged as above. The resulting supernatants were filtered and an aliquot of 20 µl was injected into the HPLC with electrochemical detection (Waters, Milford, MA, USA) for neurochemical analysis of norepinephrine (NE), dopamine (DA) and its metabolites, 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) and 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). The components were separated on a cation exchange column (MD-150 × 3.2 column, ESA Biosciences Inc.) using isocratic mobile phase (MD-TM, ESA Biosciences Inc.) containing 2.2 mM NaCl pumped at a constant flow rate of 0.5 ml/min. The

compounds were quantified by electrochemical detection (flow cell, 2 mm GC WE, ISAC, Waters, Milford, MA, USA).

Western immunoblotting: Western blots were used to quantify the amount of the dopamine transporter (DAT), tyrosine hydroxylase (TH), the vesicular monoamine transporter 2 (VMAT2), and syntaxin present in samples of striatal tissue from wild-type and knockout mice as described previously (Caudle, Richardson et al. 2005). Briefly, samples (15 μg protein) were subjected to polyacrylamide gel electrophoresis on 4-12% precast NuPage gels (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene difluoride membrane. Membranes were then incubated overnight with a rat monoclonal antibody to the N-terminus of the DAT (Millipore, Temecula, CA, 1:5000). DAT antibody binding was detected using a goat anti-rat horseradish peroxidase secondary antibody and enhanced chemiluminescence. The luminescence signal was captured on an Alpha Innotech Fluorochem imaging system and stored as a digital image prior to densitometric analysis. Membranes were stripped with Pierce Stripping Buffer and sequentially reprobed with rabbit anti-VMAT2 polyclonal antibody (Millipore, 1:5000), rabbit anti-TH polyclonal antibody (Millipore, 1:1000) and monoclonal anti-syntaxin (Sigma, St. Louis, MA, 1:50,000). Syntaxin levels were used to normalize data among samples.

Statistical analysis: All data were analyzed using the SPSS statistics package. Behavioral analysis was performed using analysis of variance (ANOVA or repeated-measures ANOVA when the data was measured across days or trials) with Tukey's post hoc test where appropriate. Brain chemistry was assessed using multifactorial ANOVA s including genotype and age as main factors with Tukey's post hoc test where appropriate. Since

initial results showed no significant main effects or interaction effects for sex in any test other than body weight, sex was excluded from all behavioral analyses.

2.3 Results

2.3.1 Changes in body weight in ephrin-A5$^{-/-}$ mice

A repeated measurement ANOVA revealed that there was a significant effect of genotype [$F(1,53) = 64.59$, $p < 0.001$], sex [$F(1,53) = 12.98$, $p < 0.001$], and postnatal day (P)[$F(15,795) = 2646.644$, $p < 0.001$], as well as significant interactions: sex x genotype [$F(1,53) = 5.45$, $p = .0234$], day x sex [$F(15,795) = 12.356$, $p < 0.001$], day x genotype [$F(15,795) = 27.807$, $p < 0.001$], and day x sex x genotype [$F(15,795) = 3.130$, $p < 0.001$] (Figure 2-2A and B). Post hoc tests revealed that there were no differences in birth weight regardless of genotype or sex. In wild-type mice, a sex difference developed at P29 and persisted through P60 with males weighing significantly more than females. However, in knockout mice there were no significant sex differences at any time point. In male animals, a genotypic difference in weight manifested at P13 with wild-type mice weighing significantly more than knockout animals (Figure 2-2A). In female mice, this same genotypic difference was observed slightly later at P17 (Figure 2-2B). To determine whether the decrease in body weight was due to decreased food and water consumption, food and water were monitored daily for a period of 4 days in adult mice. There were no significant differences for total food and water intake between the genotypes (Figure 2-3). Since food and water intake could only be measured following weaning, it is unclear whether there were genotypic differences in caloric intake at the time when initial body weight differences emerged.

2.3.2 Motor developmental delays in ephrin-A5$^{-/-}$ mice

Loss of ephrin-A5 significantly altered motor function and sensory development in mouse pups starting as early as P13. Approximately 65 percent of the wild-type pups exhibited eye opening by P13 compared to only 37.5 percent of the ephrin-A5$^{-/-}$ pups [$F(1,66) = 4.5$, $p = 0.037$] (Figure 2-4). Likewise, time to complete the negative geotaxis test as well as day to reach test criteria were significantly longer in ephrin-A5$^{-/-}$ mice compared to wild-type controls [$F(1,64) = 1471.87$, $p < 0.001$] (Figure 2-5A and B respectively). In addition, latency to complete the negative geotaxis test significantly decreased with age in both genotypes [$F(8,512) = 32.402$, $p < 0.001$]. Approximately eighty percent of the wild-type pups reached criteria by P13 and by P15 all wild-type animals completed the test. In contrast, only ten percent of the ephrin-A5$^{-/-}$ mice had reached criteria by P13 and it was not until P17 that all had achieved this level (Figure 2-5B). There were no significant differences for mid-air righting between wild-type and ephrin-A5$^{-/-}$ mice (data not shown).

Hanging wire performance significantly increased over the seven days of testing in both genotypes [$F(6,396) = 94.676$, $p < 0.001$] (Figure 2-6). However, starting on P14, ephrin-A5$^{-/-}$ mice fell from the wire significantly faster than wild-type animals [$F(1,66) = 13.42$, $p < 0.001$]. This poor performance continued until the sixth day of testing (P18) and by P19 no differences were observed between the genotypes. All motor and developmental milestone testing was done in both male and female pups, however, no sex differences were found.

Overall motor activity was assessed in young (P21) and adult (P60) male mice under both social and non-social conditions (Figure 2-7A and B). When examined in the

non-social test, the total number of horizontal movements over six 5-min bins was significantly higher for ephrin-A5$^{-/-}$ mice compared to wild-type mice [$F(1,52) = 17.58$, $p < 0.001$] (Figure 2-7A). In addition there was a significant effect of bin [$F(5,260) = 98.36$, $p < 0.001$] without bin x genotype or bin x genotype x age interactions. This increased activity persisted as adults and was also observed under social conditions. In the social behavior test, a pair of non-sibling null and wild-type mice was observed for social interactions for a 30 min session. Ephrin-A5$^{-/-}$ mice again exhibited a significant increase in motor activity during the 30 minute paired testing period [$F(1,16) = 6.37$, $p = 0.02$] (Figure 2-7B), although no differences were observed for face sniffs, crawl-under/over behaviors, self-grooming, and allogrooming (data not shown).

2.3.3 Decreased monoamine concentrations in the brain of ephrin-A5$^{-/-}$ mice

There was an overall decrease in the levels of monoamines in ephrin-A5$^{-/-}$ mice (Table 2-1, Figure 2-8 and Figure 2-9) with lower levels of DA in the striatum [$F(1,37) = 9.25$, $p = 0.004$] (Figure 2-8A) and the hypothalamus [$F(1,34) = 10.2$, $p = .003$] (Figure 2-8B), 5-HT in the hippocampus [$F(1,35) = 5.2$, $p = .028$] (Figure 2-9A) and the cerebellum [$F(1,34) = 6.5$, $p = .015$] (Figure 2-9B) and NE in the frontal cortex [$F(1,34) = 14.4$, $p = .001$] (Figure 2-9D). These reductions were consistent in both 15 and 30 day old mice. In addition, DA turnover in the striatum (Figure 2-8D), as assessed by comparing the ratio of DOPAC to DA, was significantly higher in the null mice compared to wild-types. These results are consistent with the decreased DA and increased DOPAC [$F(1,37) = 4.58$, $p = 0.038$] (Figure 2-8A, and C) seen in the striatum. The 5-HT metabolite 5-HIAA was also significantly lower in the cerebellum of null mice at both ages [$F(1,34) = 8.9$, $p = .005$] (Figure 2-9C).

2.3.4 Decreased DAT and VMAT2 protein levels the striatum of ephrin-A5$^{-/-}$ mice

DAT, VMAT2 and TH protein levels in the striatum of 30 day old wild-type and ephrin-A5$^{-/-}$ were measured. There was a decrease in both DAT and VMAT2 protein levels in the striatum of ephrin-A5$^{-/-}$ mice. DAT levels were decreased by 38 percent (t =-2.914, p =0.03; Figure 9) and VMAT2 levels by 41 percent (t =-3.670, p =0.01; Figure 2-10). These results are consistent with our HPLC striatal findings. TH protein levels were reduced by 25 percent between wild-type and ephrin-A5$^{-/-}$ animals, but this did not reach statistical significance (p = 0.20; Figure 2-10).

2.4 Discussion:

Previously, we have reported that ephrin-A5 and one of its receptors, EphA5, are important in regulating the proper targeting of dopaminergic axons during neuronal development (Cooper, Kobayashi et al. 2009). As such, disruption of dopaminergic targeting may lead to loss of dopamine and alterations of psychomotor and sensorimotor behaviors which are highly dependent on monaminergic function. Here, we focused on assessing the effects of loss of ephrin-A5 on neurobehavioral development. Our data reveal that loss of ephrin-A5 leads to developmental delays, deficits in sensorimotor development, increased locomotor activity, and global decreases in several monoamines and their metabolites in the brain.

Ephrin-A5$^{-/-}$ mice were found to have delayed eye opening and exhibited deficits in their performance of the negative geotaxis and the hanging wire grip strength tests. These developmental delays could be attributed in part to decreased body weight. However, since mice of both genotypes ate the same amount of food as adults, we speculate that the reduction in body weight seen in ephrin-A5$^{-/-}$ mice is most likely attributable to alteration

in metabolism and/or level of activity, but can also be due to differences in suckling behavior of the pups.

Ephrin-A5$^{-/-}$ mice exhibited significant deficits in their performance in the negative geotaxis and hanging wire tests. While ephrin-A5$^{-/-}$ mice were able to reach wild-type performance levels by P19 in the hanging wire test, they were never able to reach wild-type performance levels in the negative geotaxis testing period, suggesting a more lasting deficit in certain aspects of motor control. Our neurochemical results indicate that these mice also exhibited decreases in cerebellar 5-HT and its metabolite (5-HIAA) and, therefore, it may be possible to attribute these functional deficits to the alterations in cerebellar monoaminergic system development, although other possibilities exist. In addition to cerebellar monoaminergic changes, ephrin-A5$^{-/-}$ mice exhibited decreases in 5-HT in the hippocampus, DA in the striatum and hypothalamus, and NE in the frontal cortex.

Dramatic alteration in central monoaminergic systems such as those observed in ephrin-A5$^{-/-}$ mice have been linked to depression, schizophrenia and attention deficit-hyperactivity disorder (ADHD) (Maletic, Robinson et al. 2007; Parkitna, Bilbao et al. 2010). In particular, the alterations in regional monoaminergic levels including the cerebellum, frontal cortex and striatum are evident in the same regions most affected in ADHD (Bush 2011). For example, neuroimaging studies show that individuals with ADHD have brain volume reductions in the cerebellum, prefrontal cortex, striatum, corpus collosum and the dorsal anterior cingulate cortex as compared to healthy controls (Emond, Joyal et al. 2009). These findings indicate that ADHD symptoms are most likely due to structural and functional alterations that encompass a large portion of the central nervous system and not one isolated brain region. More recently, data from genome-wide

association studies have identified a neurodevelopmental network involved in neurite outgrowth to be altered in ADHD (Poelmans, Pauls et al. 2011). In addition, evidence for the involvement of cell adhesion/axon guidance molecules, in ADHD was reported (Lesch, Timmesfeld et al. 2008; Sandau, Alderman et al. 2012). Based on these findings and on the role of ephrin-A5 in neurite outgrowth (Gao, Shinsky et al. 1998) and targeting of dopaminergic axons (Cooper, Kobayashi et al. 2009), these data suggest that loss of ephrin-A5 may result in neurodevelopmental alterations that may contribute to behavioral phenotypes such as the ones observed in ADHD.

Many of the core symptoms used for ADHD diagnosis fall under the hyperactivity category. Excessive running and difficulty in engaging in playing or quiet leisure activity falls under this category. In addition, it was found that children diagnosed with ADHD have delays in motor skill development that was reflected in both the physical and neurological examination for subtle signs and in short interval cortical inhibition in the motor cortex (Gilbert, Isaacs et al. 2011). These share some similarity to the developmental delays and hyperlocomotion observed in the ephrin-A5$^{-/-}$ mice. Additionally, identified ADHD susceptibility genes are known to be involved in the regulation of monoamine activity (Faraone and Mick 2010) and stimulant therapy for ADHD act as broad-spectrum monoaminergic agonists (Zametkin and Rapoport 1987; Meijer, Faber et al. 2009). Given that ephrin-A5$^{-/-}$ mice have decreases in DA, NE and 5-HT in the same brain regions implicated in ADHD pathology and that persistent hyperactivity is observed, our data suggest that the ephrin-A5$^{-/-}$ mouse may be an appropriate animal model for the study of hyperactivity associated with ADHD. However, further study is needed to assess additional behavioral domains, such as sustained attention and impulsive behaviors.

Monoamines are essential for normal brain development and postnatal survival (Zhou, Quaife et al. 1995; Takahashi, Miner et al. 1997). Monoamine levels are controlled through a combination of synthesis, storage, and re-uptake, which, if altered, can have significant consequence on proper development. Indeed, significant reductions in monoamines and developmental delays have been observed in vesicular monoamine transporter (VMAT2) and dopamine transporter (DAT) knockout mice. Although VMAT2 knockout is lethal, VMAT2 hypomorphs that express approximately 5 percent VMAT2 have certain similarities with ephrin-A5$^{-/-}$ mice, because of the global decreases in brain monoamine levels and the decreased levels of VMAT2. VMAT2 deficient animals have diminished levels of DA, NE and 5-HT and increased monoamine turnover in the striatum, cortex and hippocampus, three of the brain regions in which ephrin-A5$^{-/-}$ mice have monoamine decreases (Caudle, Richardson et al. 2007; Taylor, Caudle et al. 2009). Similarly, DAT knockout mice have decreased levels of DA, developmental delays, and are hyperactive (Giros, Jaber et al. 1996). Thus, alterations in monoamine transporter levels can have significant consequences on monoamine levels, neuronal development, and behavioral function. Here, we found that ephrin-A5$^{-/-}$ mice had decreased DAT and VMAT2 levels. Decreased DAT levels have also been observed by neuroimaging in some ADHD patients (Volkow, Wang et al. 2007) and VMAT2 levels have been found to be decreased in platelets of ADHD patients in one small study (Toren, Rehavi et al. 2005).

In summary, ephrin-A5$^{-/-}$ mice exhibited increased motor activity and developmental delays as compared to wild-type mice. This increase in motor behavior was replicated under several different testing conditions, both in social and non-social conditions. Decreased brain region levels of monoamines, increased locomotor activity and

developmental delays are all key components in the diagnosis of ADHD. Future studies should examine the sensitivity of these mice to psychomotor stimulants and whether these mice have alterations in working memory, attention, and impulsive behavior to better assess the relevance of ephrin-A5$^{-/-}$ mice to ADHD behavior.

Figure 2-1: Developmental and behavioral assessment-timeline

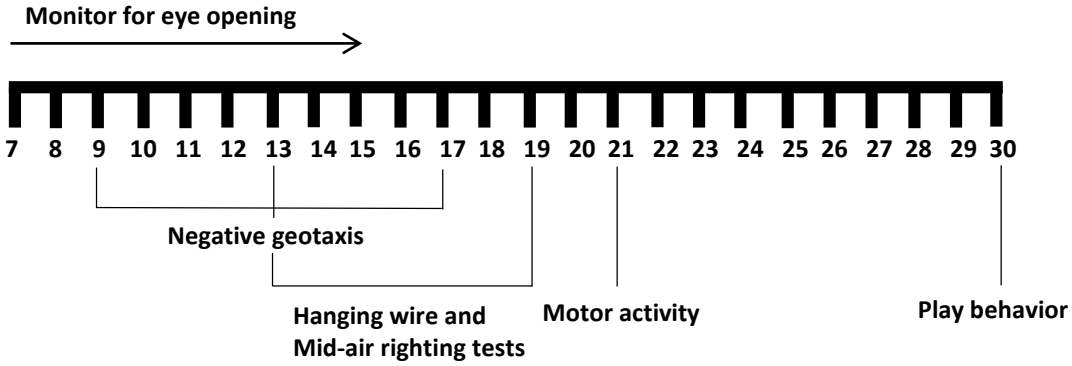
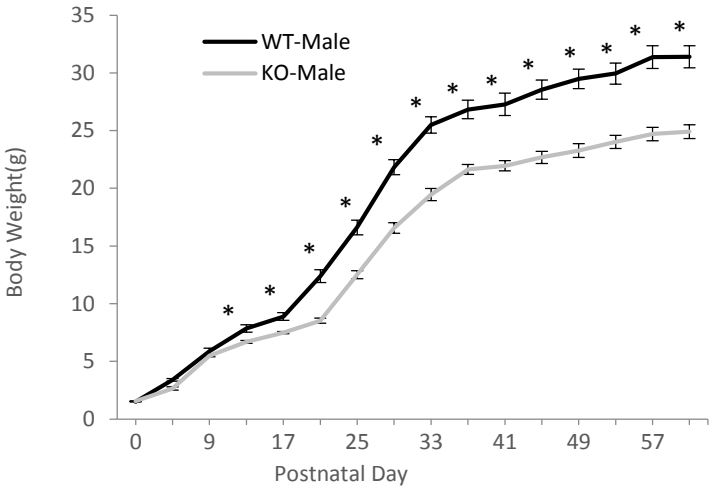


Figure 2-2: Changes in body weight during development
(A, B) Male and female ephrin-A5$^{-/-}$ and wild-type pups were weight at day of birth (P0), and following P5, body weight was measured every 4 days until P60.
Data are presented as mean body weight (g) ±SEM.
* indicates significantly different from ephrin-A5$^{-/-}$ mice; $p < 0.05$.

A.



B.

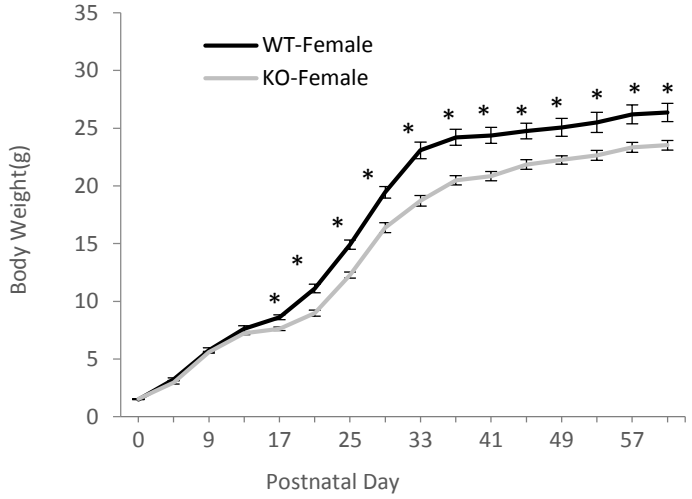


Figure 2-3: Food and water consumption
Starting on P60, food and water intake were measured daily for four days in individually housed ephrin-A5$^{-/-}$ and wild-type animals (n=10). There were no significant differences between the genotypes.
Data are presented as mean food (g) and water (ml) intake +SEM.

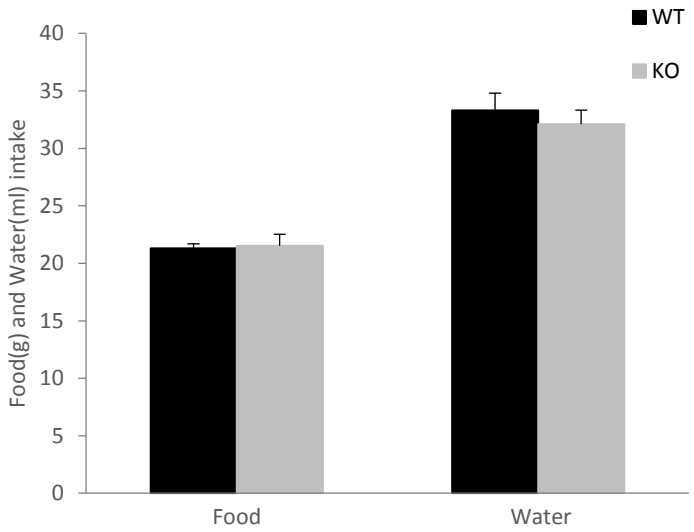


Figure 2-4: Delayed eye opening in ephrin-A5$^{-/-}$ mice
Starting at P9, ephrin-A5$^{-/-}$ (n=32) and wild-type (n=36) mice were observed daily to determine the first day in which both eyes were open.
* indicates significantly different from wild-type mice; $p < 0.05$.

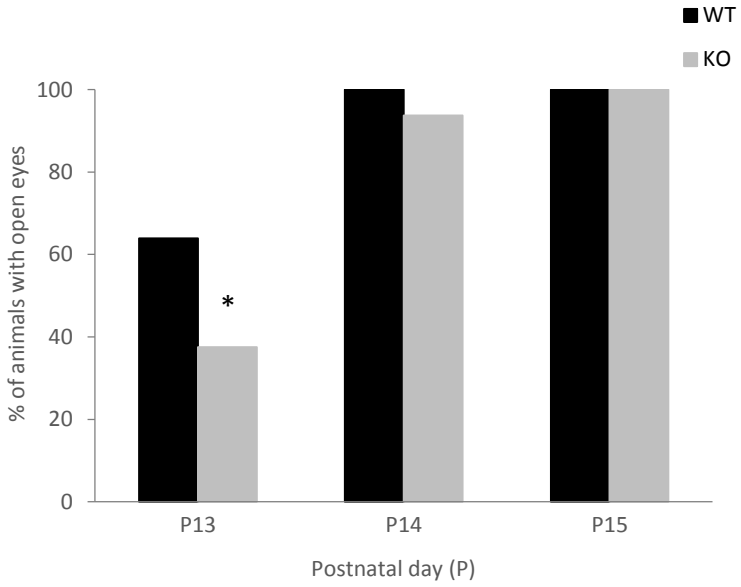


Figure 2-5: Decreased performance and delay in reaching criteria by ephrin-A5$^{-/-}$ mice in the negative geotaxis developmental test

The ability of ephrin-A5$^{-/-}$ (n=32) and wild-type (n=36) mice to display the negative geotaxis response was tested on P9-17.

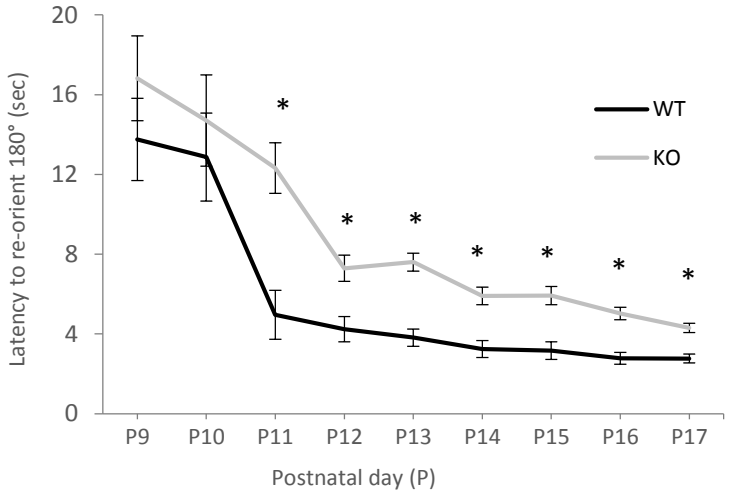
(A) The mean latency of ephrin-A5$^{-/-}$ and wild-type mouse pups to re-orient themselves 180° across nine days of testing. Starting at day three of testing, ephrin-A5$^{-/-}$ mice were significantly slower to re-orient themselves compared to wild-type animals.

Data are presented as latency to re-orient 180° ±SEM.

(B) Wild-type mice reached test criteria two days earlier then ephrin-A5$^{-/-}$ mice.

* indicates significantly different from wild-type pups, $p<0.05$.

A.



B.

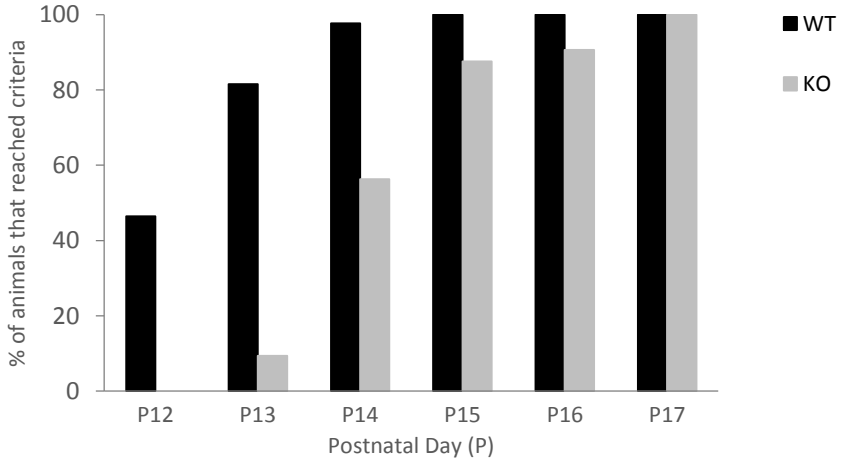


Figure 2-6: Delayed development of grip strength in ephrin-A5$^{-/-}$ mice
Grip strength ability was measured using the hanging wire test on P13-19 in ephrin-A5$^{-/-}$ (n=32) and wild-type (n=36) mouse pups.
Data are presented as mean latency to fall ±SEM.
* indicates significantly different from ephrin-A5$^{-/-}$ pups; $p<0.05$.

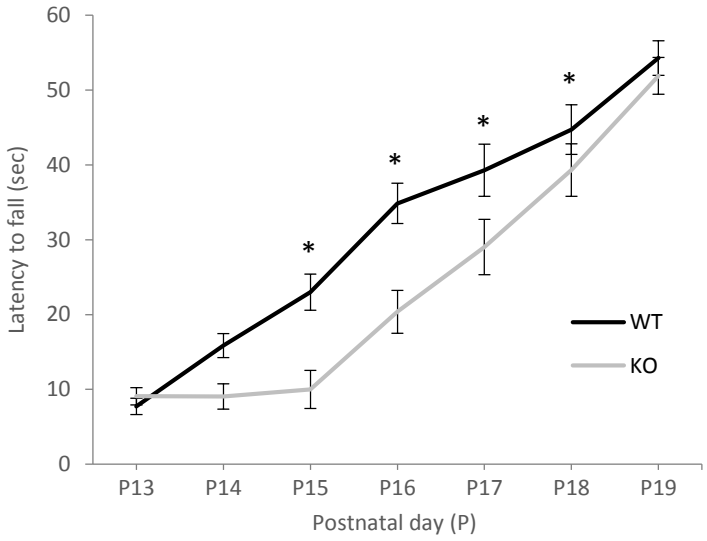


Figure 2-7: Ephrin-A5$^{-/-}$ mice showed persistent increased locomotor activity
(A) Horizontal locomotor activity of young (P23) and adult (P60) ephrin-A5$^{-/-}$ and wild-type mice were monitored for 30 min each. Ephrin-A5$^{-/-}$ mice exhibited increased locomotor activity at both time points.
(B) Social behavior was assessed between genotype, sex and age matched non-sibling pairs during a 30 minutes open field observation session. These observation sessions were run on P30. The number of horizontal motor movements made by the pair of mice was recorded. Data are presented as mean ±SEM.
* indicates significantly different from wild-type mice; $p<0.05$.

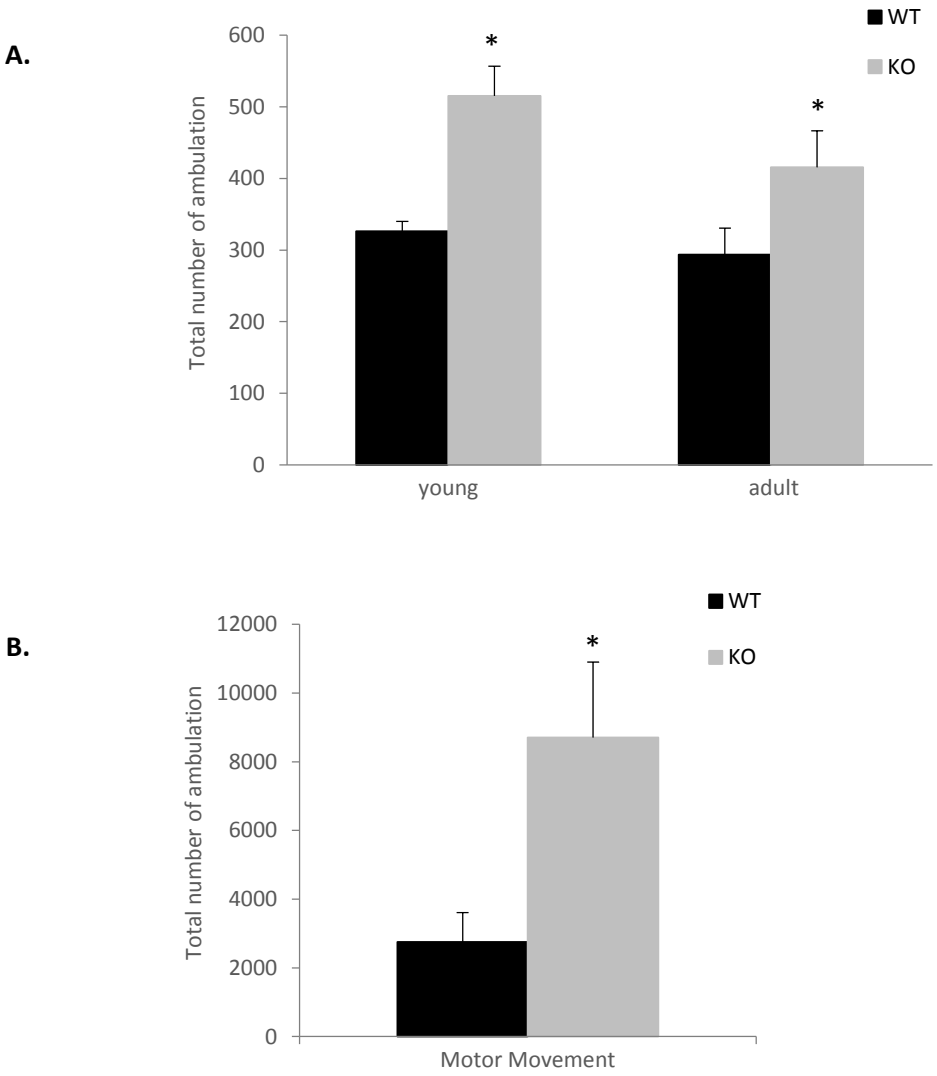


Figure 2-8: Alterations in the dopamine system in the brain of ephrin-A5$^{-/-}$ mice
Ephrin-A5$^{-/-}$ and wild-type mice were sacrificed on P15 and 30 and different brain regions were dissected and analyzed for dopamine (DA) and its metabolites using HPLC.
(A, B) Reduction of DA levels in the striatum (A) and the hypothalamus (B) were observed in null mice.
(C, D) Ephrin-A5$^{-/-}$ pups had reduced DOPAC and increased DOPAC/DA turnover rate in the striatum. Data are presented as mean ng of neurotransmitter /mg of protein +SEM
* indicates significantly different from wild-type mice; $p < 0.05$.
^ indicates significant different from P15 within strain; $p < 0.05$.

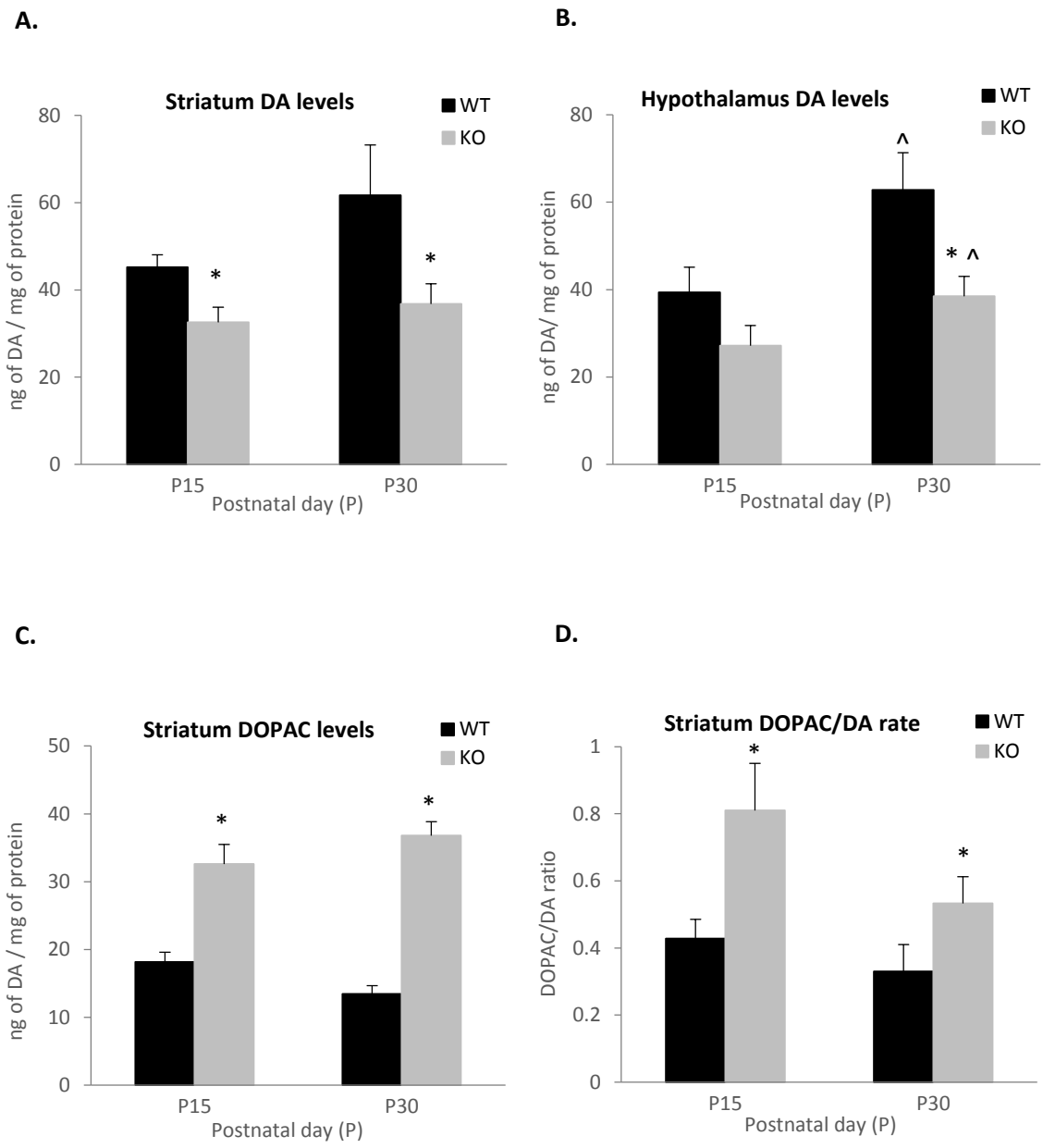


Figure 2-9: Alterations in serotonin, its metabolite and norepinephrine levels in the brain of ephrin-A5$^{-/-}$ mice

Ephrin-A5$^{-/-}$ and wild-type mice were sacrificed on P15 and 30 and different brain regions were dissected and analyzed for norepinephrine (NE), serotonin (5HT) and its metabolites using HPLC.

(A, B) Reduced 5HT levels the hippocampus (A) and the cerebellum (B) of null mice.

(C) Reduced 5HIAA levels in the cerebellum of the null mice.

(D) NE levels in the frontal cortex were decreased in the null mice.

Data are presented as ng of neurotransmitter /mg of protein +SEM.

*indicates significantly different from wild-type mice; $p < 0.05$.

^ indicates significant different from P15 within strain; $p < 0.05$.

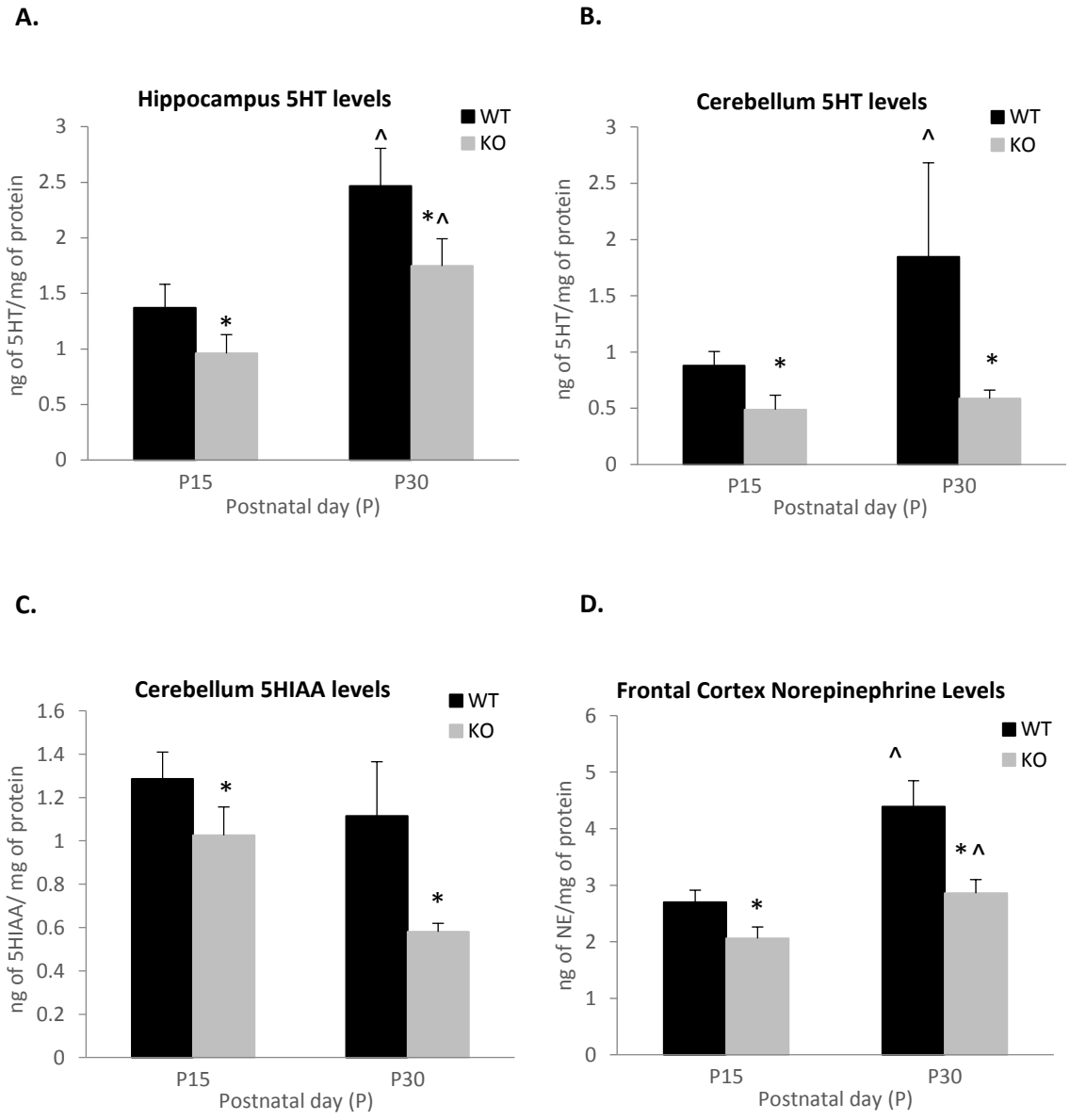


Figure 2-10: Decreased DAT and VMAT2 protein levels in ephrin-A5$^{-/-}$ mice
Ephrin-A5$^{-/-}$ and wild-type mice (n=4 per group) were sacrificed on P30 and the striatum was dissected and analyzed for dopamine transporter (DAT), tyrosine hydroxylase (TH), the vesicular monoamine transporter 2 (VMAT2), and syntaxin protein levels using western immunoblotting.
DAT and VMAT2 levels were reduced in the striatum of ephrin-A5$^{-/-}$ mice.
Data are presented as protein levels (percent of control) +S.E.M.
Syntaxin levels were used to ensure equal protein loading across samples.
*indicates significantly different from wild-type mice; $p < 0.05$.

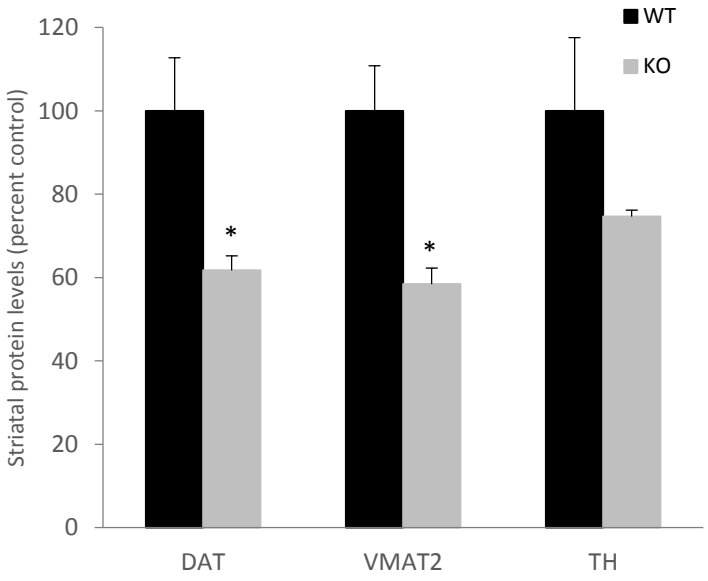


Table 2-1: Neurotransmitter and metabolite levels in developing ephrin-A5$^{-/-}$ and wild-type mice
Neurotransmitter and metabolite levels detected in the cerebellum, hippocampus, striatum, frontal cortex and hypothalamus of male p15 and p30 ephin-A5$^{-/-}$ and wild-type mice.
Values are expressed in ng/mg of protein ±SEM.
* indicates significantly different from wild-type mice; $p < 0.05$.
^ indicates significant different from P15 within strain; $p < 0.05$.

	WT 15	***KO 15***	***WT 30***	***KO 30***
Cerebellum				
5-HIAA/5-HT	2.37 ± 0.96	4.81 ± 1.15	0.83 ± 0.12	1.16 ± 0.17
NE	13.7 ± 0.99	11.6 ± 1.67	14.4 ± 1.59	12.4 ± 0.40
Hippocampus				
5-HIAA	1.26 ± 0.20	1.02 ± 0.09	1.59 ± 0.10	1.07 ± 0.12
5-HIAA/5-HT	0.97 ± 0.09	1.33 ± 0.26	0.55 ± 0.10 ^	0.78 ± 0.20 ^
NE	8.59 ± 1.01	8.02 ± 0.55	8.55 ± 0.61	7.46 ± 0.63
Striatum				
HVA	11.2 ± 0.45	12.8 ± 0.69	9.24 ± 0.73 ^	9.56 ± 0.92 ^
DOPAC/HVA	0.25 ± 0.02	0.43 ± 0.05 *	0.21 ± 0.04 ^	0.28 ± 0.03 ^*
5-HT	2.11 ± 0.50	1.79 ± 0.33	3.64 ± 0.75	2.10 ± 0.50
5-HIAA	3.50 ± 0.28	4.26 ± 0.41	2.62 ± 0.31 ^	3.03 ± 0.46 ^
5-HIAA/5-HT	1.56 ± 0.56	1.73 ± 0.33	1.58 ± 0.76	1.56 ± 0.47
NE	5.87 ± 0.91	4.86 ± 0.94	6.59 ± 0.91	7.15 ± 0.98
Frontal Cortex				
DA	7.84 ± 0.93	11.0 ± 1.97	25.1 ± 4.01 ^	19.9 ± 3.94 ^
DOPAC	1.91 ± 0.24	3.00 ± 0.40	4.17 ± 0.87 ^	3.95 ± 0.78 ^
HVA	3.20 ± 0.52	3.28 ± 0.37	4.42 ± 0.78	4.26 ± 0.68
DOPAC/DA	0.30 ± 0.08	0.32 ± 0.07	0.16 ± 0.01 ^	0.20 ± 0.02 ^
DOPAC/HVA	0.43 ± 0.06	0.33 ± 0.02	0.17 ± 0.01	0.22 ± 0.01
5-HT	2.33 ± 0.26	1.79 ± 0.23	5.49 ± 0.81 ^	4.32 ± 0.69 ^
5-HIAA	1.07 ± 0.11	1.22 ± 0.12	1.11 ± 0.19	1.35 ± 0.13
5-HIAA/5-HT	0.49 ± 0.08	0.76 ± 0.11 *	0.22 ± 0.03 ^	0.36 ± 0.05 *^
Hypothalamus				
DOPAC	58.0 ± 10.5	43.3 ± 4.58	48.8 ± 10.0	43.5 ± 4.10
HVA	101 ± 13.3	85.0 ± 8.91	61.5 ± 10.3 ^	50.8 ± 4.79 ^
DOPAC/DA	1.48 ± 0.19	2.62 ± 0.80	0.77 ± 0.10 ^	1.34 ± 0.20 ^
DOPAC/HVA	2.97 ± 0.53	4.25 ± 0.86	0.98 ± 0.10 ^	1.44 ± 0.14 ^
5-HT	53.4 ± 7.20	57.1 ± 13.0	64.4 ± 3.81	45.0 ± 6.84
5-HIAA	62.6 ± 10.3	57.5 ± 4.99	40.8 ± 5.30 ^	41.9 ± 2.79 ^
5-HIAA/5-HT	1.30 ± 0.22	2.28 ± 0.47	0.64 ± 0.56	1.24 ± 0.43
NE	16.9 ± 2.00	17.0 ± 1.93	18.7 ± 2.26	13.8 ± 4.83

CHAPTER 3: DECREASED MATERNAL BEHAVIOR AND ANXIETY IN EPHRIN-A5$^{-/-}$ MICE

3.1. Introduction:

Proper maternal behavior is essential for the survival of the offspring (Numan and Insel 2003). It is important for growth and normal development of the young and can influence their physiological, behavioral and cognitive functions in adult life (Franklin, Saab et al. 2012). For example, early life stress in the form of maternal neglect or separation, produced behavioral and emotional changes as well as alterations in cognitive functions later in life [reviewed in (Meaney 2001; Veenema 2009)]. Veenema *et al*. (Veenema, Blume et al. 2006) found that adult rats that were separated from their mothers earlier in life had exaggerated inter-male aggression, increased depression-like behavior and altered levels of arginine vasopressin (AVP) and serotonin (5-HT) in the hypothalamus. In addition, differences in the hypothalamic-pituitary-adrenal (HPA) response to stress were found in the offspring of low *vs* high licking and grooming (LG) rats (Liu, Diorio et al. 1997). Adult rats that were born and raised by high–LG mothers had decreased plasma corticosterone levels in response to restraint stress compared to those raised by low–LG mothers (Liu, Diorio et al. 1997).

In addition, maternal care can affect the development of maternal behavior in the female offspring (Melo, Lovic et al. 2006; Shoji and Kato 2009). Shoji *et al*. (Shoji and Kato 2009) compared the maternal behavior of the offspring of two inbred mice, CBA/Ca and BALB/c, which differ in their levels of maternal care; CBA/Ca female exhibit nursing and pup licking more frequently and retrieve their pups faster than BALB/c female. They found that low levels of maternal care earlier in life (by BALB/c dams) decreased maternal

behavior in the offspring. However, since no maternal effect was found in CBA/Ca offspring, the author suggested that maternal behavior is governed by genes to environment interaction. The effects of environmental conditions on maternal care and consequently on the development of the offspring is probably due to alteration in the level of anxiety of the mother (Meaney 2001). However the direction and nature of the anxiety-maternal care relationship is not clear; some studies suggest that a positive relationship exists (Maestripieri, Badiani et al. 1991), others found negative correlations (Kessler, Bosch et al. 2011), and still others found none (Bosch and Neumann 2008; Curley, Jensen et al. 2012).

In rodents, a wide range of behaviors directed toward the care of the young have been reported. These include nest building, pup retrieval, aggression toward a male intruder, nursing, pups' licking and grooming, and crouching over the pups to provide thermoregulation (Weber and Olsson 2008). Interestingly, these behaviors can be seen in non-parental virgin, female rodents. When initially exposed to pups virgin female rats will avoid them. Yet, after continuous daily exposure of about 7 days the female will start caring for the young. This pup-induced behavior is referred to as sensitized maternal behavior (Numan 2014). By contrast, other mammalian species such as mice do not require the "sensitization period" and show spontaneous maternal behavior immediately when presented with pups (Numan 2014). Here, the level of maternal care is higher in postpartum female mice which are faster to retrieve the pups, spend more time crouching over them (Gandelman, Zarrow et al. 1970; Stolzenberg and Rissman 2011) and are able to build a better, more complex nest than virgin female mice (Bond, Neumann et al. 2002). In addition, maternal motivation is lower in virgin compared to postpartum female.

Interestingly, these differences can be reduced when the female is being repeatedly exposed to the pups (Stolzenberg and Rissman 2011). Recently, the use of gene knockout mice has made significant contribution to our understanding on the neuronal regulation of maternal behavior. Mice carrying a null mutation for the prolactin receptor gene (Lucas, Ormandy et al. 1998), the dopamine β-hydroxylase gene (Thomas and Palmiter 1997), and the forebrain Gq/11 gene (Wettschureck, Moers et al. 2004) exhibit defects in maternal behavior compared to wild-type controls. In addition, several brain regions and neural circuits have been implicated in the development of maternal behavior, specifically the medial amygdala (MeA), the ventral tegmental area (VTA) of the midbrain, the medial prefrontal cortex (mPFC) and the hypothalamus (Gammie 2005; Pereira and Morrell 2011; Numan 2014). Since maternal care encompassed repertoire of behaviors, and each involve unique motor movement different circuits will control distinct maternal behavior, although some overlapping exists (Gammie 2005). The medial preoptic area (MPOA) of the hypothalamus, has been shown to be essential for the onset and maintenance of many aspects of maternal behavior (Gammie 2005; Tsuneoka, Maruyama et al. 2013; Wu, Autry et al. 2014). Here, hormonal activation stimulates maternal behavior, whereas interruption of neuronal activity decreases maternal responsiveness to pups [reviewed in (Numan 2014)]. In addition, damage to the MPOA by knife cut or electrical lesions severally disrupt pup retrieval and nest building, [reviewed in (Numan and Insel 2003)]. Furthermore, a recent study (Wu, Autry et al. 2014) found a subset of galanin-expressing neurons within the MPOA that act as a switch to turn on and off parenting behavior in mice; ablation of these neurons in lactating female mice reduced pup retrieval, whereas its activation triggered pup grooming (Wu, Autry et al. 2014).

The involvement of hormones and neurotransmitters in regulating maternal care has also been shown, specifically the neuropeptides arginine vasopressin (AVP) and oxytocin (OXY) both which are activated around parturition and in lactation (Bosch and Neumann 2012). Blockade of AVP-Va1 receptor within the MPOA decreased maternal care, whereas chronic intracerebroventricularly (i.c.v.) Infusion of AVP improved it in lactating rats (Bosch and Neumann 2008). In addition, mice that were selectively bred for low anxiety levels have lower AVP expression in the hypothalamus and displayed less maternal care (Kessler, Bosch et al. 2011). Finally, maternal aggression is reduced in lactating AVP-V1b-receptor knockout mice (Wersinger, Caldwell et al. 2007) but not AVP-V1a-receptor knockout mice (Wersinger, Caldwell et al. 2007). The involvement of OXY in maternal care has been widely studied in rats (reviewed in (Bosch and Neumann 2012)), where i.c.v infusion of OXY into the lateral ventricle induced maternal behavior in steroid-primed virgin female rats (Pedersen and Prange 1979). In mice, it is suggested that OXY is important for the initiation, but not the maintenance of this behavior (Insel and Harbaugh 1989; Rich, deCardenas et al. 2014).

Ephrin-A5 is expressed in areas of the brain that are central for normal maternal behavior specifically the hypothalamus (Zarbalis and Wurst 2000; Deschamps, Morel et al. 2010). Here, ephrin-A5 transcripts were detected in the preoptic area, the suprachiasmatic nucleus (SCN), the paraventicular nucleus (PVN) and the arcute nucleus (AR) (Zarbalis and Wurst 2000). We sought to determine if maternal behavior is affected by ephrin-A5 deletion. Specifically, we tested ephrin-A5$^{-/-}$ and wild-type control lactating females for their ability to build a nest, retrieve their pups and display maternal aggression toward a male intruder. In addition different tests for anxiety were used to assess the impact

of maternal care on adult behavior. Finally, immunohistochemistry was used to analyze levels of arginine vasopressin (AVP), and GnRH within the hypothalamus. We found that ephrin-A5 inactivation resulted in decreased maternal and anxiety-like behavior as well as alteration in AVP-immunoreactivity (AVP-ir) in the hypothalamus of the mutant female.

3.2 Materials and Methods:

Animals: Ephrin-A5 animals were described earlier (see chapter 2). Animals used in this study were generated using heterozygous crosses. To test for maternal behavior (pup retrieval, maternal aggression, nest building and the Bruce effect), ephrin-A5$^{-/-}$, heterozygous and wild-type littermate females were bred with heterozygous males. Pregnant females were housed separately until time of birth. Day of birth was recorded as postnatal (P0) and the total number of pups born, number of pups dead, and number of pups alive were recorded. Pup body weights were measured every 4 days until P20 and again at P60. All pups were labeled for individual identification by marking their tails.

Genotyping was performed by tail DNA polymerase chain reaction (PCR) analyses as described previously (Frisen, Yates et al. 1998). Primers 1: 5'TCCAGCTGTGCAGTTCTCCAAAACA3' and 2 5'ATTCCAGAGGGGTGACTACCACATT3' were used for amplification of wild-type sequences (397 bp) and primers 1 and 3 5'AGCCCAGAAAGCGAAGGAGCAAAGC3' for amplification of the null sequences (513 bp).

Nest building: The test was performed as described previously (Deacon 2006) with some modifications. For maternal nest building ephrin-A5$^{-/-}$, heterozygous and wild-type virgin female mice (n=5 per genotype) were housed with a male mouse and checked for the

presence of a sperm plug; once a plug was observed, the date was recorded as embryonic day 0.5 (E0.5) and the male was removed from the cage. On E17.5 the pregnant mice were individually housed overnight with food, water and new bedding but no environmental enrichment. The next morning (the first stage of the dark cycle), mice were provided with nesting material (nestlets; Ancare; UK agent, Lillico) in the home cage. Nests were observed 1, 6 and 24 hours later and assessed on a rating scale of 1-5 (Deacon 2006). A score of 1 was given if there was no nest and a score of 5 represents a perfect, fully enclosed nest, by two observers who were blind to the genotype and time of the nest. In order to assess nest build by nulligravid mice, virgin females (n=6 per genotype) were individually housed overnight with food water and new bedding. The next morning they were provided with the nesting material and the procedure was repeated as described above.

Maternal aggression and pup retrieval test: Ephrin-A5$^{-/-}$ (n=9), heterozygous (n=10) and wild-type (n=8) lactating female mice were exposed to a wild-type intruder male in their home cage for 5 minutes on postpartum day 4 (P4) and 6. The pups were removed from the cage 2 minutes before the behavioral test, and the test was recorded for subsequent analysis. The intruder males were sexually naïve and group housed. After the test, the pups were randomly distributed throughout the cage and the time to retrieve the 1st and 3rd pup was recorded. Retrieval counted only when the pup brought into the nest completely and, a score of 180 sec was assigned if the dam failed to retrieve her pups in 3 minutes.

Olfactory memory for pheromones (the Bruce Effect): The test was performed as previously described (Thomas and Palmiter 1997; Wersinger, Temple et al. 2008). Briefly, ephrin-A5$^{-/-}$ and wild-type (n=18 per genotype) control females were housed with an ephrin-A5 heterozygous male. Females were checked for the presence of a sperm plug

every day and once the plug was detected (embryonic day 0.5, E0.5), the male was removed from the cage. Twenty four hours later, the sire male (familiar) or a FVB/NJ male (unfamiliar) was placed in the female's cage for 72 hours, after which the female was housed alone for the reminding of the gestation.

Elevated-plus maze (EPM): The maze was constructed of black Plexiglas with four arms in the form of a "plus", 30 cm above the floor. Two opposing arms of the maze (65 cm long) were enclosed in 8 cm high, black Plexiglas walls, while the two remaining arms (30 cm long) were left open. Two experiments were conducted using the EPM. In experiment 1 we tested male ephrin-A5$^{-/-}$ (n=7) mice that were born and reared by ephrin-A5$^{-/-}$ mother and wild-type (n=6) control mice that were born and reared by wild-type mother. In experiment 2, male and female ephrin-A5$^{-/-}$ (n=10 per sex), heterozygous (n=11 per sex) and wild-type (n=10 per sex) littermates that were born to heterozygous mothers were tested. Mice in the second experiment were born and reared by the same mother, and thus the effect of rearing are removed. The test begin when each mouse was individually placed in the center (5 cm x5 cm) of the maze facing an open arm and the number of times each animal enter an arm (either closed or open) as well as the duration in each arm were recorded for 5 minutes. Increases in the number of entrances and/or the time spent in the open arms provide indications of anxiolytic-like behaviors, and the total number of entrances (into both open and closed arms) is a measure of locomotor activity. EPM testing was carried under dim light and an arm entry was recorded only when all four paws crossed into the arm.

Light–dark box test: The test was performed as described previously (Rossi-George, LeBlanc et al. 2004), with some modifications. Briefly, the plexiglas box (47 x 24 x 21 cm,

L x W x H) was divided into two compartments, one black-walled fully opaque (14 cm long), and the other (33 cm long) lit from the compartment ceiling by a 20 W bulb. Free passage was allowed between the compartments by a small 4 x 5 cm opening. Male and female ephrin-A5$^{-/-}$ (n=10 per sex), heterozygous (n=10 per sex) and wild-type (n=9 male and 10 female) mice were individually placed in the dark compartment and allowed to freely explore the box for 5 minutes. All trials were videotaped for subsequent analysis. Latency to emerge from the dark compartments, light-dark transitions, time in the light compartment and a risk assessment, in which the head and fore-paws extend into the lighted area but the remainder of the body stays in the dark compartment (Bailey and Crawley 2009) was recorded.

Open-field test: The apparatus was described previously (Brodkin, Frank et al. 2014) and consisted of a 40 cm by 40 cm square arena enclosed at a height of 45 cm. The mouse behavior was captured by infrared beams which detect motor movement for 30 minutes with six, five minute bouts per session and the time and distance traveled in the center of the box were measured.

Corticosterone ELISA: Blood samples were collected by tail bleeding from male and female ephrin-A5$^{-/-}$ (n=5 per sex) and wild-type (n=5 per sex) littermates. Briefly, the tail was dipped in warm water (37 °C) for 30 seconds after which the tip of the tail (<0.5cm) was removed using surgical blade. Blood (20ul) was collected into a microvette tubes (Kent Scientific, Torrington CT, cat no. MCVT200-SER) and allowed to clot at room temperature for 1 hour. The blood was then centrifuged (10,000 x g) for 5 minutes at 20 °C to isolate upper layer serum and the corticosterone levels were measured by a competitive enzyme immunoassay (Arbor assay, Ann Arbor, MI, cat No. K014). Blood collections were done

in <3 min so that sampling is completed before activation of the HPA axis (Vahl, Ulrich-Lai et al. 2005). All standard and samples were run in triplicates. In order to compare corticosterone levels under both basal and stress conditions blood was collected twice from the same mice. Basal (unstressed) levels were compared by collecting blood in the morning (first stage of the dark cycle) and a week later blood was collected again after a 5 minutes exposure to the elevated plus maze test (stressor).

Immunohistochemistry: Lactating (postpartum day 7) ephrin-A5$^{-/-}$ and wild-type females (n=3 per genotype) were deeply anesthetized and transcardially perfused with 0.9 % saline followed by 4 % paraformaldehyde (PFA) (Sigma, St. Louis, MO) in phosphate buffer. Once fixed, brains were removed, post fixed in the same solution at 4 °C and cryoprotected in 30% sucrose overnight. The brains were cut into 30 µm coronal sections and labeled overnight using the rabbit anti-[Arg^{8}]-vasopressin (1:10,000; Peninsula, San Carlos, CA), followed by three washes with phosphate buffer and detection using goat secondary antibodies conjugated with Alexa Fluor 488 (1:200; Invitrogen, Grand Island, NY).

In order to quantitatively analyze AVP immunoreactivity (AVP-ir), a total of 8 sections per animals (30 µm x 8) containing the suprachiasmatic nucleus (SCN) and the paraventricular nucleus (PVN) were used and the number of AVP-ir cells were manually counted.

Statistical analysis: The following tests were used:

Mann-Whitney U test	Nest building
chi-square test	Bruce effect
Student t-test	EPM
	Immunohistochemistry
	Litter size
	Gestation time and latency to detect sperm plug
ANOVA	Anxiety tests
	Maternal behavior-pup retrieval and aggression
	Corticosterone (CORT) levels

3.3 Results:

3.3.1 Altered nesting behavior in primigravid (pregnant) ephrin-A5$^{-/-}$ mice

Nest building was assessed as described previously on a scale of 1-5 (Deacon 2006). Female mice were individually housed and provided with nesting material and the nest was quantified 1, 6 and 24 hours later.

Primigravid ephrin-A5$^{-/-}$ mice showed impairment of nesting behavior compared to heterozygous and wild-type littermates (Figure 3-1A, right panel). Null mice achieved significantly lower nesting scores at six hours ($U=.5$, $p=0.012$) as well as twenty four hours ($U=2$, $p=0.028$) compared to wild-type, suggesting a lower quality nest. One hour after providing the nesting material wild-type mice were already starting to make a nest, with the nesting square partially torn compared to the null mice that left the nesting square mostly intact. By six hours wild-type mice built a nearly perfect nest with more than 90 percent of the nesting square torn, whereas null mice left most of the material intact. Although by twenty four hours the null mice shredded most of the nesting square, a well

formed nest was usually not found in the cage (Figure 3-1B). There were no significant differences in nest score between heterozygous and wild-type mice in all three times tested. However, heterozygous mice had a significant higher nest score then null mice at twenty four hours (U=3, p=0.047), suggesting that single deletion of the gene in not sufficient to decrease nesting performance in these mice. Since the rating scale that was used to analyze the nest is non-linear, these data can alternatively be presented as the percentage of mice that received a specific score (1-5) for each testing time as shown in the appendix (appendix-1). At 1 hour 100 percent of the null and heterozygous mice received a score of 1 compared to only 40 percet of the wild-type mice. By 24 hours 80 percent of the wild-type built a perfect nest compared to only 20 percent of the null mice, suggesting an impairment in nesting behavior.

Since non-pregnant, nulligravid mouse are also able to build nests (Sherwin 1997; Bond, Neumann et al. 2002) and nesting requires normal sensorimotor behaviors (Gaskill, Gordon et al. 2012), we analyzed nests built by nulligravid, virgin female mice (Figure 3-1A, left panel). There were no significant differences in nest score between the genotypes at all three time point suggesting that ephrin-A5 deletion does not affect the motor ability of the mice to build a non-maternal nest. However, twenty four hours after the introducing of the nesting material, the nests of primigravid wild-type females rated significantly higher than those of nulligravid females (U=3, p=0.028). This difference was not observed in ephrin-A5$^{-/-}$ females where both primigravid and nulligravid mice built equal quality nests.

3.3.2 No changes in maternal aggression between ephrin-A5$^{-/-}$ and wild-type mice

We examined maternal aggression in lactating female mice on postpartum days 4 and 6 because it has been shown that aggression is highest during the early lactation period

(Svare, Betteridge et al. 1981). Pups were removed from the cage 2 min before the introduction of a wild-type male intruder into the home cage and the behavior of the dam was recorded for 5 minutes. A repeated measure ANOVA was used to analyze the latency to first attack as well as the number of attacks made by the dam. There were no statistically significant differences between the genotypes on either measurements (Figure 3-2A and B).

3.3.3 Reduced pup retrieval in lactating ephrin-A5$^{-/-}$ mice

To further evaluate the role of ephrin-A5 on maternal behavior, we examined post-partum female mice in the pup retrieval task at the end of the aggression test (Figure 3-3). Pups were randomly distributed throughout the cage and the latency to retrieve the 1st and 3rd pup were recorded. If a dam failed to retrieve her pups in 3 minutes, a score of 180 sec was assigned. Null mice showed an impaired performance to retrieve the pups back to their nest on both days. A repeated measure ANOVA tests showed a significant genotypic difference on postpartum day 4 [$F(2,23)=6.563$, $p=0.006$] (Figure 3-3A) as well as day 6 [$F(2,23)=9.652$, $p=0.001$] (Figure 3-3B) were ephrin-A5$^{-/-}$ female mice took longer to retrieve the pups back to the nest compared to wild-type and heterozygous littermates. With the exception of one heterozygous female which did not retrieve her pups on P4, all wild-type and heterozygous mice retrieved their pups into the nest, whereas only about 45 percent of ephrin-A5$^{-/-}$ female mice completed the task (Figure 3-3C, Fisher Exact Test $p<0.05$). To test whether consecutive pup exposure improved maternal behavior in null mice, the pup retrieval test was repeated two days later on postpartum day 6. Repeated exposure to the pups did not improve retrieval in the null mice nor in wild-type or

heterozygous females [F(2,22)=0.137, p=0.870] (Figure 3-3D), although genotypic difference was still observed [F(2,22)=14.958, p<0.0001].

3.3.4 Reduced pup survival in ephrin-A5$^{-/-}$ mice

We observed decreased survival rate in pups that were born and reared by ephrin-A5$^{-/-}$ females. Although litter size was comparable between the genotype (Figure 3-4A, *t*=-1.636, p=0.120) within the first postpartum week pups reared by null female had 55 percent survival rate compared to 80 percent survival rate for pups reared by wild-type females (Figure 3-4B). It is important to note that survival rate was highly correlated to the shape of the nest, and to the level of pup gathering by the mother on the day of parturition; when a well formed nest was not observed in the cage, and the mother was not gathering the pups after birth, the pups would not survive (Figure 3-4C).

3.3.5 Pup body weights were not affected by the level of maternal care

We sought to determine whether growth was affected by the dams' genotype. Body weights of heterozygous pups born and reared by ephrin-A5$^{-/-}$ (n=8), heterozygous (n=6), and wild-type (n=8) dams were measured every 4 days until P20 and again at P60 (Figure 3-5A). The rationale here is that if heterozygous pups that were reared by null dams weigh less than those reared by wild-type or heterozygous control dams, then nursing is probably different between the genotypes. In addition, since differences were observed in body weights between null and wild-type mice (chapter 2, Figure 2-2), we wanted to determine whether maternal behavior (in the form of nursing) attributes to those changes. There were no genotypic differences between the groups [F(2,61)=1.444, p=0.244] (Figure 3-5B). However, there was a significant effect of sex [F(1,61)=43.674, p<0.001], postnatal day [F(5,305)=11096.859, p<0.001], and postnatal day x sex interaction [F(10,305)=0.724,

p=0.70]. *Post hoc* testing revealed that on P60 male mice weigh significantly more than female mice. The fact that heterozygous pups from all 3 dams (ephrin-A5$^{-/-}$, heterozygous and wild-type) had similar body weights (Figure 3-5B), suggest that nursing is not affected by ephrin-A5 deletion and that the growth differences between the null and wild-type mice (chapter 2, Figure 2-2) are not due to differences in maternal behavior.

Finally, we wanted to confirm our previous results (chapter 2, Figure 2-2) and see if the differences between null and wild-type mice are consistent with the current breeding scheme: ephrin-A5$^{-/-}$ and wild-type females bred with heterozygous males (See Materials and methods and Figure 3-5A). As expected, there was a significant effect of genotype [$F(1,27)=22.719$, $p<0.0001$] and postnatal day [$F(5,135)=4666.440$, $p<0.0001$] as well as postnatal day x genotype [$F(5,135)=18.431$, $p<0.0001$] and postnatal day x sex [$F(5,135)=35.739$, $p<0.0001$] interaction. *Post hoc* tests revealed that null mice weight significantly less than wild-type mice in all the days that were tested (Figure 3-5C). In addition, a sex difference was observed on P60 with female mice weighing less than male mice. These data support our previous results (chapter 2, Figure 2-2) and show that null mice weigh less than wild-type mice and that the decreased level of maternal care is not responsible for this difference.

3.3.6 Olfactory memory for pheromones (the Bruce effect) is intact in ephrin-A5$^{-/-}$ mice

To examine whether the Bruce effect, a pheromonal effect in which signals presented from an unfamiliar male terminates pregnancy, is affected by ephrin-A5 deletion, we exposed naïve ephrin-A5$^{-/-}$ and wild-type control females to familiar or unfamiliar males 24 hours after the detection of a sperm plug and monitored for pregnancy block.

Exposure to an unfamiliar male produced pregnancy block in almost 90 percent of wild-type and ephrin-A5$^{-/-}$ females (Figure 3-6A). In contrast, when the females were re-exposed to the familiar male pregnancy block was observed in only 10 percent of wild-type females and 33 percent of null mice; this difference in the percentage of pregnancy block between the genotypes was not significant (Chi Square test, p=0.20). However, within each genotype the proportion of females that exhibit pregnancy block in response to an unfamiliar male was significantly higher than the ones with the familiar male (Chi Square test, p=0.0006 for the wild-type female and p=0.016 for the null female) (Figure 3-6A). Finally, the number of days it took to detect the sperm plug as well as the gestation period was not significantly different between the genotypes (Figure 3-6B; p>0.05). These results suggest that both ephrin-A5$^{-/-}$ and wild-type control females exhibit normal pregnancy block in response to olfactory cues from an unfamiliar male.

3.3.7 Altered anxiety-like behavior in ephrin-A5$^{-/-}$ mice

3.3.7 A. Elevated plus test (EPM)

To examine whether the level of maternal care affects the offspring anxiety in adult life, adult ephrin-A5$^{-/-}$ mice that were born and raised by ephrin-A5$^{-/-}$ dams and wild-type mice that were born and raised by wild-type females were examined on the elevated plus maze (EPM) (Figure 3-7). The EPM test is one of the most commonly used assays to study the psychological and neurochemical basis of anxiety behavior in rodents (Bourin, Petit-Demouliere et al. 2007). It takes advantage of the normal preference of mice for a dark and protected space (the closed arm) over an open and exposed area (the open arm). Student's t-test was used to analyze the absolute number and the percentage of open arm entries, as well as the time and percentage of time spent in the open arm and the total number of

entries (into both open and closed arm). Ephrin-A5$^{-/-}$ mice showed anxiolytic-like behaviors in the EPM test. There were genotypic differences where ephrin-A5$^{-/-}$ mice had a higher number of open arm entries (t=3.807, p=0.003) (Figure 3-7A), and percent of open arm entries (t=4.068, p=0.002) (Figure 3-7B), as well as increased time spent in the open arm (t=4.829, p=0.0005) (Figure 3-7C) and percent of time spent in the open arm (t=4.619, p=0.007) (Figure 3-7D) compared to wild-type mice. In addition, null mice had an increased number of total entries (t=4.169, p=0.002) (Figure 3-7E) into both arms.

In order to examine whether the differences in anxiety-like behavior are due to differences in maternal care, we repeated the above study using male and female ephrin-A5$^{-/-}$, heterozygous and wild-type littermate mice that were born and reared by the same heterozygous mother (Figure 3-8). There were an overall genotypic differences where ephrin-A5$^{-/-}$ mice had a higher number of open arm entries [$F(2,56)=21.550$, $p<0.0001$] (Figure 3-8A), and percentage of open arm entries [$F(2,56)=12.657$, $p<0.0001$] (Figure 3-8B) as well as increased time spent in the open arm [$F(2,56)=9.365$, $p=0.0003$] (Figure 3-8C) and percentage of time spent in the open arm [$F(2,56)=7.330$, $p=0.002$] (Figure 3-8D) compared to heterozygous and wild-type littermates. In addition, null mice had an increased number of total entries [$F(2,56)=23.056$, $p<0.001$] (Figure 3-8E) into both arms. There were no significant effect of sex or genotype x sex interaction in the absolute number and the percent of open arm entries as well as in the time and percent of time spent in the open arm. However a genotype x sex interaction was found in the total entries [$F(2,56)=4.446$, $p=0.020$]. *Post hoc* tests revealed that ephrin-A5$^{-/-}$ male mice had increased total entries compared to ephrin-A5$^{-/-}$ female mice ($p=0.030$).

These data suggest that both male and female null mice are less anxious as revealed by increased entries and time spent in the open arm, which is usually avoided by rodents. In addition, the increase in total entries that was observed in the null mice supports our previous results that showed increase locomotor activity in the null mice (Chapter 2, Figure 2-7). Finally, since decreased anxiety was observed regardless of maternal behavior, we conclude that this change in behavior is the result of genetic factors and not exposure to early life stressors.

3.3.7 B. Light-dark box test

The light-dark box test was used to examine whether the anxiolytic-like behaviors of ephrin-A5$^{-/-}$ mice were specific to the EPM. The test based on the mice tendency to avoid the light unprotected area. Both male and female ephrin-A5$^{-/-}$ mice showed anxiolytic-like behavior in the tests (Figure 3-9). A multivariate ANOVA test showed a significant genotypic differences where ephrin-A5$^{-/-}$ mice spent significantly more time in the light chamber of the box [$F(2,53)=11.361$, $p<0.0001$] (Figure 3-9A), and had an increased number of head pokes [$F(2,53)=8.367$, $p=0.0007$] (Figure 3-9B), compared to wild-type and heterozygous mice. In addition, there was a sex differences were male mice had increased head poke compared to female mice [$F(1,53)=8.367$, $p=0.0014$]. There were no statistical differences between the genotypes in the latencies to initially enter the light or the number of transitions between the two compartments (Figure 3-9C and D, respectively). However, there was a significant effect of sex [$F(1,53)=30.613$, $p<0.0001$] where female mice had a consistently lower number of transitions between the two compartments across all genotypes. In addition, a genotype x sex interaction was found [$F(2,53)=3.809$, $p=0.030$], where *post hoc* test revealed that ephrin-A5$^{-/-}$ null male initially

enter the light faster than ephrin-A5$^{-/-}$ null female ($p=0.014$). These results support our previous data from the EPM test and suggest that ephrin-A5 deletion decreases anxiety in mice.

3.3.7 C. Open –field test

In the open field test, the mouse was placed in an unfamiliar squared arena and was allowed to explore it for 30 minutes. Since rodents normally stay close to the walls in the periphery, a behavioral known as thigmotaxis, an increase in time spent as well as increased activity in the central zone of the field is often used as an indicator of lower anxiety and vice versa (Bourin, Petit-Demouliere et al. 2007). Since male and female mice behave similarly in the EPM and the light-dark box test (see above), we decided to test males only in the open field test. Ephrin-A5$^{-/-}$ heterozygous and wild-type male mice were introduced to the same area of the arena (top left corner) and were allow to explore for 30 minutes. The percentages of time as well as the distance in the center were analyzed in 6, 5 minute bins. There were no significant differences between the genotypes in either measurement (Figure 3-10).

3.3.8 Corticosterone levels are comparable between the genotypes

Activation of the hypothalamic-pituitary-adrenal (HPA) axis has been shown to have important role in anxiety. In response to stress, corticotrophin-releasing hormone (CRH) is released from the hypothalamus, which leads to the secretion of adrenocorticotrophic (ACTH) hormone form the pituitary into the blood. ACTH, in turn induces the release of glucocorticoid stress hormones from the adrenal (Miller and O'Callaghan 2002). In order to test whether the alteration in anxiety is due to changes in the activation of the HPA axis, we determined corticosterone (CORT) concentrations, the

major stress steroid produced in mice. Levels were measured under baseline conditions and immediately after exposure to a mild stressor (the elevated plus maze). Serum CORT concentration was not significantly different between the genotypes [$F(1,16)=0.963$, $p=0.34$] nor was there a significant effect of sex [$F(1,16)=0.121$, $p=0.73$]. However, CORT levels were overall significantly higher under stress conditions compared to baseline [$F(1,16)=22.379$, $p=0.0002$] (Figure 3-11).

3.3.9 Altered AVP levels in the brain of lactating ephrin-A5$^{-/-}$ mice

As described earlier, AVP has been shown to play a role in maternal behavior. To examine the levels of AVP in the hypothalamus, the number of AVP-ir positive cells in the SCN and PVN were compared in lactating ephrin-A5$^{-/-}$ and wild-type control females. There were no significant differences in AVP-ir in the PVN. However, in the SCN, ephrin-A5$^{-/-}$ females had significantly more AVP-ir cells compared to wild-type control females (t=4.172, $p=0.014$) (Figure 3-12).

3.4 Discussion:

3.4.1 Nesting behavior in lactating mice

Here, we demonstrate that genetic deletion of ephrin-A5 significantly decreased maternal behavior. Nesting behavior was reduced in lactating ephrin-A5$^{-/-}$ females at 6 and 24 hours after providing the nesting material. Since mice are born without the ability to regulate their body temperature, the construction of the nest is important for their survival (Weber and Olsson 2008). It is therefore not surprising that pups born to ephrin-A5$^{-/-}$ females had a lower survival rate compared to those born to wild-type controls. Although maternal nest building behavior is important for the lifetime reproductive success of the

mouse (Bult and Lynch 1997) and primigravid mice tend to build the most complex quality nests (Bond, Neumann et al. 2002), non-pregnant, nulligravid mouse are also capable of building a nest (Sherwin 1997; Bond, Neumann et al. 2002). Here nesting is a spontaneous behavior (Deacon 2012). It provides shelter from predators and is essential for thermoregulation. Non-maternal nesting behavior has been shown to be effected by hippocampal damage and can be used to assess the mouse well-being (Jirkof 2014). In addition, it requires sensorimotor behaviors such as carrying, digging, sorting and pushing (Gaskill, Gordon et al. 2012). In order to examine whether the impairment in nesting seen in the primigravid ephrin-A5$^{-/-}$ mice are due to sensorimotor defects we analyzed nesting in nulligravid, virgin females. We found no differences in non-maternal nesting behavior between the genotype suggesting that the sensorimotor behaviors required for nest building are intact in the null mice. Since the difference in nesting was only observed between primigravid null and wild-type females, the defect is most likely due to responses to maternal factors such as changes in hormones during pregnancy and/or maternal motivation. Specifically, a state of high progesterone and low estrogen have been suggested to control maternal nest-building in primigravid mice (Lisk, Pretlow et al. 1969). Interestingly, in collaboration with Bonnie Deroo from the University of Western Ontario, it has been shown that progesterone levels in the ephrin-A5$^{-/-}$ mice are lower, suggesting that it may play a role in decreased maternal care in these mice.

3.4.2 Maternal aggression and care

In the pup retrieval test more than 50 percent of the lactating ephrin-A5$^{-/-}$ females failed to retrieve their pups back to the nest, and those that did retrieve took longer than wild-type and heterozygous littermates. It has been shown that the latency of pup retrieval

decreases in virgin females with repeated exposure to pups (Stolzenberg and Rissman 2011), as well as across the first week of postpartum in primiparous mice (Feierstein, Lazarini et al. 2010). In order to test whether maternal responsiveness to pups in the form of pup retrieval improves in ephrin-A5$^{-/-}$ females across the first postpartum week, pup retrieval was measured twice (4 and 6 days postpartum). We did not detect differences in retrieval latency across the days tested in any genotype, suggesting that repeated exposure to pups did not induce retrieval behavior in the null mice and was not sufficient to improve the retrieval deficit.

Differences with respect to maternal aggression were not observed between the genotypes, but since it has been shown that different neuronal circuits influence separate maternal behaviors (Gammie 2005), it is possible that ephrin-A5 deletion affects circuits that control pup retrieval and nest building but not those that regulate maternal aggression. Consistent with this notion, lesion studies have shown that defects in maternal aggression do not affect pup retrieval in rats (Hansen 1989; Factor, Mayer et al. 1993).

Nursing is considered a major aspect of maternal care. During the first postpartum weeks, the dam spend almost 50 percent of the time nursing the pups (Champagne, Curley et al. 2007). Thus for the first two weeks of life, pups are fully dependent on nursing for development (at P17 the pups start to eat solid food and wean from nursing) (Weber and Olsson 2008). Previously we reported that ephrin-A5$^{-/-}$ mice have decreased body weight compared to wild-type control (Sheleg, Yochum et al. 2013). Here, we confirmed these results and showed that the differences in body weight were not due to differences in maternal care since heterozygous pups that were born and raised by ephrin-A5$^{-/-}$ females

had similar body weight to those that were born and raised by heterozygous and wild-type control females.

3.4.3 Olfaction and maternal care

Normal olfaction is essential for the onset of maternal behavior in mice (Levy and Keller 2009). Chemosensory signals emanating from the pups are important for pup recognition by the mother and female mice with deficiency in olfaction activity such as the adenylyl cyclase 3 (AC3) deficient mice and the cyclic nucleotide gated channel alpha 2 (Cnga2) deficient mice are impaired in several maternal behaviors, including pup retrieval and maternal aggression (Wang and Storm 2011; Fraser and Shah 2014). Finally, it has been shown that pregnancy in mice stimulates the production of new interneurons in the olfactory bulb (Shingo, Gregg et al. 2003). The fact that ephrin-A5$^{-/-}$ females were able to block pregnancy when presented with an unfamiliar male and maintained it in the presence of the familiar male, suggests that they were able to smell and form olfactory memory for pheromone cues from male mice. These data imply that some olfaction, when tested under the above conditions, is normal in the null mice. However, further studies involving olfactory cues, specifically from pups, are needed to determine whether the neuronal pathways for pup olfaction is normal.

3.4.4 Anxiety

Ephrin-A5$^{-/-}$ mice had lower levels of anxiety-like behavior as revealed by increased entrance and time spent in the open arm of the EPM, as well as increased time in the light compartment of the light-dark box and decreased number of head pokes. Although it has been suggested that the quality and quantity of early life care influences anxiety (Meaney 2001), we did not find correlation between the two. Decreased anxiety in the EPM

test was observed in the null mice regardless of the levels or quality of maternal care. Additionally, in response to mild stress (exposure to the EPM) both ephrin-A5$^{-/-}$ and wild-type controls showed similar activation of the HPA axis; blood CORT concentration was increased under stressful condition in both genotype with no significant differences between the genotypes.

3.4.5 Molecular mechanism of maternal care

The interaction between the medial preoptic area (MPOA) and the mesolimbic system plays a pivotal role in regulating maternal behavior in rats (Numan and Stolzenberg 2009). Numan *et al*. (Numan 2014) proposed a model in which MPOA projections to the VTA cause the release of DA into the nucleus accumbens (NA). This release stops the inhibitory effect of the NA on the ventral pallidum (VP) which becomes responsive to the stimulation from the pups. Since the mesolimbic DA system is involved in motivated behavior, it is suggested that the MPOA-VTA connection governs the appetitive aspect of maternal responses to pup stimuli which includes retrieval behavior (Stolzenberg and Numan 2011; Numan 2014). Previously, we have shown that ephrin-A5 and one of its receptors, EphA5, are expressed in the mesolimbic DA system and are required for the proper formation and maintenance of this dopaminergic pathway (Cooper, Kobayashi et al. 2009). Both *In vivo* and *in vitro* data suggested that ephrin-A5 has an adhesive effect on EphA5-expressing dopaminergic neurons from the midbrain, and its absence led to decrease in neuronal targeting to the striatum. Thus, it is possible that the decreased maternal behavior seen in the null mice is due to alteration in the mesolimbic DA system.

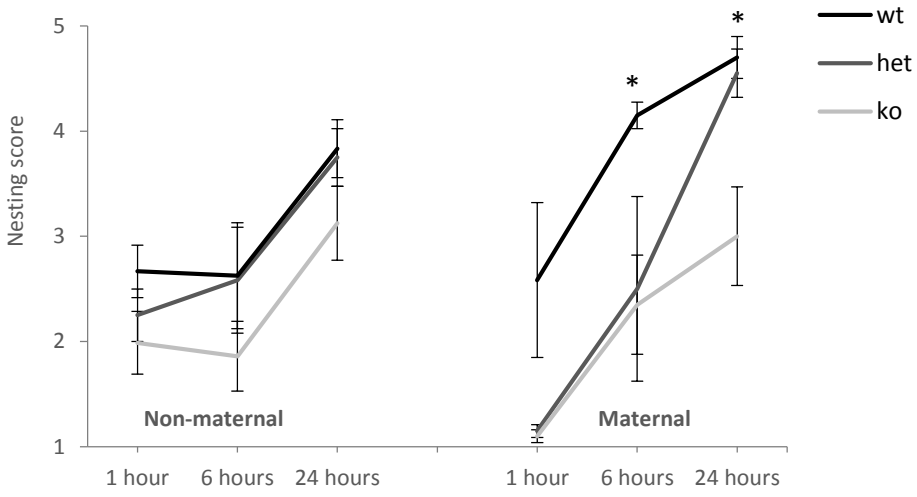
Finally, increased AVP immunoreactivity (AVP-ir) in the SCN of lactating ephrin-A5$^{-/-}$ females compared to lactating wild-type controls was found. We hypothesize that this

increase is not due to differences in the number of AVP producing cells but rather differences in AVP release pattern. Supporting this is the fact that a similar staining pattern was observed in ephrin-A5$^{-/-}$ male mice (chapter 4 Figure 4-9). Further, when neuronal release was inhibited in the wild-type control by colchicine injection, the number of AVP-ir did not differ between the genotype (chapter 4 Figure 4-9).

AVP plays a role in maternal behavior, i.c.v injection of AVP to estrogen primed, ovariectomized virgin female rats induced the onset of maternal behavior (Pedersen, Ascher et al. 1982), whereas anti-AVP antiserum injection delayed it (Pedersen, Caldwell et al. 1985). In addition, mice (Kessler, Bosch et al. 2011) and rats (Bosch and Neumann 2008) that were selectively bred for high anxiety-like behavior (HAB) had elevated hypothalamic AVP activity and were intrinsically more maternal than those with low AVP activity. Finally, higher density of AVP-V1a-receptor is correlated with increased pup licking and grooming in postpartum mice (Curley, Jensen et al. 2012), and manipulation of V1a-receptor expression within the MPOA resulted in alteration of maternal behavior (reviewed in (Bosch and Neumann 2012)). Given the above data, the fact that AVP innervation from the SCN is found in the MPOA (Rood, Stott et al. 2013), and the presence of AVP-V1a receptor mRNA in the SCN (Li, Burton et al. 2009), it is possible that ephrin-A5 deletion caused reduced AVP projection from the SCN to the MPOA, or AVP release in the MPOA, which altered AVP activity in the MPOA, and in turn decreased maternal behavior.

Figure 3-1: Deficits of nesting behavior in primigravid (pregnant) ephrin-A5$^{-/-}$ mice
(A) Nest building was assessed in ephrin-A5$^{-/-}$, heterozygous and wild-type pregnant (n=5 per genotype) and non-pregnant females (n=6 per genotype). Nest rating was lower in pregnant mutant mice compared to wild-type controls starting 6 hours after providing the nesting material (A, right panel). There were no significant differences in nest rating between the non-pregnant females (A, left panel). Data are presented as mean nest score ±SEM.
(B) Representative pictures of nests build by pregnant wild-type and ephrin-A5$^{-/-}$ mice 24 hours after providing the nesting material.
* indicates significantly different from wild-type mice; $p<0.05$.

A.



B.

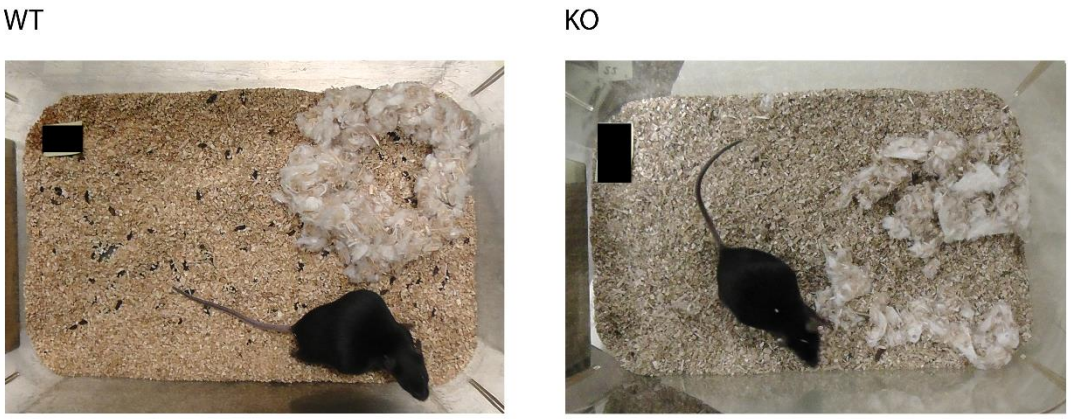


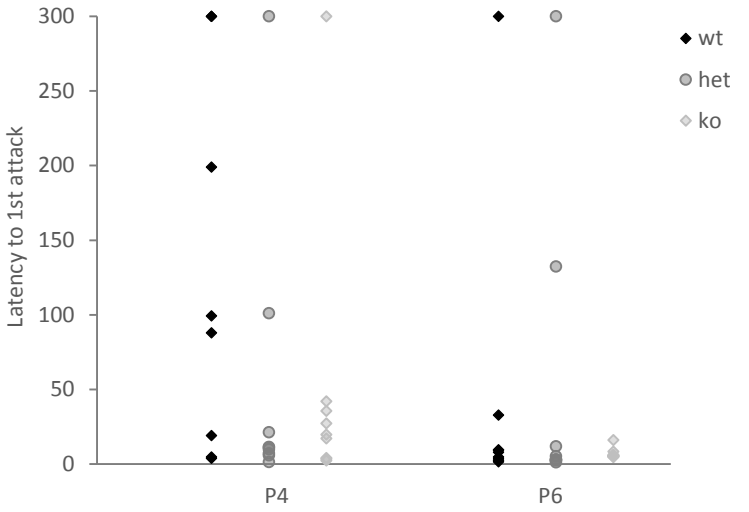
Figure 3-2: No changes in maternal aggression between ephrin-A5$^{-/-}$ and wild-type mice

(A) The latencies to first attack by the dam on the male intruder were not significantly different between the genotypes. Data are presented as individual latencies to first attack.

(B) The cumulative number of attacks made by the dam during the 5 minute aggression test were not significantly different between the genotypes.

Data are presented as mean number of fights +SEM.

A.



B.

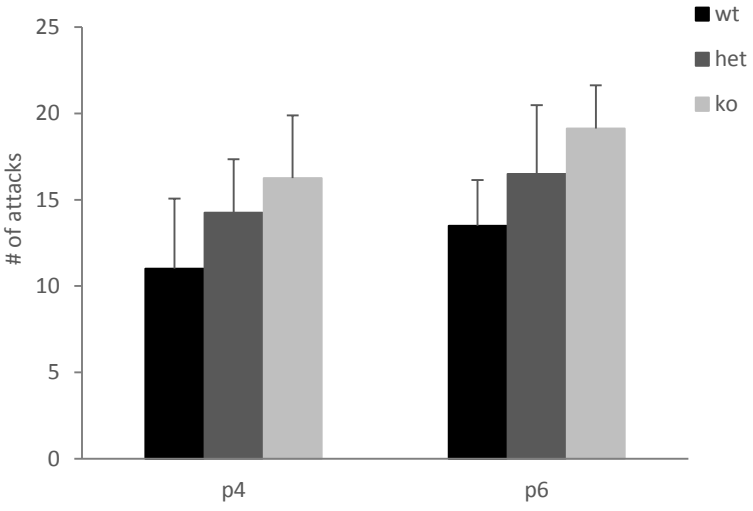


Figure 3-3: Pup retrieval is impaired in lactating ephrin-A5$^{-/-}$ mice

(A, B) The latencies to retrieve the 1st and 3rd pup on postnatal day 4 (A) and 6 (B) were tested in ephrin-A5$^{-/-}$ (n=9 per test day), heterozygous (n=10) and wild-type (n=8) lactating female mice. There were significant differences between the genotypes on both test days where the mutant females took longer to retrieve their pups than both the wild-type and the heterozygous mice. Data are represented as mean latency to retrieve the x pup +SEM.

(C) The percentage of females that retrieve their pups were significantly lower in ephrin-A5$^{-/-}$ mice compared to wild-type and heterozygous control females.

(D) No differences were observed across the days tested. Data are presented as mean latency to retrieve the 1st pup ±SEM.

* indicates significantly different from wild-type mice; $p<0.05$.

** indicates significantly different from heterozygous mice; $p<0.05$.

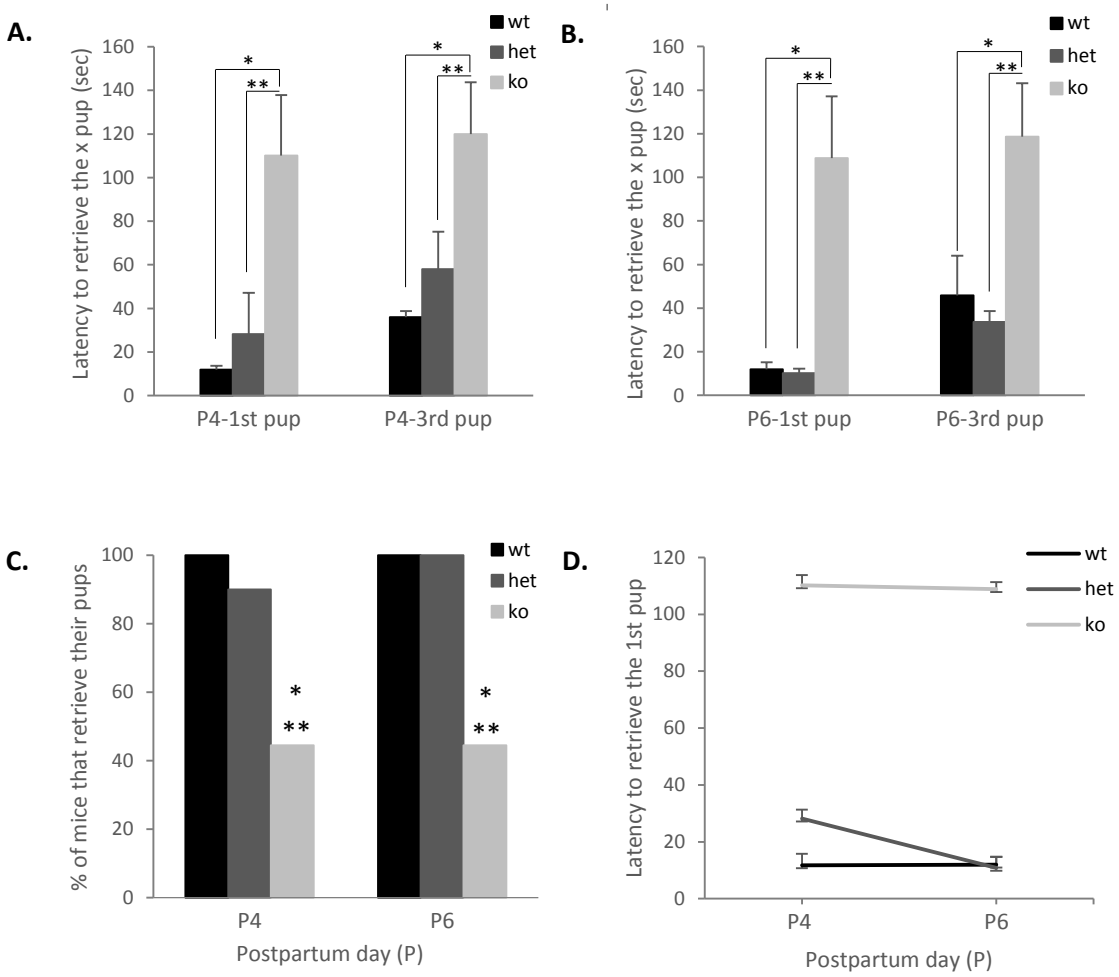
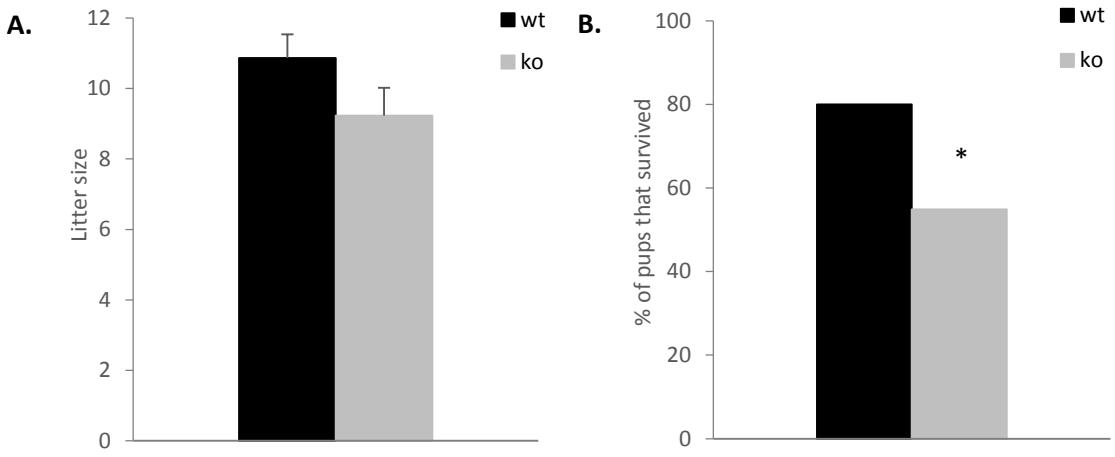


Figure 3-4: Decreased survival rate of pups that were born and reared by ephrin-A5$^{-/-}$ dams

(A) Litter size was similar for ephrin-A5$^{-/-}$ (n=13) and wild-type (n=15) dams. Data are presented as mean litter size +SEM.

(B) Pup survival rate was lower for pups that were born and reared by ephrin-A5$^{-/-}$ mothers. Data are presented as percentage of pups that survived per genotype.

(C) Representative pictures of ephrin-A5$^{-/-}$ and wild-type dams within the first few hours after birth.



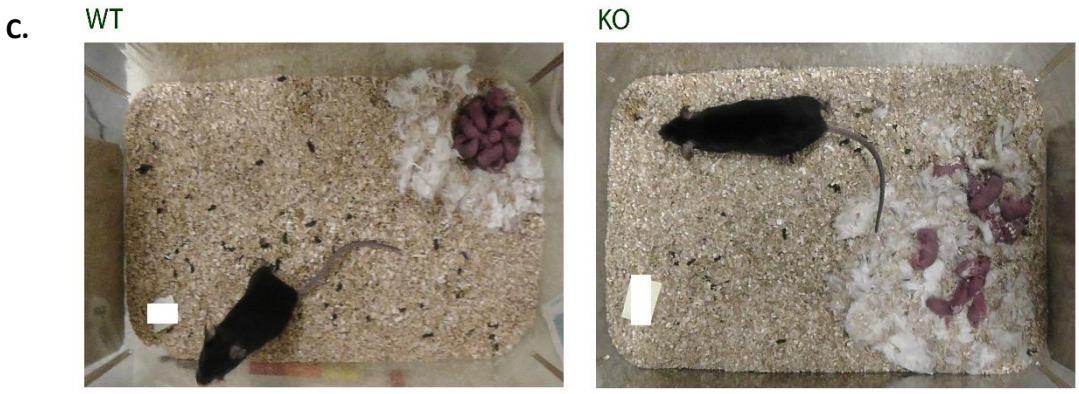


Figure 3-5: Changes in body weights between genotypes were not due to differences in maternal behavior
(A) Breeding scheme.
(B) Body weights of heterozygous pups that were born and reared by ephrin-A5$^{-/-}$, heterozygous and wild-type dams were measured. There were no significant differences between the groups.
(C) Ephrin-A5$^{-/-}$ mice had decreased body weight compared to wild-type controls.

A.

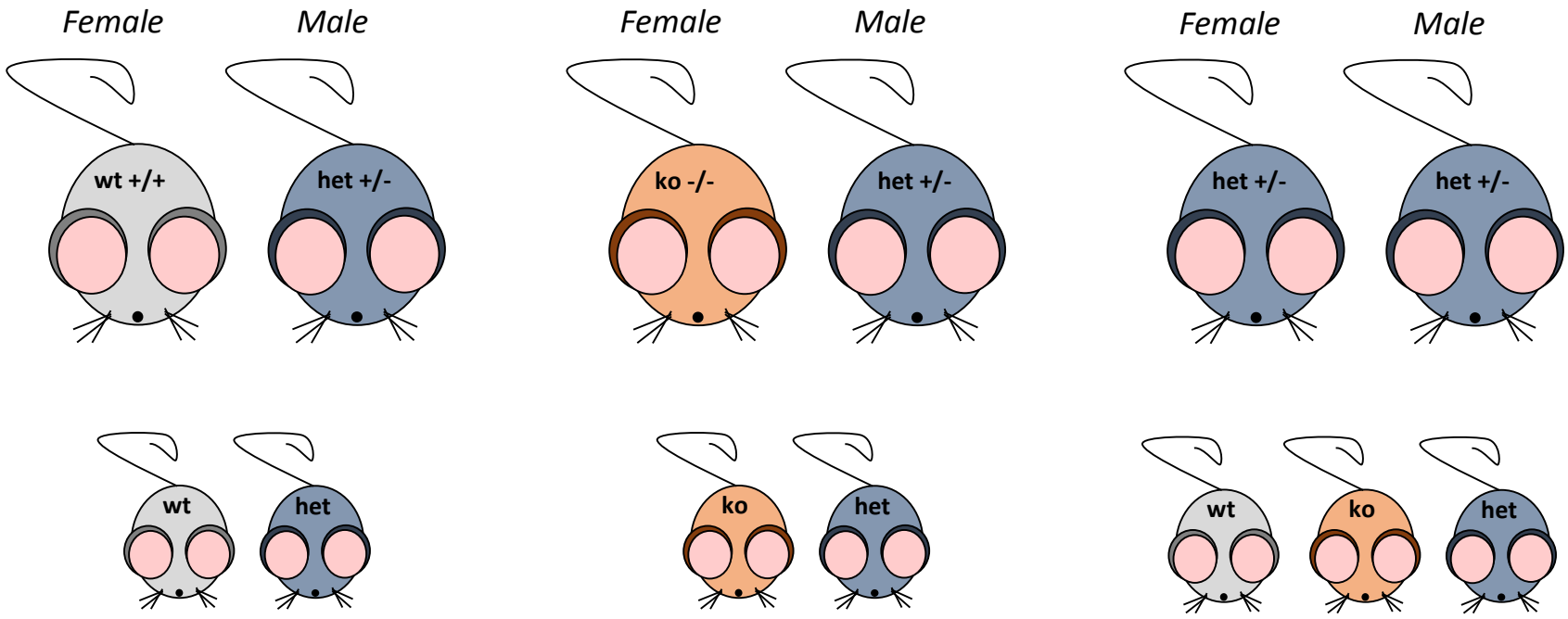
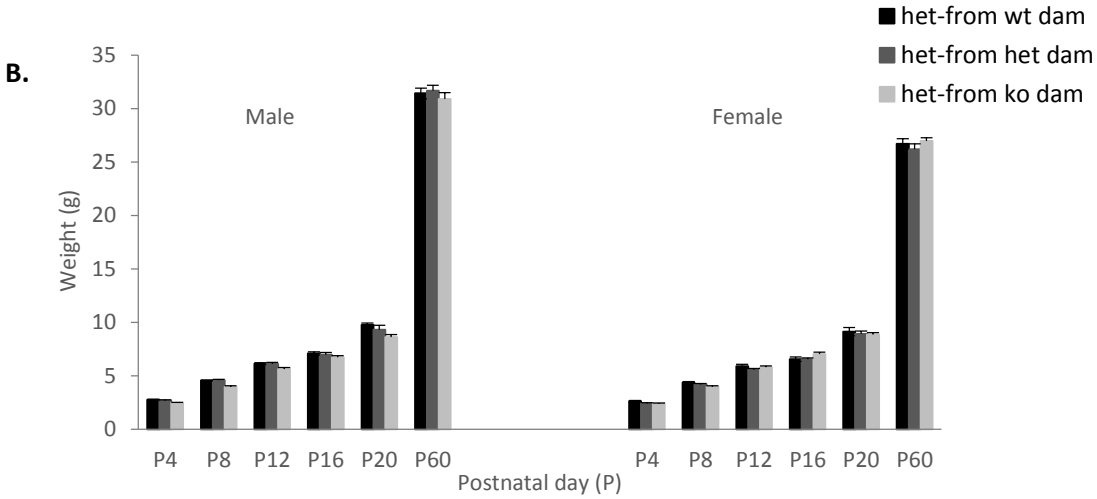


Figure 3-5: Changes in body weights between the genotyped were not due to differences in maternal behavior

(A) Breeding scheme.
(B) Body weights of heterozygous pups that were born and reared by ephrin-A5$^{-/-}$, heterozygous and wild-type dams were measured. There were no significant differences between the groups.
(C) Ephrin-A5$^{-/-}$ mice had decreased body weight compared to wild-type controls.



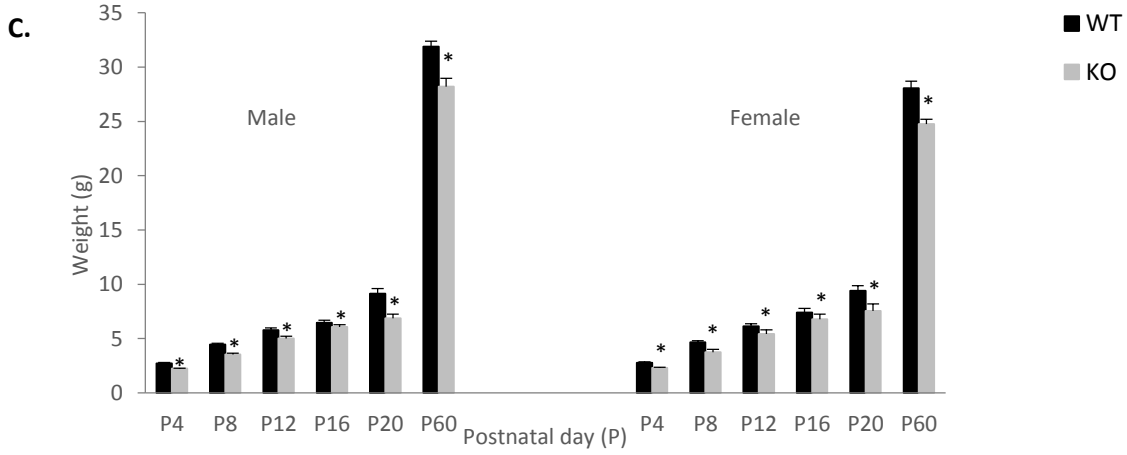


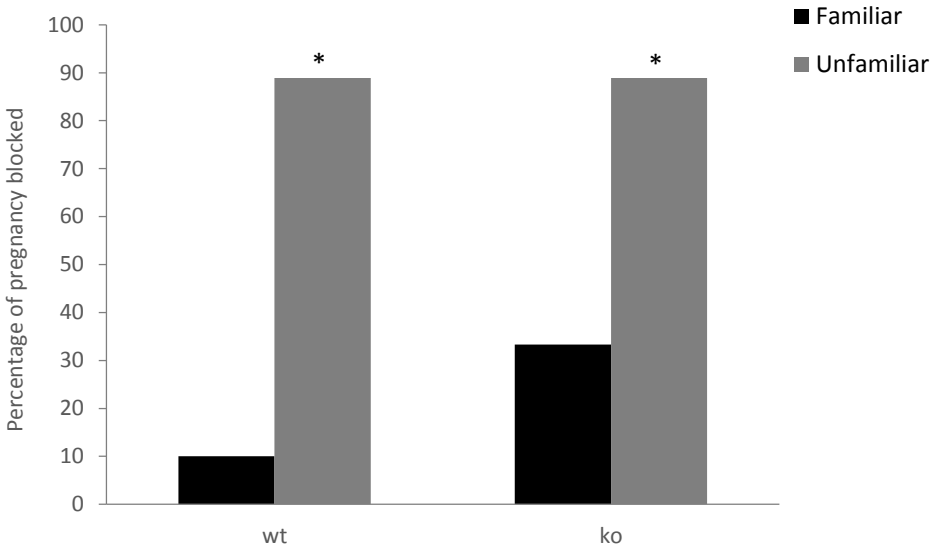
Figure 3-6: Ephrin-A5 deletion does not affect male-induced pregnancy block (the Bruce effect) in mice

(A) The percentage of pregnancy blocks in response to familiar or unfamiliar males were assessed in ephrin-A5$^{-/-}$ and wild-type females (n=18 per genotype). Both genotype exhibited pregnancy block in response to the unfamiliar male and remained pregnant after re-exposure to their mate (familiar).

(B) The mean number of days until sperm plug was observed as well as the gestation period was not affected by ephrin-A5 deletion.

*indicates significantly different from the percentage of pregnancy block in response to familiar male; $p<0.05$.

A.



B.

Genotype	Treatment	Days until plug observed (mean±SEM)	Gestation period (mean±SEM)
WT	Familiar	3.20±0.73	19.57±0.23
WT	Unfamiliar	3.25±0.59	na
KO	Familiar	4.22±0.86	19.79±0.42
KO	Unfamiliar	3.89±0.65	na

Figure 3-7: Decreased anxiety-like behavior of ephrin-A5-/- mice that were born and reared by null mice in the elevated plus maze (EPM)

Anxiety behavior was measured on the EPM in male ephrin-A5$^{-/-}$ (n=7) and wild-type (n=6) mice. (A, B, C, D) Number and percentage of open arm entries (A and B) as well as time and percentage of time (C and D) spent in the open arm were higher in null mice compared to wild-type controls. (E) Total number of entries into both open and closed arm was significantly higher in ephrin-A5$^{-/-}$ mice. Data are presented as mean +SEM.

* indicates significantly different from wild-type mice; $p<0.05$.

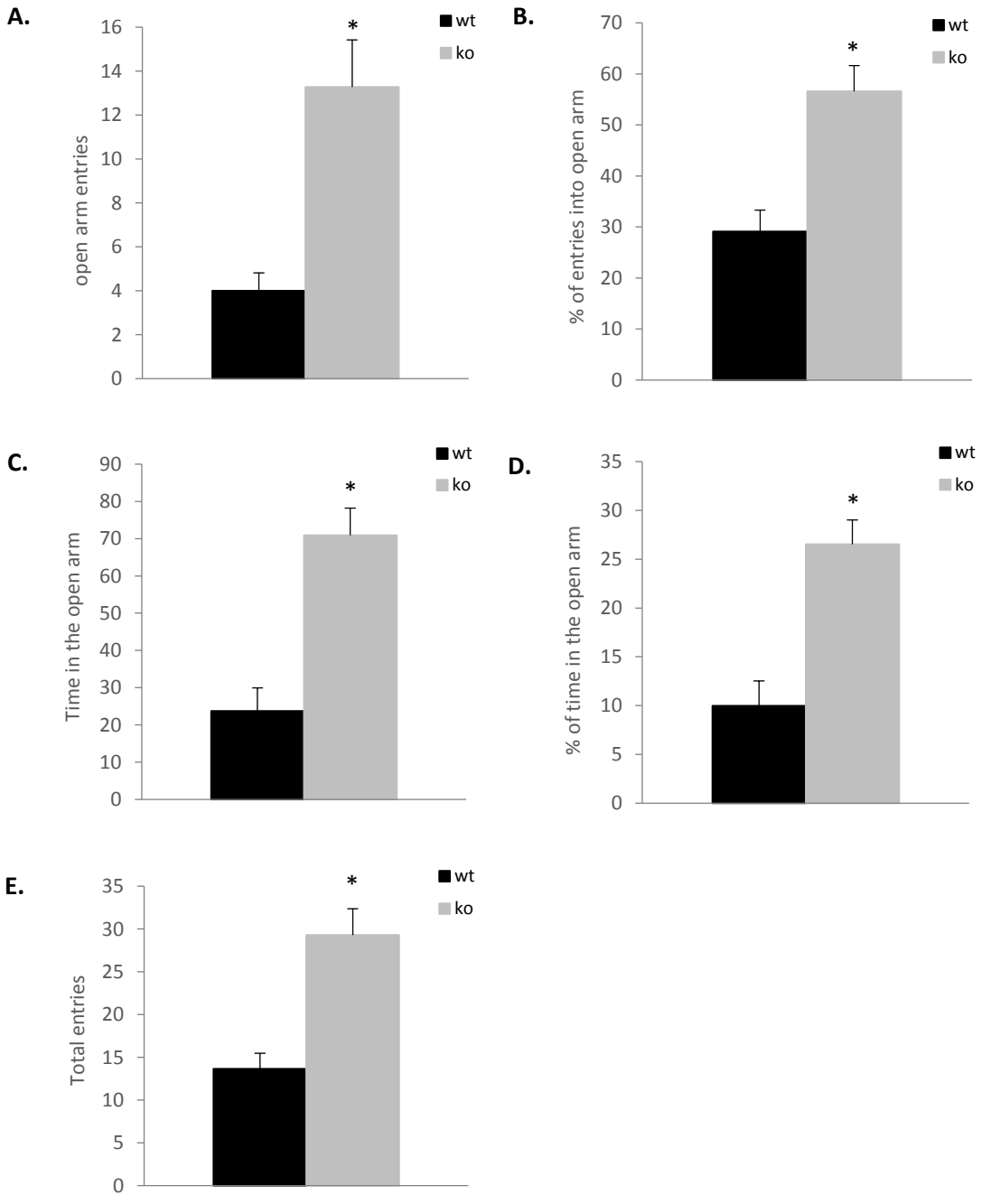


Figure 3-8: Decreased anxiety-like behavior of ephrin-A5-/- mice in the elevated plus maze

Anxiety-like behavior was measured on the elevated plus maze in male and female ephrin-A5$^{-/-}$ (n=10 per sex), heterozygous (n=11 per sex) and wild-type (n=10 per sex) mice.

(A, B, C, D) Number and percentage of open arm entries (A and B respectively) as well as time and percentage of time spent in the open arm (C and D respectively) were higher in both male and female null mice compared to heterozygous and wild-type controls.

(E) Total number of entries into both open and closed arm was significantly higher in male and female ephrin-A5$^{-/-}$ mice.

Data are presented as mean +SEM.

* indicates significantly different from wild-type mice; $p<0.05$.

** indicates significantly different from heterozygous mice; $p<0.05$.

Figure 3-8: Decreased anxiety-like behavior of ephrin-A5$^{-/-}$ mice in the elevated plus maze

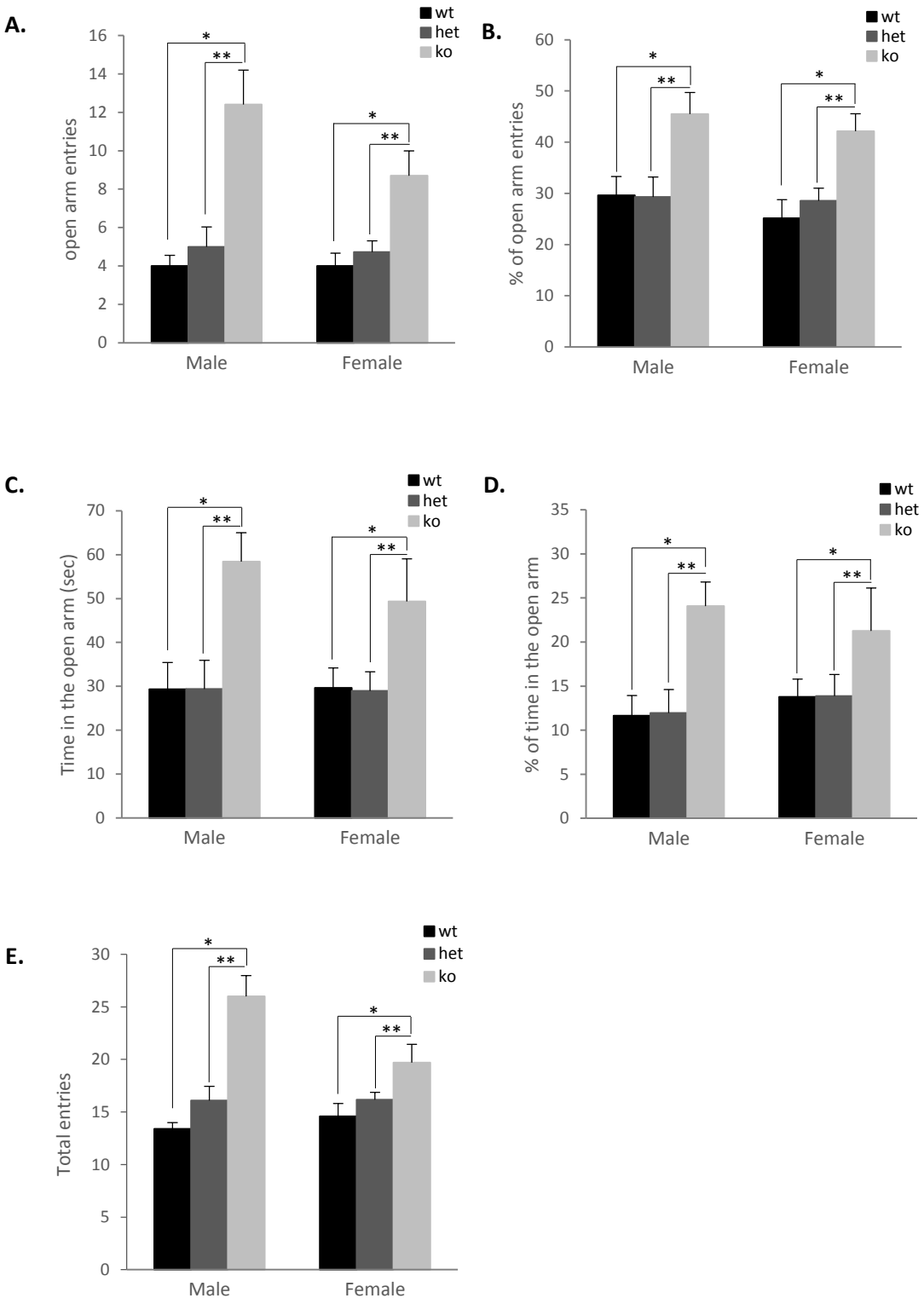


Figure 3-9: Decreased anxiety-like behavior of ephrin-A5$^{-/-}$ mice in the light-dark box
Anxiety-like behavior was measured in male and female ephrin-A5$^{-/-}$ (n=10 per sex), heterozygous (n=10 per sex) and wild-type (n=9 male and 10 female) mice in the light-dark box.
(A, B) Ephrin-A5$^{-/-}$ mice spend more time in the light compartment (A) and had increased number of head pokes (B) compared to heterozygous and wild-type controls.
(C, D) There were no genotypic differences in the latency to the first exit (C) and the number of transition between the two compartments (D).
Data are presented as mean +SEM.
* indicates significantly different from wild-type mice; $p<0.05$.
** indicates significantly different from heterozygous mice; $p<0.05$.
*** indicates significant sex differences; $p<0.05$.

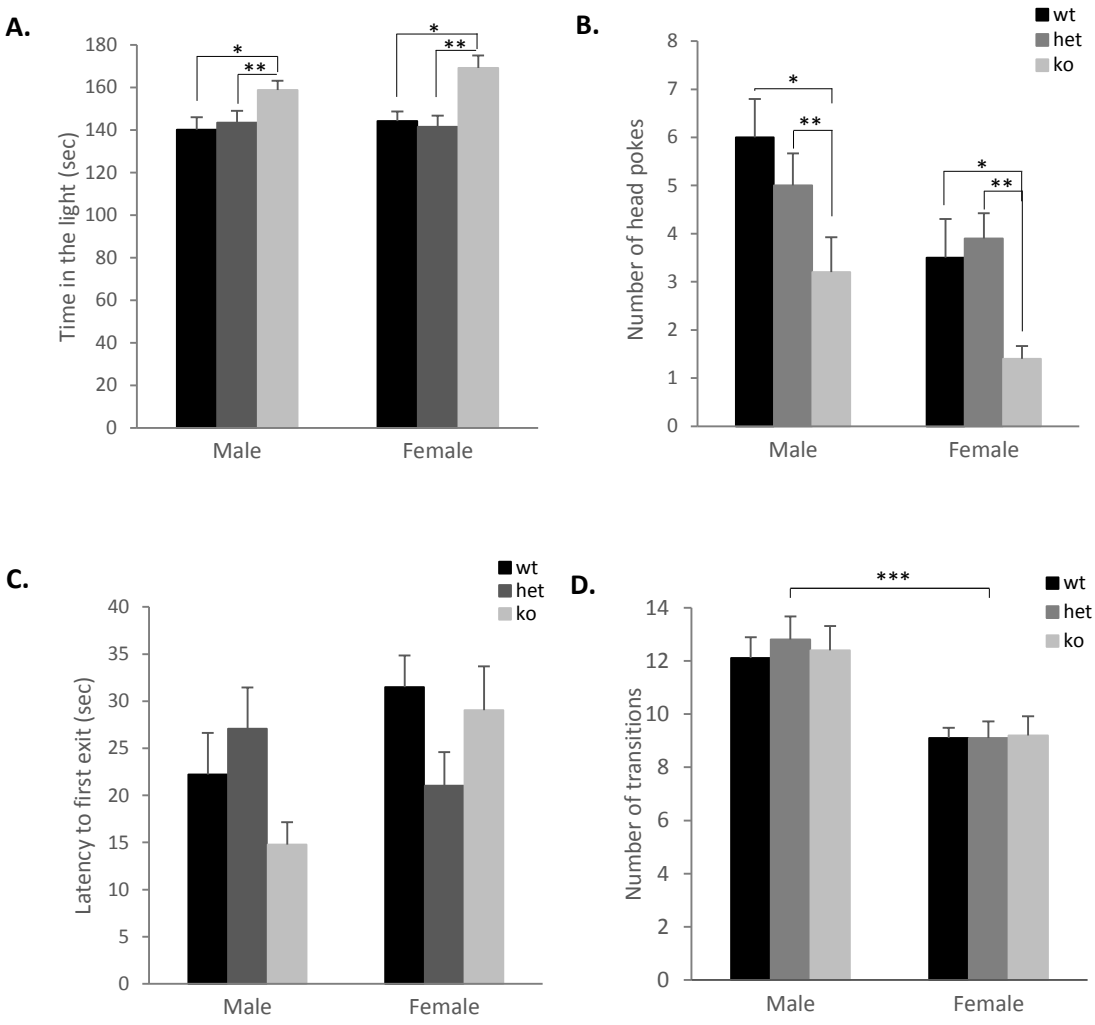
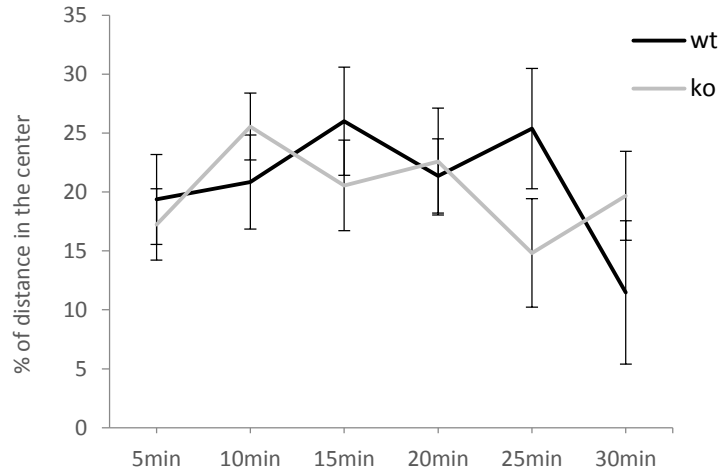


Figure 3-10: No differences in anxiety-like behavior in the open field test
Anxiety-like behavior was measured in male ephrin-A5$^{-/-}$ (n=10) and wild-type (n=9) male mice in the open field test.
(A, B) No significant differences were found in the % of distance (A) and the time spent (B) in the center of the box.

A.



B.

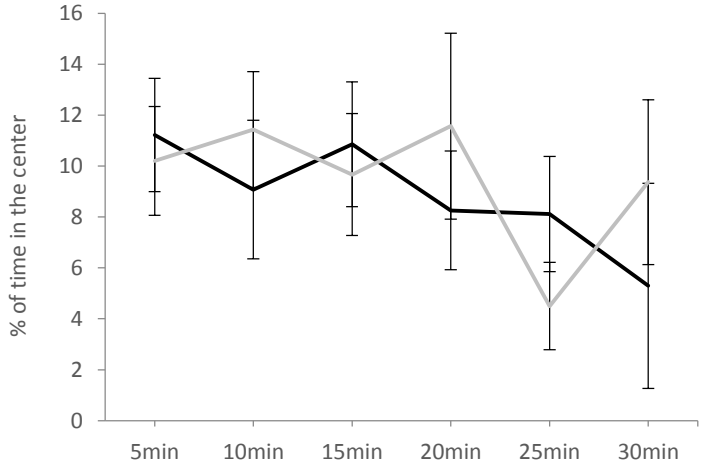


Figure 3-11: No differences in corticosterone levels between ephrin-A5$^{-/-}$ and wild-type mice

Corticosterone (CORT) levels were measured in male and female ephrin-A5$^{-/-}$ and wild-type mice (n=5 per sex and genotype) under baseline and mild stress (the elevated plus) conditions. There were no significantly differences between the genotypes. However, CORT levels were significantly higher under stress conditions compared to baseline.
Data are presented as mean CORT levels (ng/ml) +SEM.
* indicates significantly different from baseline conditions; $p<0.05$.

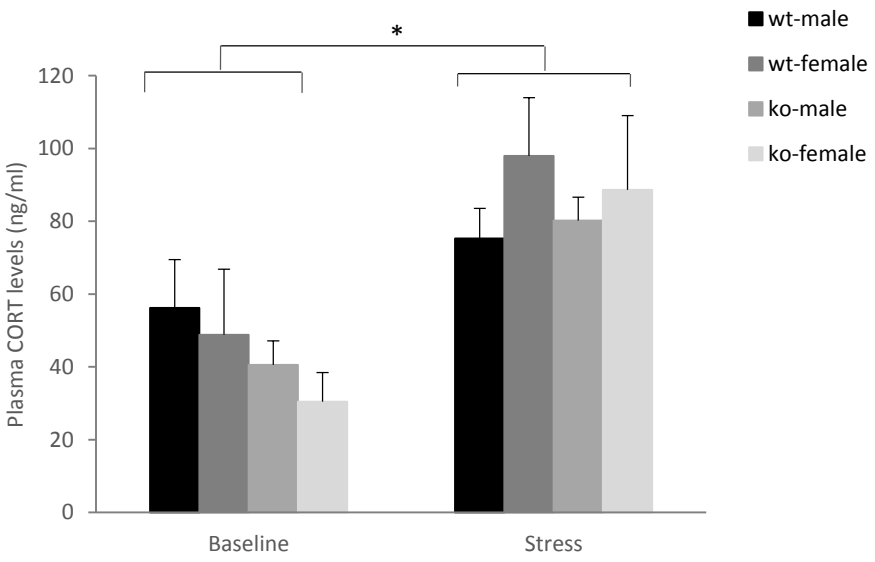
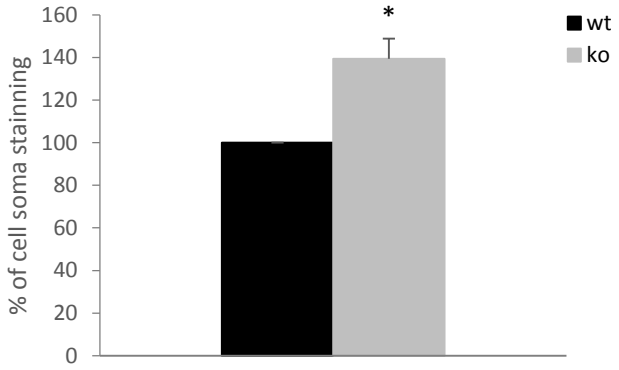
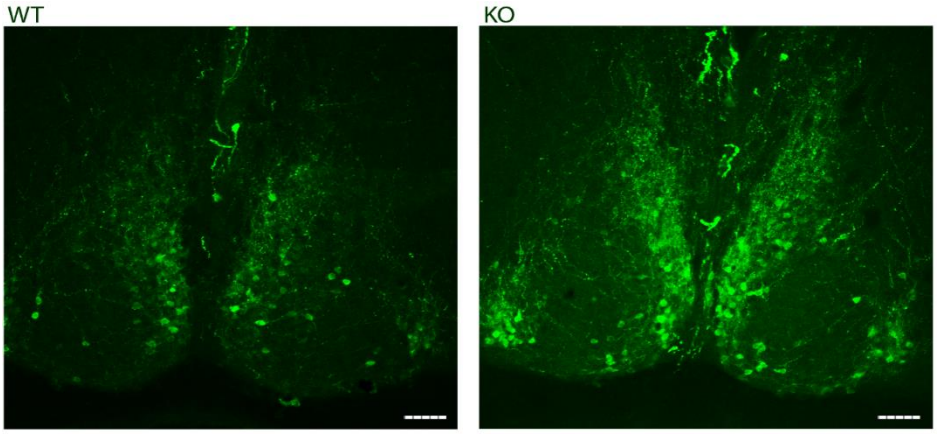


Figure 3-12: Increased AVP-ir in the cell soma of lactating ephrin-A5$^{-/-}$ females
AVP-ir in the SCN is higher in lactating ephrin-A5$^{-/-}$ females compared to wild-type controls.
(A) Quantification of AVP-ir in the SCN (n=3 per genotype). Data are presented as percent of cell soma staining +SEM.
(B) Representative immunohistochemistry images of the SCN of lactating wild-type (WT) and ephrin-A5$^{-/-}$ (KO) mice. Scale bar =500µm.
* indicates significantly different from wild-type controls; $p<0.05$.

A.



B.



CHAPTER 4: EPHRIN-A5 REGULATES INTER-MALE AGGRESSION IN MICE

4.1. Introduction:

Aggressive behavior is defined as behavior that occurs when a conflict between the interest of two individuals exist (Nelson and Trainor 2007; Numan 2014). Appropriate levels of aggression can be viewed as a universal fitness trait which enables survival, whereas exaggerated levels can physically harm or even cause death (Anholt and Mackay 2012). Animal studies classified male aggression into two major categories: offensive and defensive aggression, which differ in their motive, site and intensity of attack, and outcomes (Siegel, Roeling et al. 1999; Blanchard, Wall et al. 2003). Offensive aggression is also known as inter-male aggression and occurs in response to challenge over resources (i.e., territory). It involves attack toward the back and flanks of the opponent, where wounds are less likely to result in death (Blanchard, Wall et al. 2003; Nelson 2006). Given the site of the attack, it is suggested that this behavior serves to develop social dominance as opposed to causing serious damage (Siegel 2005; Nelson 2006). Indeed, its successful outcomes are termination of the challenge and the gain of possession over resources. Finally, it has been proposed that the motives and outcomes of offensive aggression in rodents may correspond to those in human anger-aggression (Blanchard and Blanchard 2003).

Under natural conditions, both mice and rats live in reproductive groups, and offensive aggression against intruders is used to gain dominant status and access to sexually active females (Numan 2014). In laboratory research, the resident-intruder (RI) model is the most commonly used test to study this kind of aggression (Nelson and Chiavegatto 2000). The test involves the placement of a non-aggressive group-housed male mouse (the

intruder) in the home cage of another mouse, the resident, which has been single housed for several weeks (Nelson and Chiavegatto 2000). This isolation will enhance the establishment of a territory by the resident and as a consequence of territoriality, the resident will attack the intruder (Koolhaas, Coppens et al. 2013). The level of aggression is then measured in terms of latency to first attack and number of attacks made by the resident on the intruder.

Defensive aggression, also known as fear-induced aggression (Nelson and Chiavegatto 2000) occurs in the presence of a stimuli that is considered dangerous to the animal. Here, the animal will first try to avoid the threat and will attack only if escape is not possible. This type of aggression is characterized by submissive posture and attacks directed toward the nearest offending body parts, which are usually the head and snout (Siegel 2005). The successful outcome of this behavior is the discontinuation of the threat and its attack. The target biting test has been used to measure this type of aggressive behavior in rodents (Miyakawa, Yagi et al. 2001; Johnson, Carlson et al. 2003). In this test, the animal is confined to an open-ended tube and an electric shock is delivered to its tail. Since the animal cannot escape, it will bite an inanimate target that is placed near its snout. The number of target biting reflects the level of aggressiveness.

Different brain regions and signaling molecules have been linked to aggression, specifically, the hypothalamus, medial amygdala (MEA), lateral septum (LAS), periaqueductal grey (PAG) and the bed nucleus of the stria terminalis (BNST) (Siegel, Roeling et al. 1999; Nelson and Trainor 2007; Numan 2014). Studies in rats identified a broadly distributed “hypothalamic attack area” (HAA) from which electrical and pharmacological stimulation elicited attacks, and lesions reduced it (Kruk, van der Poel et

al. 1979; Siegel, Roeling et al. 1999). The HAA includes the lateral part of the anterior hypothalamus (AH), the ventromedial nucleus of the hypothalamus (VMN) and the ventral part of the lateral hypothalamus (Numan 2014). It has been suggested that under normal conditions, this area controls whether agonist behavior is appropriate or not, but when stimulated the animal will attack even when not suitable (Kruk 1991). For example, in the laboratory, a resident male will attack a male intruder but not a receptive or unreceptive female, however, when the HAA is stimulated the male will attack the female as well (Numan 2014). Recently, Lin *et al*. (Lin, Boyle et al. 2011) have identified an aggression locus in the mouse ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) that corresponds to the HAA of the rat.

Finally, the activation of specific molecular signals such as hormones and neurotransmitters has also been implicated in the manifestation of aggression, specifically, testosterone, serotonin (5-HT) and arginine vasopressin (AVP) (reviewed in (Nelson and Trainor 2007; Anholt and Mackay 2012)).

In this study, we analyzed offensive and defensive aggression in ephrin-A5$^{-/-}$ and wild-type male mice. In addition, given the essential role of the olfactory system in aggression, we sought to determine whether loss of ephrin-A5 affects olfactory function and in turn, affects aggression. Finally, brain neurochemistry and AVP levels in the brain were measured using HPLC and immunohistochemistry respectively in order to determine their roles in the aggression.

4.2. Materials and Methods:

Resident-intruder aggression test: Adult (p>60 days) ephrin-A5$^{-/-}$ and wild-type (n=9 per genotype) male mice were used as the residents and were individually housed for two weeks. Since territoriality is strongly based on the presence of olfactory cues (Koolhaas, Coppens et al. 2013), bedding was not changed a week before testing. Each resident was than confronted in its home cage with a grouped housed (4-5 mice per cage) male intruder, that was age and genotype matched to the resident, for 10 minutes. During the test, the latency to the first attack as well as the number of attacks were recorded. For the resident intruder test using a zinc sulfate treated intruder, a new set of adult (p>60 days) ephrin-A5$^{-/-}$ and wild-type (n=10 per genotyped) male mice were used as the residents and a grouped housed (4-5 mice per cage) zinc-sulfate-treated wild-type male were used as the intruders. In addition, we measured the number of time the resident spent in non-aggressive exploratory face and anogenital sniffing of the intruder.

Zinc sulfate treatment: Intranasal instillation was described previously (Czarnecki, Moberly et al. 2011). Briefly, animals were lightly anesthetized with ketamine and an Eppendorf microloader (Eppendorf Hauppauge, NY) attached to a Hamilton syringe containing 0.15 ul of 5% zinc sulfate (Sigma, St. Louis, MO) was inserted 7 mm into one naris. The mouse was placed on its back for five minutes and then rotated to its side for another 20 min. The procedure was then repeated for the other naris.

Target biting defensive aggression test: Adult (p>60 days) ephrin-A5$^{-/-}$ (n=9) and wild-type (n=10) male mice were tested in the target biting test as described previously (Wagner, Nabert et al. 1983) Briefly, mice were confined in a plastic cylinder (2.8 cm inner diameter; 9.8 cm long) with their tails passed through a slot at the rear of the cylinder and taped to 2

brass bar electrodes. The cylinder was placed in a chamber with a biting target in front of the mouse. The test session lasted 20 minutes with 10 two minute trials. During the test the mice received a tone-conditioned stimulus (CS) for 15 seconds which terminated with the onset of a 2 mA, 0.15 sec tail shock. The number of times the mouse bit the target was collected in eight 15 sec bins over the two minutes trial and cumulated over the 20 minutes session. The number of times the animal bit the target was recorded per bin. The number of bites from bins 2-7 was averaged and the data was analyzed as target bites in 3 bins (bin1, bin2-7 and bin8).

Olfactory-guided foraging test: The test was performed as described previously (Ferguson, Young et al. 2000; Yang and Crawley 2009) with some modification. Briefly, individually housed ephrin-A5$^{-/-}$, and wild-type male mice (n=8 per genotype) were provided with flavored cereal in their home cage for 5 days. Food was then withheld and testing began 24 hours later. Each mouse was then transferred to a holding cage and a piece of flavored cereal was placed on the surface of the cage bedding. The latency to locate the cereal was recorded and the mouse was returned to his home cage. This procedure was then repeated three times with the cereal buried in different positions of the cage about 2 cm beneath the bedding.

Olfactory habituation-dishabituation test: The olfactory habituation-dishabituation test was adapted from (Ferguson, Young et al. 2000; Trinh and Storm 2003; Yang and Crawley 2009). Briefly, ephrin-A5$^{-/-}$ (n=9), and wild-type (n=10) mice were presented in their home cage with a cotton swab dipped in water. The animal was allowed to explore it for 2 minutes and the procedure was repeated 2 more times at 1 minute intervals. On the fourth trial, the cotton swab was laced with urine from male mice and the procedure was repeated for 2

more times for a total of 6 trials. During each 2 minute presentation the total number of investigatory sniffs (defined as nasal contact with the cotton swab) was recorded. Urine was collected from ten gonadally intact males by holding the mouse by the scruff of the neck over a funnel and applying pressure on the abdomen. Samples were pooled and stored at -80° C until use.

Male-female recognition test: Male ephrin-A5$^{-/-}$, and wild-type mice (n=9 per genotype) were tested for their preference to male verses female mice using a social chamber. The chamber was a 40 cm×40 cm×36.6 cm Plexiglas chamber with a stainless steel grid floor. On two opposite corners of the chamber there were two cylinders, 11 cm in diameter and 13 cm tall, made of the same stainless steel grid as the floor. An adult wild-type male mouse was placed in one of the cylinders and an adult wild-type female mouse in the second one. Each mouse was given a 15 minutes habituation period to explore the empty chamber before the start of the trial. The test begins when a mouse was placed in the center of the chamber and allowed to explore it for 15 minutes. Each time the subject placed one or both paws on a cylinder a contact was recorded. The number of contacts as well as the time spent near either the male or female-containing cylinder was recorded.

Real time RT-PCR: RNA was prepared from testes of adults (p=60 days) wild-type male mice using the RNeasy, Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Removal of chromosomal DNA contamination was performed and the RNA was quantitated by absorbance at 260 nm and 280 nm. Reverse transcription of the resulting RNA into cDNA was performed using SuperScript II Reverse Transcriptase (Invitrogen, Grand Island, NY) and the PCR reactions were performed using gene-specific primers as described previously (van Eyll, Passante et al. 2006). Duplicate wells were

included for each primer pair and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as an internal control. RNA levels were analyzed using the ABI PRISM 7000 system.

Testosterone ELISA: Blood samples were collected by cardiac puncture from adults (p=70 ±5 days) ephrin-A5$^{-/-}$ (n=13), and wild-type (n=15) male mice and allowed to clot. The blood was then centrifuged to isolate the serum, which was extracted using diethyl ether (Sigma, St. Louis, MO). Testosterone concentrations were measured using a commercially available competitive ELISA kit (Cayman Chemical Ann Arbor, MI, Cat No.582701).

Immunohistochemistry: Adult (p>60 days) ephrin-A5$^{-/-}$ and wild-type control male mice (n=3 per genotype) were deeply anesthetized and transcardially perfused with 0.9 % saline followed by 4 % paraformaldehyde (PFA) (Sigma, St. Louis, MO) in phosphate buffer. Once fixed, brains were removed, post fixed in the same solution at 4 °C and cryoprotected in 30% sucrose overnight. The brains were cut into 40 µm coronal sections and labeled overnight using rabbit anti-[Arg^{8}]-vasopressin (1:10,000; Peninsula, San Carlos, CA) followed by three washes with phosphate buffer and detection using goat secondary antibodies conjugated with Alexa Fluor 488 (1:200; Invitrogen, Grand Island, NY). In order to quantitatively analyze AVP immunoreactivity (AVP-ir), a total of 10 sections per animals (40 µm x 10) containing the entire suprachiasmatic nucleus (SCN) were used and the relative optical density (OD) was calculated using Photoshop (Adobe Systems, Mountain View, CA, USA).

Colchicine injections: Colchicine (Sigma, St. Louis, MO) was used in order to prevent the transport of AVP to the terminals and therefore to induce the accumulation of the peptide in the cell body (Liu, Kwok et al. 1991). Wild-type mice (n=3) were anesthetized and

injected with colchicine (20 µg) into the lateral ventricle. 48 hours later the animals were used for immunohistochemistry as described above.

HPLC analysis: Adult (p>60 days) male and female ephrin-A5$^{-/-}$ and wild-type mice (n=8 per group) were sacrificed and the brains removed. Selected brain regions were dissected on ice, snap frozen, and stored at -80 ^{o}C until analysis, as described previously (Schuh, Richardson et al. 2009). Briefly, frozen samples were sonicated in 500 µL of 0.1 N perchloric acid and centrifuged at 15,000 x g for 20 min at 4 ^{o}C. The pellets were kept for protein assay and the supernatants were re-centrifuged as above. The resulting supernatants were filtered and an aliquot of 20 µl was injected into the HPLC with electrochemical detection (Waters, Milford, MA, USA) for neurochemical analysis of norepinephrine (NE), dopamine (DA) and its metabolites, 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) and 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). The components were separated on a cation exchange column (MD-150 × 3.2 column, ESA Biosciences Inc.) using isocratic mobile phase (MD-TM, ESA Biosciences Inc.) containing 2.2 mM NaCl pumped at a constant flow rate of 0.5 ml/min. The compounds were quantified by electrochemical detection (flow cell, 2 mm GC WE, ISAC, Waters, Milford, MA, USA).

Statistical analysis: The following tests were used:

Student t-test	Resident intruder (RI)
	Investigatory sniffs
	Olfactory foraging test
	Olfactory habituation-dishabituation test
	Male-female recognition test
	Testosterone ELISA
ANOVA	Target biting
	HPLC

4.3 Results:

4.3.1 Reduced inter-male (offensive) aggression in ephrin-A5$^{-/-}$ mice

In order to evaluate the effects of ephrin-A5 on inter-male, offensive aggression we tested the mice in the RI model using age and genotype matched resident and intruder. Our data revealed a striking absence of fighting in ephrin-A5$^{-/-}$ mice (Figure 4-1). Wild-type mice took an average of 90 seconds to initiate a fight (Figure 4-1A) and fought about 8 times per 10 min test session (Figure 4-1B), whereas null mice did not fight at all. Since it has been shown that individual differences can elicit different responses from the resident (Mugford and Nowell 1970; Nelson and Chiavegatto 2000), we wanted to confirm that the lack of aggression is indeed due to behavioral changes in the resident and not differences in the non-test intruder. Therefore, we repeated the RI test using a zinc-sulfate treated, anosmic, wild-type mice as an intruder (Figure 4-2). Supporting our previous results, ephrin-A5$^{-/-}$ mice displayed a large decrease in inter-male aggression toward an anosmic intruder. Only two out of ten null mice engaged in aggressive behavior compared to eight out of ten wild-type mice (Figure 4-2A) and those that did fight had a significant increase

in the latency to the first attack (t=4.305, p=0.0004) (Figure 4-2B) and a significant decrease in the number of fights (t=-5.198, p<0.0001) (Figure 4-2C). Taken together, our data show that loss of ephrin-A5 caused significant decrease in inter-male aggression. However how ephrin-A5 regulates aggression is still unknown.

4.3.2 Investigatory sniffs during the resident intruder test

In order to confirm that the null mice are able to recognize the presence of another animal in their home cage, investigatory behavior in the form of face and anogenital sniffs were monitored during the RI test. As illustrated in figure 4-3, both genotype had high levels of investigatory sniffs, but the null mice showed a significant increase in sniffs [F(1,18)=16.118 , p=0.0008]. This increase in non-aggressive behavior and decrease in aggressive fights was reported previously (Patel, Siegel et al. 2010) and might be due to differences in social interactions between the genotypes. While the wild-type mice engaged in aggressive attacks, the null mice were involved in investigatory sniffs.

4.3.3 No differences in olfactory-guided foraging and habituation-dishabituation tasks between ephrin-A5$^{-/-}$ and wild-type mice

The olfactory-guided foraging test is based on the mouse ability to use olfactory cues for foraging (Yang and Crawley 2009). In the test the animal uses odor cues to locate food hidden underneath the bedding. An inability or a delay in the time it takes to locate the food is used as an indicator of dysfunctional olfactory behavior (Yang and Crawley 2009). The test began when the mouse was transferred to a holding cage and a piece of flavored cereal was placed on the surface of the cage bedding. The latency to locate the cereal was recorded and the mouse was returned to his home cage. This procedure was then repeated three times with the cereal buried in different positions of the cage underneath the

bedding. There were no significant differences between the genotypes [$F(1,14)=0.191$, $p=0.67$]. Both ephrin-A5$^{-/-}$ and wild-type mice located the food placed on the surface as well as buried underneath the bedding as rapidly (Figure 4-4A and B), suggesting that the general ability to smell is intact in the null mice.

In the habituation-dishabituation test, investigatory sniffs were recorded in six trials consisted of three presentations of water, followed by three presentations of male urine. Upon initial presentation of the cotton swab (dipped in water) both genotype showed high levels of investigatory sniffs (Figure 4-5). This exploratory activity was induced by the novelty of the cotton swab (since water doesn't have an odor) and declined across the second and third exposure to the water (habituation). Next, the introduction of male urine on the swab elicited significantly higher number of sniffs (dishabituation) then the water ($p<0.0001$) suggesting that the animals were able to smell and distinguish between the water and the urine. Finally, both genotyped habituated to the urine odor, indicated by a decline in the number of sniffs over the last three trials. Although there was no genotypic effect across all six trials [$F(1,17)=0.003$, $p=0.96$], a significant effect of trial was found [$F(5,85)=45.745$, $p<0.0001$] suggesting that both ephrin-A5$^{-/-}$ and wild-type mice were able to recognize the new odor.

4.3.4 No defects in male-female recognition task in ephrin-A5$^{-/-}$ mice

To investigate whether the loss of aggression in ephrin-A5$^{-/-}$ mice is due to loss of sex discrimination, we simultaneously introduce a female and a male mouse to each genotype and monitor their preference using a social chamber. Both ephrin-A5$^{-/-}$ and wild-type mice spent on average about 65 percent of the time investigating a female. There were no significant differences in the percentage of number of female cylinder touches (t=0.672,

p=0.51) nor in the percentage of time touching a female cylinder (*t*=-0.465, p=0.65) between the genotypes (Figure 4-6). These data suggest that both genotypes are able to distinguish between a male and a female.

4.3.5 Ephrin-A5 expression in the hypothalamic-pituitary-gonadal (HPG) axis

Testosterone is a steroid hormone produced by the hypothalamic–pituitary–gonadal (HPG) axis. The process starts in the brain when gonadotropin releasing hormone (GnRH) is secreted from the hypothalamus and transported to the anterior pituitary gland. Here, binding of GnRH to its receptor stimulate the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which act on the gonads to stimulate gametogenesis and the production of steroid sex hormones in particular testosterone.

Previously it was reported that ephrin-A5 is expressed in the hypothalamus and the pituitary during both development and postnatal stages (Zarbalis and Wurst 2000). Specifically, ephrin-A5 is expressed in areas of the hypothalamus such as the arcuate nucleus and the median eminence from which GnRH is secreted from (Glick 1991). We further examined the HPG axis for the expression of ephrin-A5 in the testes using real time RT-PCR. Testes were dissected from adult wild-type mice and extracted for RNA after which the relative expressions of individual Eph receptors as well as ephrin ligands were determined. Our result indicates that although ephrin-A5 is expressed in the mouse testes, its expression is relatively low compared to other members of the family, most notably ephrin-A2, ephrin-B2 and EphA2 (Figure 4-7).

4.3.6 Testosterone levels are comparable between ephrin-A5$^{-/-}$ and wild-type mice

Testosterone levels in serum were measured in male ephrin-A5$^{-/-}$ and wild-type mice. No differences were found between the two genotype (t=-0.604, p=0.55). However, both null and wild-type mice had high variation in blood testosterone levels, this variation was reported previously in other strains of mice (Klomberg, Garland et al. 2002; Brouillette, Rivard et al. 2005; Lacombe, Lelievre et al. 2007). Since hemolysis, the breakdown of erythrocytes with subsequent release of their intracellular contents, might interfere with the test results (Snyder, Rogers et al. 2004), we repeated the experiment using the BD Vacutainer® SST™ serum separation tubes (BD, Franklin Lakes, New Jersey). These tubes were used in order to minimize the presence of red blood cells in the sample. No differences were found in testosterone levels and/or variation when using these tubes (data not shown). Therefore our results (Figure 4-8) represent data from both experiments and show that testosterone levels are not affected by loss of ephrin-A5.

4.3.7 Increased AVP staining in the SCN of ephrin-A5$^{-/-}$ mice

Levels of AVP-ir through the entire SCN were significantly higher in the SCN of ephrin-A5$^{-/-}$ mice compared to wild-type controls (Figure 4-9). After colchicine injection, a significant higher levels of AVP-ir was observed in the SCN of the wild-type raising it to similar levels as the null mice. This increase was due to colchicine-induced inhibition of axonal transport which lead to the accumulation of the peptide in the cells bodies (Liu, Kwok et al. 1991).

4.3.8 Brain chemistry

The levels of dopamine (DA) and its metabolites DOPAC and HVA were comparable between the genotypes in all the brain regions that were tested. However differences in the serotonin system were found in the cerebellum and the hypothalamus. In the cerebellum a genotype x sex interaction was found ($F(1,28)=7.132$, $p=0.013$) where female null mice had higher levels of 5-HT compared to wild-type females. In addition, 5-HIAA levels were overall higher in the null mice ($F(1,28)=6.861$, $p=0.014$). In the hypothalamus, 5-HT levels were significantly higher in the null mice compared to the wild-type mice $F(1,28)=5.254$, $p=0.030$). In addition, in the frontal cortex, NE levels were significantly higher in the female null mice compared to the wild-type females ($p=0.048$) (Table 4-1).

4.3.9 Increased defensive aggression in ephrin-A5$^{-/-}$ mice

Defensive aggression was measured using the target-biting test (Figure 4-10). Ephrin-A5$^{-/-}$ mice had significant higher cumulative number of target biting in the 20 min session compared to wild-type mice ($t=3.706$, $p=0.002$) (Figure 4-10A). In addition, a repeated measure ANOVA was used to analyze the average number of target bites over the 3 bins and there was an overall significant effect of genotype [$F(1,17)=6.433$, $p=0.02$] and bin [$F(2,34)=18.704$, $p<0.0001$] (Figure 4-10B). *Post hoc* analysis revealed that null mice bite the target significantly more on bin 2-7 ($p=0.0005$) and bin 8 ($p=0.046$) compared to wild-type controls. In addition, target biting following the shock (bin1) was higher than target biting during the inter-shock interval (bin2-8) ($p=0.0002$) and during the tone CS (bin8) ($p<0.0001$). These three distinct rates of target biting behavior are in agreement with previously published report (Wagner, Nabert et al. 1983; Johnson, Carlson et al. 2003).

4.4 Discussion:

4.4.1 Offensive aggression

In this study we found that deletion of ephrin-A5 in male mice results in a major reduction in offensive aggressive behavior toward an intruder male. When tested with age and genotype matched intruders, none of the ephrin-A5$^{-/-}$ animals engaged in attack behavior. It has been reported that the level of aggressive behavior can be influenced by the intruder; changes in social investigation, movement and pheromones led to different responses from the resident (Mugford and Nowell 1970; Nelson and Chiavegatto 2000). For example, castrated mice do not produce the pheromones that induce aggression and therefore failed to stimulate fighting in the RI test (Mugford and Nowell 1970). As such, it is possible that the lack of aggression in the null mice was due to lack of stimuli from the null intruder that can be either behaviorally or olfactory. To eliminate these possible differences, we used a zinc sulfate treated wild-type intruders. Intranasal zinc sulfate application has been shown to cause anosmia by disrupting the connection of the olfactory epithelium to the main olfactory bulb (McBride, Slotnick et al. 2003). Rodents treated with zinc sulfate failed to initiate a fight but elicited similar responses from the resident as non-anosmic intruders (J and H 1976). Under these conditions, ephrin-A5$^{-/-}$ mice were still less aggressive then wild-type controls, suggesting that the reduced aggression is due to behavioral changes in the resident and not individual differences from the intruders.

4.4.2 Olfactory behavior

Individual recognition and gender discrimination were found to be important for the onset of aggression in rodents. An increase in investigatory sniffing often occurs before aggressive encounters in mice, suggesting that the recognition of the mouse as a “stranger”

is important (Doty 1986). In addition, the detection of male olfactory cues is essential for sex discrimination and mice that are unable to detect them cannot discriminate males from females and will not engage in inter-male aggression (Stowers, Holy et al. 2002). Thus, activation of the olfactory system by different odors has been shown to influence aggression (Guillot and Chapouthier 1996). For example, masking animals' natural odor by artificial scents increased the latency to the first attack and reduced the number of attacks in the RI assay (Ropartz 1968). In addition, surgical removal of the olfactory bulb (Ropartz 1968) as well as anosmia (Slotnick, Restrepo et al. 2010), produced by intranasal application of zinc sulfate, completely abolished the initiation of aggressive behavior in rodents. Finally, mice lacking functional cyclic nucleotide–gated channel α2 (CNGA2), which is required for the odor-evoked main olfactory epithelium signaling, or TRP2, a putative ion channel that is expressed exclusively in the vomeronasal organ, failed to display inter-male aggression in the RI test (Stowers, Holy et al. 2002; Mandiyan, Coats et al. 2005). As such, chemosensory cues are required for proper aggressive behavior in animals. These cues are detected by sensory neurons in two olfactory organs: the main olfactory epithelium (MOE) and the vomeronasal organ (VNO), and proceed to the main olfactory bulb and the accessory olfactory bulb respectively (Stowers, Cameron et al. 2013). From here the signals are sent to specific brain regions which translate them into the appropriate behavioral response (Nelson and Trainor 2007). Recently, one of these regions was identified in the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) (Lin, Boyle et al. 2011). Here, optogenetic stimulation of the VMHvl initiated aggressive behavior from a resident mice toward intruders that under unstimulated/normal conditions would not elicit attack. Moreover, genetic inhibition of VMHvl neuronal

activity prevented attacks even toward an intruder male. Furthermore, cells within the VMHvl that are activated during male aggressive behavior are mostly distinct from those that are activated during mating, suggesting that stimuli from a male intruder processed differently than those from a female intruder, and therefore produced different responses (Lin, Boyle et al. 2011). Further analysis of the VMHvl activity suggested that these neurons play a role in signaling the presence of a male olfactory cues and converting them into the appropriate social behavior, i.e. attack (Falkner, Dollar et al. 2014).

Ephrin-A5 is expressed in the olfactory system (St John, Pasquale et al. 2002; Cutforth, Moring et al. 2003; Deschamps, Morel et al. 2010) and has been shown to involve in the formation of the proper mapping into the olfactory bulb (Cutforth, Moring et al. 2003) and the accessory olfactory bulb (AOB) (Knoll, Zarbalis et al. 2001). As such, mice deficient in both ephrin-A3 and ephrin-A5 have a posterior shift in the location of two different glomerular targets in the olfactory bulb, and mice with single mutant for ephrin-A5 have misprojection of the VNO axons to AOB. In the current study we did not detect significant genotype-dependent differences in the general olfactory behavior. Olfactory-guided foraging and the habituation to a new olfactory stimulus were comparable between the genotypes. In addition, both ephrin-A5$^{-/-}$ and wild-type male mice preferred the female containing cylinder over the one with the male, suggesting that gender discrimination is intact. Thus the ability to detect chemosensory cues from the environment seem normal in the null mice. However, it is possible that specific connection to the hypothalamic aggression center is disrupted in these mice.

In contrast to olfaction, visual cues do not seem to be involved in the development of offensive aggression; blind mice initiated aggressive behavior towards an unfamiliar

male similar to a mouse without vision impairments (Doty 1986) indicating that this behavior is mediated by olfactory cues. However, since previously, we have reported that ephrin-A5$^{-/-}$ mice developed ocular abnormalities (Cooper, Son et al. 2008; Son, Sheleg et al. 2014), we wanted to confirm that the lack of aggression is not due to the inability of the mice to see the intruder. Indeed, ephrin-A5$^{-/-}$ mice, despite the vision impairments, investigated the intruder when introduced into their home cage, suggesting that they were able to sense the presence of another animal in their cage.

4.4.3 Testosterone

The influence of testosterone on male aggression has been studied extensively (Delville, Mansour et al. 1996; Simon, Cologer-Clifford et al. 1998; Frye, Rhodes et al. 2002; Nelson and Trainor 2007). In human, males between the ages of 12 to 25 are more likely to commit a crime, a pattern that was referred to as the "Young Male syndrome" in 1985 (reviewed in (Craig and Halton 2009)), and occurs in concert with puberty and the rise of testosterone levels in the blood (Craig and Halton 2009). In rodents castration reduced inter-male aggression, while testosterone supplementation restored it (Barfield, Busch et al. 1972; Luttge 1972; Barkley and Goldman 1977; Nelson 2006; Lofgren, Erdman et al. 2012). In addition, testosterone treatment of wild-type mice but not mice that lack 5α-reductase, the enzyme that convert testosterone to its metabolite, increased aggressive behavior toward an intruder male (Frye, Rhodes et al. 2002), and conditional inactivation of the androgen receptor in the nervous system reduced it (Raskin, de Gendt et al. 2009). Finally, administration of anabolic androgenic steroids (AAS), a synthetic derivative of testosterone, increased aggression in animals (Breuer, McGinnis et al. 2001; McGinnis, Lumia et al. 2002; Robinson, Penatti et al. 2012). In the current study,

testosterone levels were comparable between the two groups. However, it is important to note that there exists a critical time during puberty in which levels of testicular hormones affect the development of aggressive behavior during adult life (Shrenker, Maxson et al. 1985; Schulz, Menard et al. 2006). Shrenker *et al*. (Shrenker, Maxson et al. 1985) reported that castration at 30 days, but not 50 days after birth, decreased the aggressive behavior of the mice later in life, suggesting that the timing of castration is important for the decreased behavior. Therefore, although testosterone levels were similar between the null and wild-type mice at adulthood, it is possible that differences exist during puberty. In addition, testosterone metabolism (Toda, Saibara et al. 2001; Frye, Rhodes et al. 2002) and receptor levels (Raskin, de Gendt et al. 2009) have been shown to affect aggression and therefore may play a role in the decreased aggression in the null mice.

4.4.4 Serotonin (5-HT)

Serotonin (5-HT), a monoamine neurotransmitter, has also been implicated in the development of aggressive behavior, however the direction of this relationship is controversial (Carrillo, Ricci et al. 2009; Numan 2014). Most studies have shown a negative correlation between the two; lower levels of 5HT, and its metabolite, 5-hydroxyindole acetic acid (5-HIAA), have been found in the prefrontal cortex (PFC) of rats during aggressive encounter and in the CSF of aggressive, violent men (Brown, Ebert et al. 1982; van Erp and Miczek 2000). Furthermore, activation of the 5-HT receptors 1A and 1B have been shown to inhibit aggressive behavior in mice (Nelson and Trainor 2007). However, since these receptors exist as both inhibitory autoreceptors on the serotonergic neurons, and receptors on nonserotonergic neurons, their activation led to both decreased 5-HT activity, resulting from the autoreceptor activation, and increased 5-HT activity as a

result of direct stimulation of the nonserotonergic neurons (de Boer and Koolhaas 2005). This along with studies on the 5HT2A receptor activity in mice (Shih, Ridd et al. 1999), support a positive correlation between 5-HT and aggression. Our HPLC analysis revealed changes in 5-HT and its metabolite 5-HIAA in the cerebellum and the hypothalamus of ephrin-A5$^{-/-}$ mice compared to wild-type controls. This may in part contribute to the decreased aggression.

4.4.5 AVP

The role of arginine vasopressin (AVP) in aggressive behavior has been studied extensively (Albers 2012). AVP is a nine amino acid peptide produced in the hypothalamic nuclei [i.e., paraventicular (PVN), supraoptic (SON) and suprachiasmatic (SCN)] and extended amygdala [bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA)] (Rood, Stott et al. 2012). Its peripheral functions include regulation of blood volume by promoting water retention in the kidney, as well as controlling blood pressure through vasoconstriction. These actions are most likely controlled by AVP produced in the PVN and SON because these nuclei project to the posterior pituitary where AVP is released into the bloodstream (Taniguchi, Yoshida et al. 1988; Rood, Stott et al. 2012). In addition, AVP has been shown to regulate other forms of behavior such as social recognition, social communication, affiliation and stress (Caldwell, Lee et al. 2008; López-Larrea 2011; Albers 2012). One of the first reports on AVP and aggression was done on male hamsters where injection of V1a-receptor antagonist into the anterior hypothalamus caused a dose-dependent inhibition of inter-male aggression (Ferris and Potegal 1988). Other studies in hamsters (Ferris, Melloni et al. 1997) and voles (Gobrogge, Liu et al. 2007) have shown that AVP acts on the anterior hypothalamus to promote aggression. However the site of

origin for these neurons is unknown and some data suggests that its derive from neurons within the anterior hypothalamus (Numan 2014). Moreover, manipulation of AVP in the brain and AVP receptor knockout studies have shown to altered aggressive behavior in rodents (Koolhaas, Vandenbrink et al. 1990; Wersinger, Ginns et al. 2002; Wersinger, Caldwell et al. 2007). Specifically, deletion of the V1b- receptor which express in the hippocampus, the amygdala and the hypothalamic paraventricular nucleus (Young, Li et al. 2006), decreased aggressive behavior in male mice (Wersinger, Ginns et al. 2002) by reducing the attack component directed toward the intruder (Wersinger, Caldwell et al. 2007). Compared to wild-type controls, we found an increase of AVP- expressing cells in the SCN of ephrin-A5$^{-/-}$ mice. These differences are less likely due to an increase in the number of AVP- producing neurons, but rather due to decreased AVP release. Supporting this, is the fact that wild-type mice that were injected with colchicine, an inhibitor of axonal transport (Liu, Kwok et al. 1991), had a similar number of AVP containing neurons in the SCN as null mice. This suggests that the mice differ in transport and/or release of AVP from the SCN to its target. Given the fact that AVP innervation from the SCN is within the diencephalon (Rood, Stott et al. 2013), it is possible that AVP neurons from the SCN innervate the anterior hypothalamus to control aggression. In addition, the VMH, which recently was identified as the aggression locus in the mouse (Lin, Boyle et al. 2011), contains AVP fibers (Kent, Anisman et al. 2001). Therefore it is possible that decreased AVP innervation from the SCN, caused decreased aggression in the null mice.

4.4.6 Defensive aggression

We assessed defensive aggression, in the form of target biting in the null and wild-type mice. Here, ephrin-A5 deletion caused increased target biting compared to wild-type

controls. The presence of normal and even exaggerate levels of defensive aggression demonstrate that ephrin-A5$^{-/-}$ mice are capable of attack behavior and therefore the lack of aggression toward an intruder male is not due to their inability to attack.

Figure 4-1: Loss of inter-male aggression in ephrin-A5$^{-/-}$ mice

Inter-male aggression was tested in the resident-intruder paradigm in ephrin-A5$^{-/-}$ and wild-type mice (n=9 per genotype). Ephrin-A5$^{-/-}$ male did not exhibit aggressive behavior toward an intruder male.

(A, B) Wild-type male attacked a male intruder with an average latency to the first attack of 90 seconds (A) and an average of 8 attacks per 10 minutes test (B) compared to no attacks by the null mice.

Data are presented as mean +SEM.

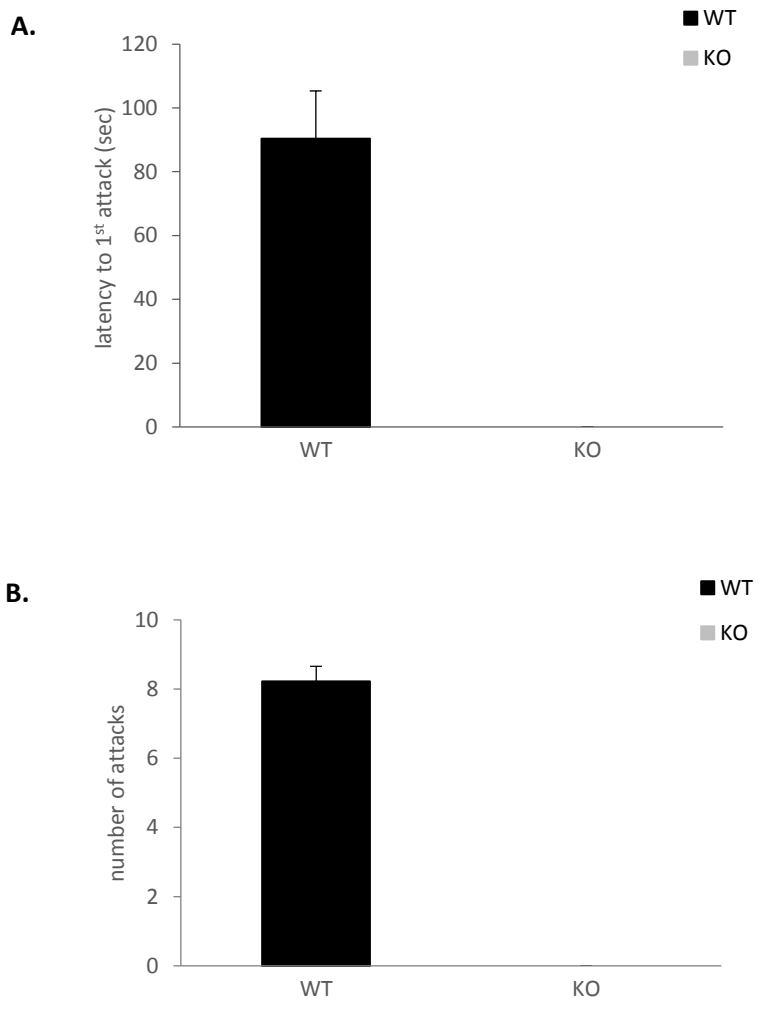


Figure 4-2: Decreased aggression toward an anosmic mice in ephrin-A5$^{-/-}$ male mice
Inter-male aggression toward a zinc sulfate treated, anosmic intruder was measured in ephrin-A5$^{-/-}$ and wild-type male mice (n=10 per genotype) in the resident intruder test.
(A) Only two out of ten null mice engaged in aggressive behavior compared to eight out of ten wild-type mice. Data are presented as percent of animals that fought.
(B, C) Ephrin-A5$^{-/-}$ mice had increased latency to the first attack (B) as well as reduced number of attacks (C) compared to wild-type control. Data are presented as mean +SEM.
* indicates significantly different from wild-type; $p<0.001$.

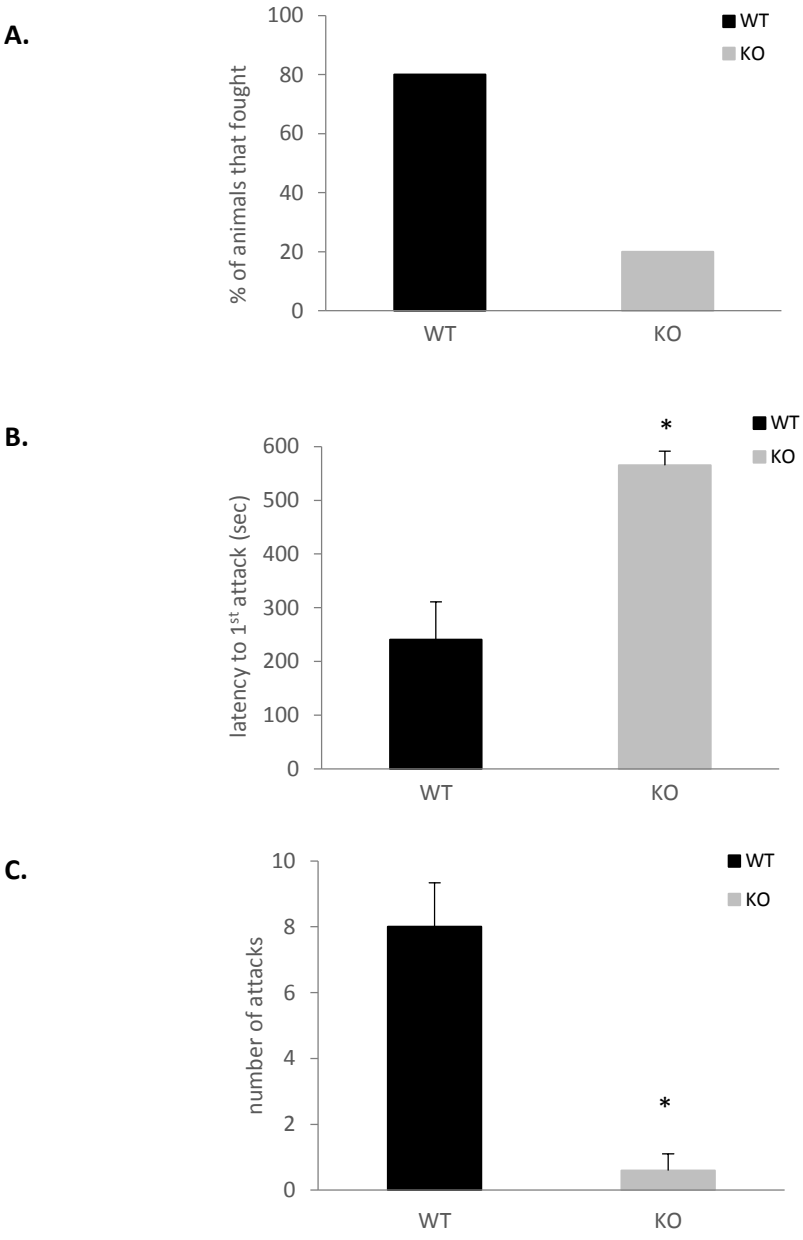


Figure 4-3: Ephrin-A5$^{-/-}$ mice showed high levels of non-aggressive investigatory sniffs during the RI test

Both genotypes were able to recognize the presence of an intruder as revealed by the number of face and ano-geno sniffs during the RI test.
Data are presented as the mean number of sniffs +SEM.
* indicates significantly different from wild-type; $p<0.05$.

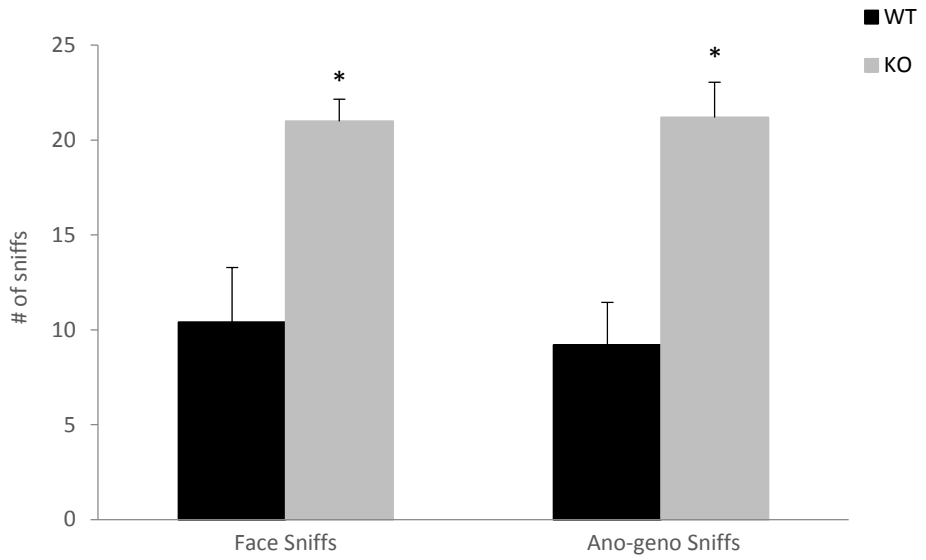


Figure 4-4: No differences in the olfactory foraging test between the genotypes
Olfactory-guided foraging was tested in ephrin-A5$^{-/-}$ and wild-type male mice (n=8 per genotype). (A, B) Both genotypes were able to locate the flavored cereal when placed on the surface (A) or buried beneath the bedding (B)
Data are presented as the time required by the mice to locate the flavored cereal (sec) +SEM.

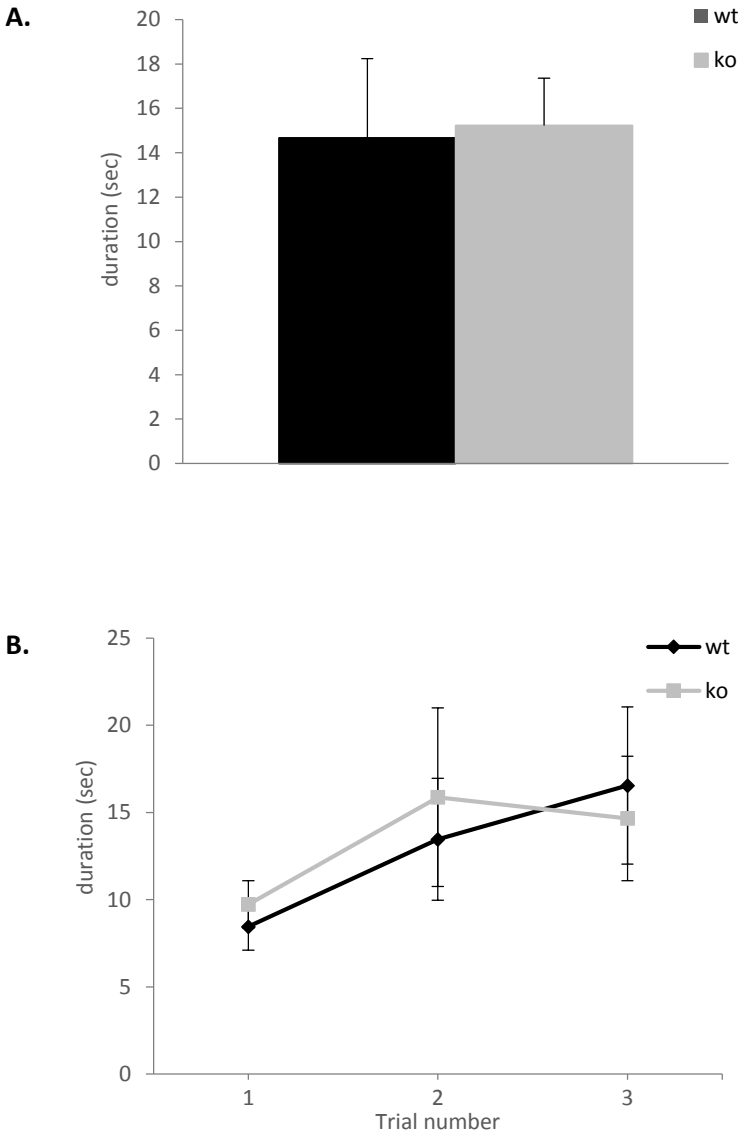


Figure 4-5: No differences in the olfactory habituation-dishabituation test between the genotypes

Both eprin-A5$^{-/-}$ (n=9) and wild-type (n=10) male mice showed increase of sniffing upon the presentation of the new odor, male urine, (dishabituation) and a decrease in subsequent presentations (habituation). This demonstrated the ability of all groups to recognize new odors. Data are presented as the mean number of sniffs +SEM.

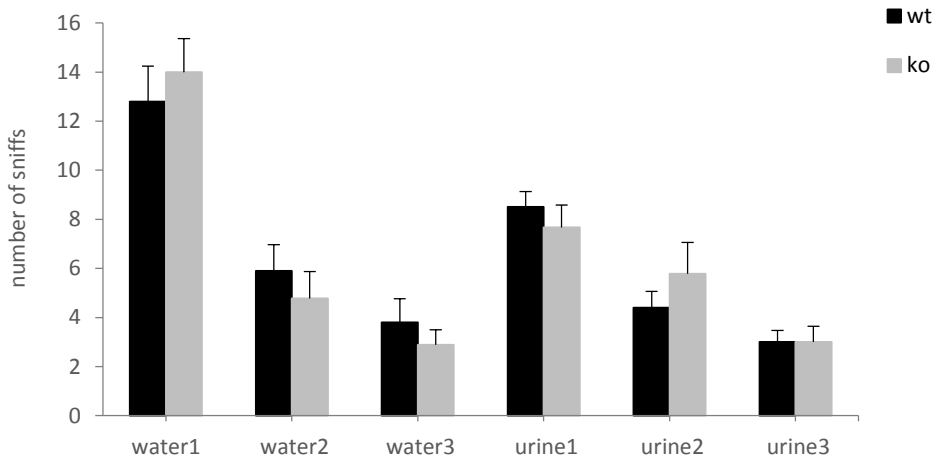


Figure 4-6: No defects in male-female recognition task in ephrin-A5$^{-/-}$ mice
Ephrin-A5$^{-/-}$ and wild-type (n=10 per genotype) male mice were tested for their preference toward a male or female mice in a social chamber. Both genotypes showed preference toward a female mice.
All male animals touched the female containing cylinder more than the one with the male and spent on average about 65 percent of the time investigating it.
Data are presented as percent of female cylinder touches (left panel) or percentage of time (right panel) +SEM.

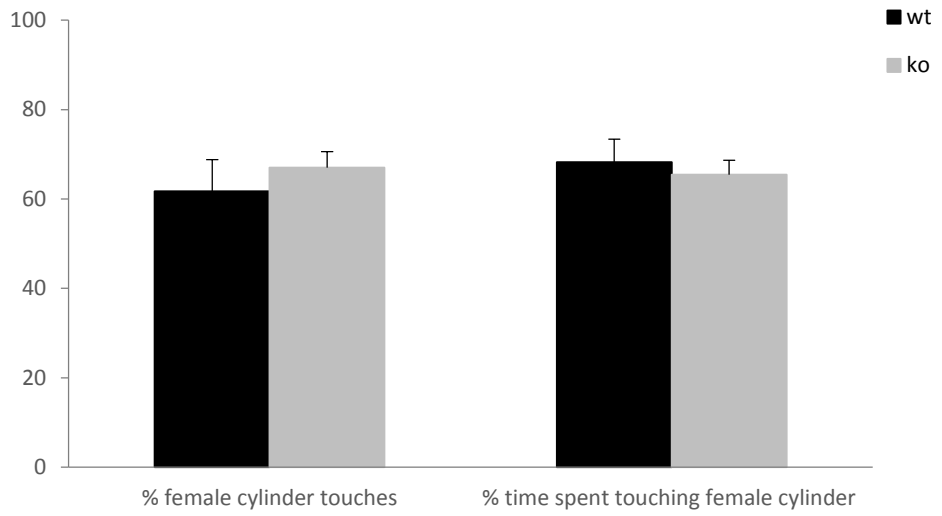


Figure 4-7: Expression of the Eph-ephrin family in the mouse testis
A relative mRNA level of members of the Eph/ephrin family in the mouse testis was measure using RT-PCR. Ephrin-A5 is moderately express in the testis.

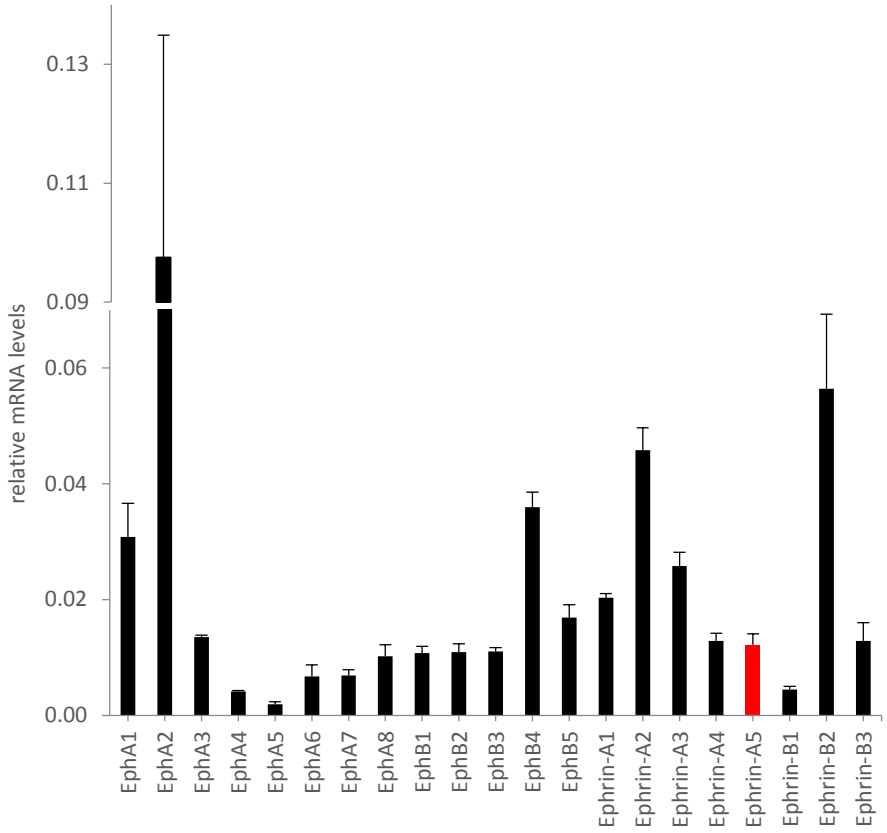


Figure 4-8: Testosterone levels in ephrin-A5$^{-/-}$ mice are within the range of wild-type control mice

Testosterone levels were measured in serum of ephrin-A5$^{-/-}$ (n=13) and wild-type (n=15) male mice. No differences were found in testosterone levels and/or variation between null and wild-type mice. Data are presented as individual testosterone levels (pg/ml).
Lines represent the mean testosterone levels ± SEM.

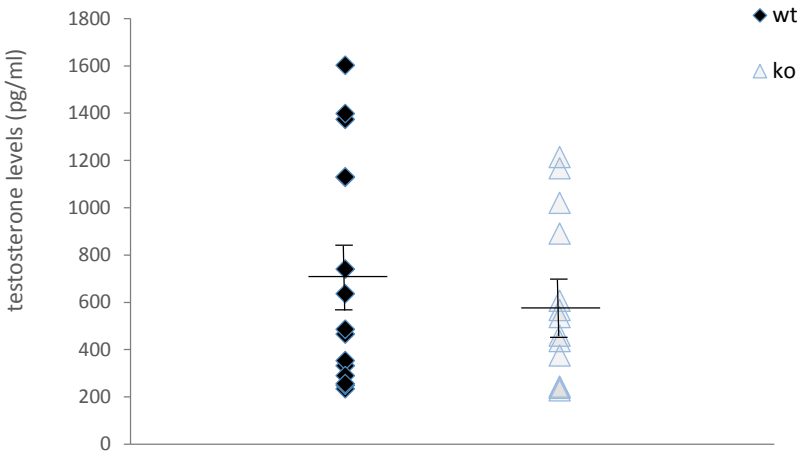


Figure 4-9: Increased AVP-ir in the cell soma of male ephrin-A5$^{-/-}$ mice
AVP-ir in the SCN is higher in male ephrin-A5$^{-/-}$ mice compared to wild-type controls.
(A) Quantification of AVP-ir in the SCN (n=3 per genotype). Data are presented as relative optical density +SEM.
(B) Representative immunohistochemistry images of the SCN of wild-type (WT), ephrin-A5$^{-/-}$ (KO) and colchicine injected wilt-type mice. Scale bar =500µm.
* indicates significantly different from wild-type mice; $p<0.05$.

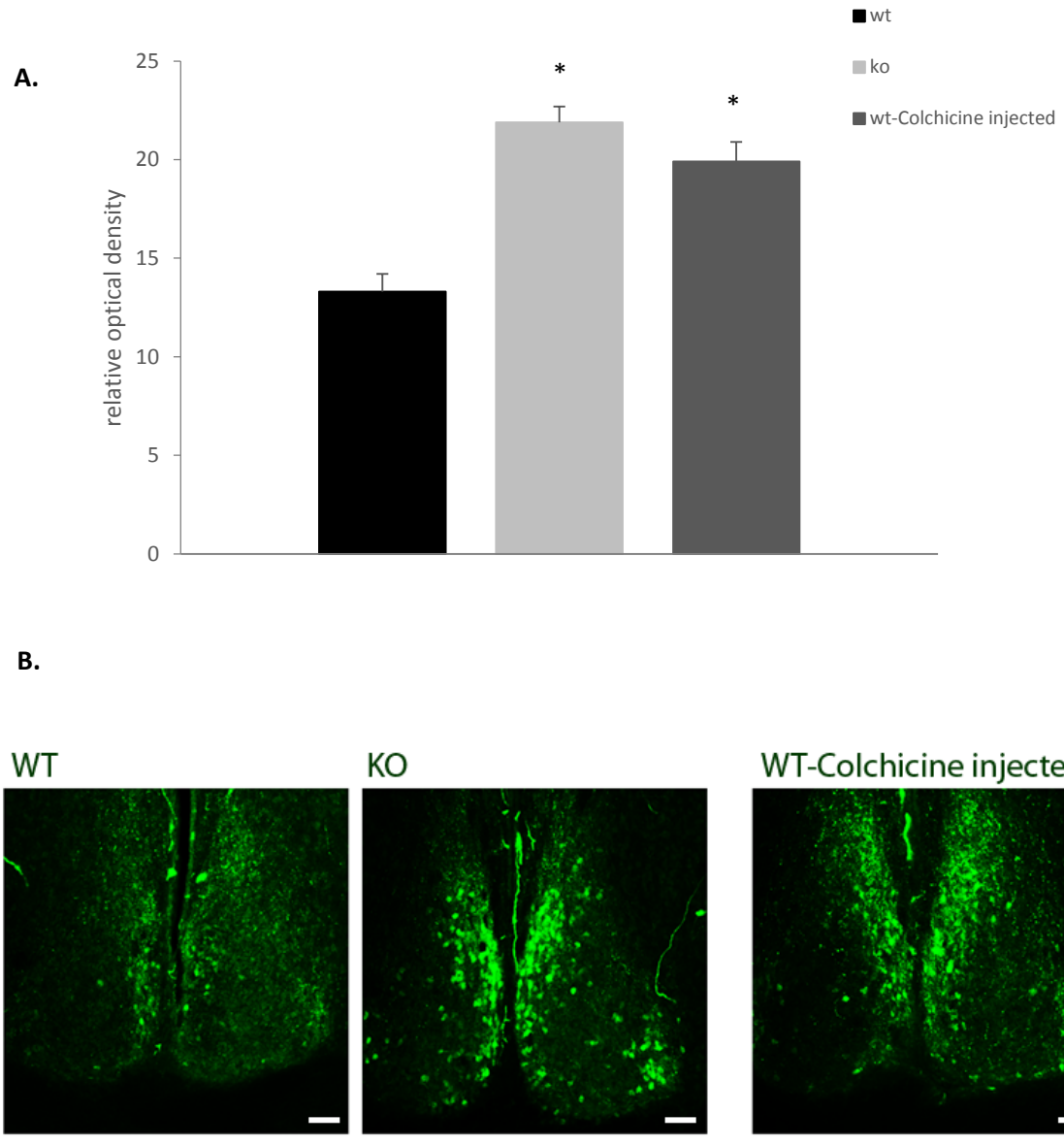


Figure 4-10: Increased target biting in ephrin-A5$^{-/-}$ mice
Defensive aggression was measured in ephrin-A5$^{-/-}$ (n=9) and wild-type (n=10) male mice in the target biting test.
(A) Ephrin-A5$^{-/-}$ mice had increased cumulative target biting during the 20 minutes test compared to wild-type controls. Data are presented as mean number of bites.
(B) Analysis of target biting per bin. Ephrin-A5$^{-/-}$ mice bite the target significantly more on bin 2-7 and bin 8 compared to wild-type controls. In addition, both genotypes bite the target more in bin 1 compared to bin 2-7 and bin 8. Data are presented as mean number of bites per bin.
* indicates significantly different from wild-type; $p<0.05$.
** indicates significantly different from bin 1; $p<0.05$.

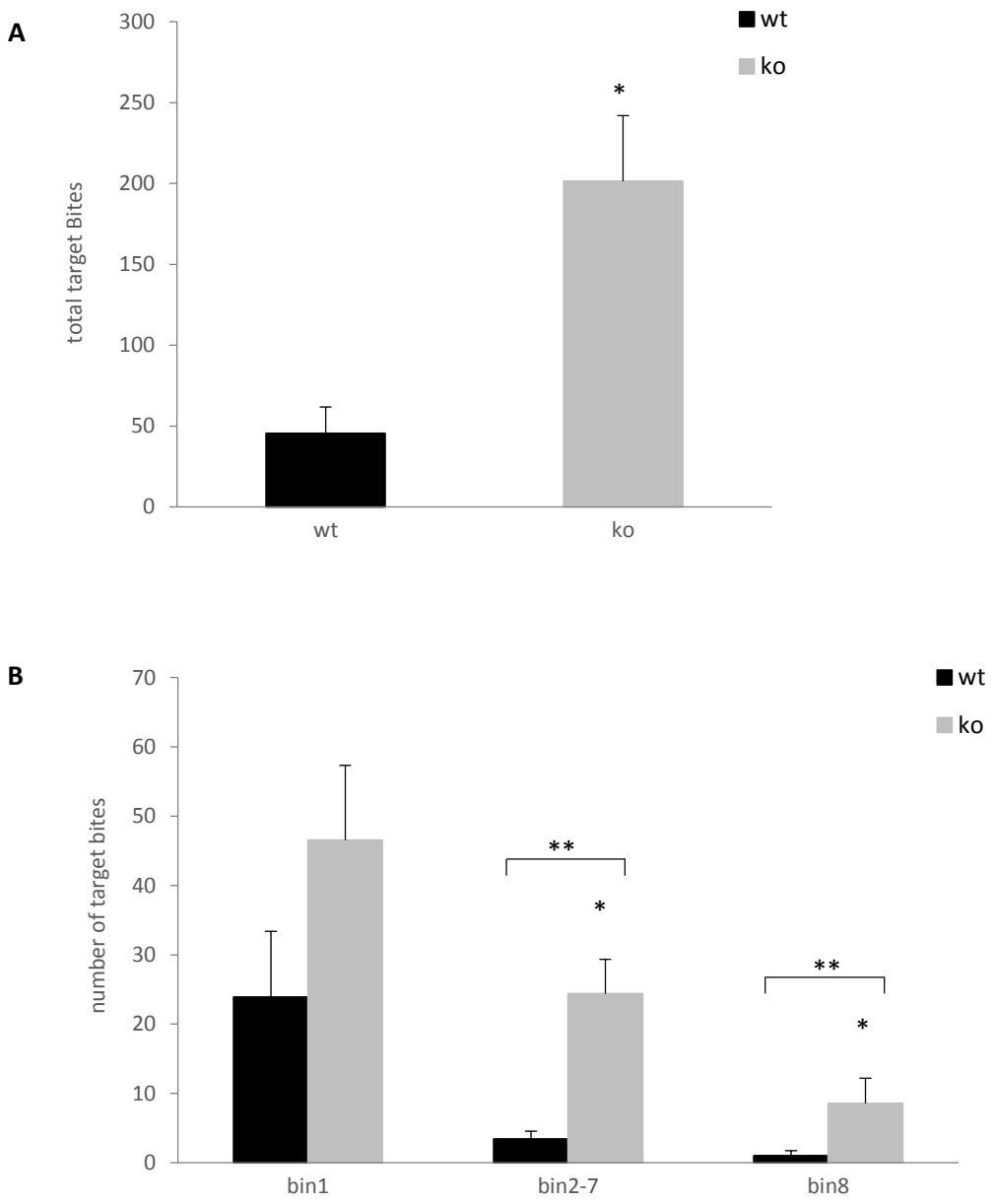


Table 4- 1: Neurotransmitter and metabolite levels in adult ephrin-A5$^{-/-}$ and wild-type mice
Neurotransmitter and metabolite levels detected in the cerebellum, hippocampus, striatum, frontal cortex and hypothalamus of male and female adult (p> 60 days) ephrin-A5$^{-/-}$ and wild-type mice. Values are expressed in ng/mg of protein ±SEM.
* indicates significantly different from wild-type mice within the same sex; $p < 0.05$.
^ indicates significant different from male mice within the same strain; $p < 0.05$.

	WT Male	***KO Male***	***WT Female***	***KO Female***
Cerebellum				
5-HT	25.33 ± 2.872	20.41 ± 1.71	17.15 ± 2.55	25.92 ± 2.96 *
5-HIAA	12.58 ± 1.65	13.90 ± 1.02	10.23 ± 0.91	16.26 ± 1.85 **
DOPAC	6.23 ± 0.45	6.69 ± 0.62	9.29 ± 0.55 ^	10.78 ± 0.60 ^
Hippocampus				
5-HT	64.61 ± 5.49	64.11 ± 7.91	63.41 ± 8.06	62.85 ± 4.88
5-HIAA	60.14 ± 7.50	62.72 ± 6.37	62.71 ± 8.33	55.48 ± 4.92
DOPAC	3.78 ± 0.38	4.53 ± 0.59	5.03 ± 0.41	4.82 ± 0.36
NE	30.40 ± 2.30	35.55 ± 5.15	31.80 ± 3.38	37.23 ± 2.06
Striatum				
DA	516.3 ± 47.90	409.5 ± 64.64	402.1 ± 76.21	404.4 ± 52.87
DOPAC	34.85 ± 2.47	32.08 ± 4.51	32.40 ± 2.80	31.21 ± 2.64
HVA	72.15 ± 4.56	67.13 ± 8.66	62.80 ± 4.20	64.22 ± 3.50
5-HT	21.30 ± 4.23	21.96 ± 1.91	24.76 ± 4.03	23.31 ± 2.30
5-HIAA	16.48 ± 2.61	14.15 ± 2.36	17.79 ± 3.34	15.84 ± 1.57
NE	97.01 ± 9.41	95.90 ± 4.25	87.12 ± 4.81	90.57 ± 4.28
Frontal Cortex				
HVA	3.20 ± 0.52	3.28 ± 0.37	4.42 ± 0.78	4.26 ± 0.68
5-HT	11.87 ± 0.46	12.9 ± 0.75	11.44 ± 0.41	11.86 ± 0.69
5-HIAA	3.14 ± 0.10	3.38 ± 0.24	2.99 ± 0.13	3.27 ± 0.09
NE	22.51 ± 1.41	21.22 ± 1.16	16.95 ± 1.17 ^	19.94 ± 0.74 *
Hypothalamus				
DA	63.20 ± 10.51	85.40 ± 16.64	106.0 ± 12.53 ^	80.15 ± 4.33
5-HT	93.89 ± 7.92	120.25 ± 14.58	105.68 ± 10.63	129.25 ± 9.28 **
5-HIAA	32.65 ± 6.02	47.27 ± 6.37	42.42 ± 5.30	40.49 ± 6.53
NE	481.73 ± 17.96	456.54 ± 27.41	486.65 ± 15.82	464.98 ± 26.02

CHAPTER 5: SUMMARY AND FUTURE DIRECTION

In this work we examined the roles of ephrin-A5, a GPI-linked ligand for the Eph family of receptor tyrosine kinases, on animal behavior using ephrin-A5$^{-/-}$ mice. Ephrin-A5$^{-/-}$ animals are smaller in weight, but otherwise morphologically normal, and are capable of breeding and producing offspring. In addition, they do not have an apparent locomotor deficit, with only slight delays in reaching developmental milestones and in maturation of motor skills. Ephrin-A5$^{-/-}$ pups have delayed eye opening and when examined during development for general coordination and strength in the negative geotaxis and the hanging wire tests, were found to be slower to complete the tasks. In addition, during the first week of life, pups that were born to ephrin-A5$^{-/-}$ dams had higher mortality rate compared to those that were born to wild-type controls. Examining the pattern of maternal care revealed that ephrin-A5$^{-/-}$ mothers are impaired in several aspects of maternal behavior, specifically, in the pup retrieval and the maternal nest building tasks. Interestingly, the ability to build a non-maternal nest was intact in the null mice, suggesting that the defect is specific to pregnant mice. We also examined the level of anxiety-like behavior in ephrin-A5$^{-/-}$ mice. Here we found differences on the elevated plus-maze, where ephrin-A5$^{-/-}$ mice spent more time and entered the open arm more than wild-type controls. These differences persist regardless of rearing conditions and were confirmed using the light-dark box as another test for anxiety. Finally, inter-male, offensive aggression is severely reduced in male ephrin-A5$^{-/-}$ mice; in the resident-intruder test most of the null mice did not attack an intruder male and those that did attack took longer to initiate and fought less. This was probably not due to inability to attack, since during the target biting test, ephrin-A5$^{-/-}$ mice

were found to have increased target biting, suggesting that they are capable of initiating aggressive behavior.

Maternal behavior, inter-male aggression and anxiety are highly dependent on proper olfactory function (Guillot and Chapouthier 1996; Levy and Keller 2009; Glinka, Samuels et al. 2012). Here, the detection of chemosensory cues by the olfactory system is processed in the brain to guide specific behavior. For example, activation of the MPOA by pup stimuli and the VMHvl by male urine, evokes maternal and aggressive behavior respectively. Our olfactory studies revealed no significant differences in general olfaction between the genotypes; both groups were able to detect flavored cereal that was hidden underneath the cage bedding, and showed increased sniffs upon presentation of a new odor. In addition, both wild-type and null male mice preferred to spend more time near a female and not a male, suggesting that both genotypes are able to discriminate between the sexes. Finally, olfactory memory for male pheromones is normal in the null females; ephrin-A5$^{-/-}$ females blocked their pregnancy in response to an unfamiliar male, but stayed pregnant in response to cues from the familiar male. Taken together, these data suggest that the ability to smell is intact in the null mice. However, how ephrin-A5 deletion affects guidance of chemosensory information in the brain is incompletely known. Although it seems that brain circuits that control foraging, sex discrimination and pregnancy block are normal, it is possible that the "maternal circuits" and the "aggression circuits" are disrupted in the null mice. Future studies will test the hypothesis that certain brain circuits are disrupted in the null mice using c-Fos as a marker for neuronal activation and specific chemosensory cues such as the major urinary proteins (MUPs) that were found to trigger

aggressive behavior in male mice (Chamero, Marton et al. 2007) and pup/odorant/pheromone, as described previously (Wang and Storm 2011).

It is also possible, that the neuronal connection is intact and the defects are due to loss of molecular signals that normally activate these circuits. Consistent with this notion, we found that the levels of arginine vasopressin (AVP) are altered in the brain of the null mice. Both male and female ephrin-A5$^{-/-}$ mice had increased cell soma expression of AVP in the SCN. We hypothesized that this increase is due to lower AVP release and therefore reduced AVP signaling in the hypothalamus. Interestingly, AVP has been shown to positively affect maternal behavior, inter-male aggression and anxiety (reviewed in (Caldwell, Lee et al. 2008)). Therefore, reduced AVP signaling may decrease these behaviors. In order to validate this hypothesis, future studies will measure AVP release in live animals using microdialysis. In addition, to determine whether alteration in the AVP system is responsible for the behavioral changes observed, rescue studies using an AVP receptor agonist (Manning, Stoev et al. 2008) and/or AVP injection into the brain should be performed.

Finally, we studied parental behavior in the form of maternal care, yet, similar nurturing behavior known as paternal behavior is presented by the father. In the laboratory, virgin male mice typically commit infanticide, however, after mating this behavior is inhibited and maternal-like behavior develops (vom Saal and Howard 1982). Here, the male parent becomes paternal and care for the pups by licking, huddling over and retrieving them into the nest (Liu, Lopatina et al. 2013). Although, paternal behavior received less attention than maternal care, common regulatory pathways exist between the two (Rilling and Young 2014). Recent studies have shown that the VMO system is important for

paternal behavior. Impairment of the VMO signaling by deletion of TRPC2, a VNO-specific ion channel, reduced pup-directed aggression and induced parental care in virgin male mice (Wu, Autry et al. 2014). Furthermore, removal of the VMO from a virgin male mice induced parental behavior and prevented pup-directed aggression (Tachikawa, Yoshihara et al. 2013). Finally, the MPOA, the central brain region that governs maternal behavior (Gammie 2005; Tsuneoka, Maruyama et al. 2013; Wu, Autry et al. 2014), controls paternal behavior as well (Wu, Autry et al. 2014). Given these observations, it will be interesting to examine whether ephrin-A5 deletion affect paternal behavior or whether it is specific to the neuronal circuits that control maternal care.

REFERENCES:

Albers, H. E. (2012). "The regulation of social recognition, social communication and aggression: vasopressin in the social behavior neural network." Horm Behav **61**(3): 283-292.

Anholt, R. R. and T. F. Mackay (2012). "Genetics of aggression." Annu Rev Genet **46**: 145-164.

Attwood, B. K., S. Patel, et al. (2012). "Ephs and ephrins: emerging therapeutic targets in neuropathology." Int J Biochem Cell Biol **44**(4): 578-581.

Bailey, K. R. and J. N. Crawley (2009). Anxiety-Related Behaviors in Mice. Methods of Behavior Analysis in Neuroscience. J. J. Buccafusco. Boca Raton (FL).

Barfield, R. J., D. E. Busch, et al. (1972). "Gonadal influence on agonistic behavior in the male domestic rat." Horm Behav **3**(3): 247-259.

Barkley, M. S. and B. D. Goldman (1977). "The effects of castration and Silastic implants of testosterone on intermale aggression in the mouse." Horm Behav **9**(1): 32-48.

Blanchard, D. C. and R. J. Blanchard (2003). "What can animal aggression research tell us about human aggression?" Horm Behav **44**(3): 171-177.

Blanchard, R. J., P. M. Wall, et al. (2003). "Problems in the study of rodent aggression." Horm Behav **44**(3): 161-170.

Bochenek, M. L., S. Dickinson, et al. (2010). "Ephrin-B2 regulates endothelial cell morphology and motility independently of Eph-receptor binding." J Cell Sci **123**(Pt 8): 1235-1246.

Bolz, J., D. Uziel, et al. (2004). "Multiple roles of ephrins during the formation of thalamocortical projections: maps and more." J Neurobiol **59**(1): 82-94.

Bonanomi, D. and S. L. Pfaff (2010). "Motor axon pathfinding." Cold Spring Harb Perspect Biol **2**(3): a001735.

Bond, T. L., P. E. Neumann, et al. (2002). "Nest building in nulligravid, primigravid and primiparous C57BL/6J and DBA/2J mice (Mus musculus)." Physiol Behav **75**(4): 551-555.

Bosch, O. J. and I. D. Neumann (2008). "Brain vasopressin is an important regulator of maternal behavior independent of dams' trait anxiety." Proc Natl Acad Sci U S A **105**(44): 17139-17144.

Bosch, O. J. and I. D. Neumann (2012). "Both oxytocin and vasopressin are mediators of maternal care and aggression in rodents: from central release to sites of action." Horm Behav **61**(3): 293-303.

Bourin, M., B. Petit-Demouliere, et al. (2007). "Animal models of anxiety in mice." Fundam Clin Pharmacol **21**(6): 567-574.

Bouzioukh, F., G. A. Wilkinson, et al. (2007). "Tyrosine phosphorylation sites in ephrinB2 are required for hippocampal long-term potentiation but not long-term depression." J Neurosci **27**(42): 11279-11288.

Breuer, M. E., M. Y. McGinnis, et al. (2001). "Aggression in male rats receiving anabolic androgenic steroids: effects of social and environmental provocation." Horm Behav **40**(3): 409-418.

Brodkin, J., D. Frank, et al. (2014). "Validation and implementation of a novel high-throughput behavioral phenotyping instrument for mice." J Neurosci Methods **224**: 48-57.

Brouillette, J., K. Rivard, et al. (2005). "Sex and strain differences in adult mouse cardiac repolarization: importance of androgens." Cardiovasc Res **65**(1): 148-157.

Brown, G. L., M. H. Ebert, et al. (1982). "Aggression, suicide, and serotonin: relationships to CSF amine metabolites." Am J Psychiatry **139**(6): 741-746.

Bult, A. and C. B. Lynch (1997). "Nesting and fitness: lifetime reproductive success in house mice bidirectionally selected for thermoregulatory nest-building behavior." Behav Genet **27**(3): 231-240.

Bush, G. (2011). "Cingulate, frontal, and parietal cortical dysfunction in attention-deficit/hyperactivity disorder." Biol Psychiatry **69**(12): 1160-1167.

Caldwell, H. K., H. J. Lee, et al. (2008). "Vasopressin: behavioral roles of an "original" neuropeptide." Prog Neurobiol **84**(1): 1-24.

Carmona, M. A., K. K. Murai, et al. (2009). "Glial ephrin-A3 regulates hippocampal dendritic spine morphology and glutamate transport." Proc Natl Acad Sci U S A **106**(30): 12524-12529.

Carrillo, M., L. A. Ricci, et al. (2009). "The effect of increased serotonergic neurotransmission on aggression: a critical meta-analytical review of preclinical studies." Psychopharmacology (Berl) **205**(3): 349-368.

Carvalho, R. F., M. Beutler, et al. (2006). "Silencing of EphA3 through a cis interaction with ephrinA5." Nat Neurosci **9**(3): 322-330.

Caudle, W. M., J. R. Richardson, et al. (2005). "Perinatal heptachlor exposure increases expression of presynaptic dopaminergic markers in mouse striatum." Neurotoxicology **26**(4): 721-728.

Caudle, W. M., J. R. Richardson, et al. (2007). "Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration." J Neurosci **27**(30): 8138-8148.

Chamero, P., T. F. Marton, et al. (2007). "Identification of protein pheromones that promote aggressive behaviour." Nature **450**(7171): 899-U823.

Champagne, F. A., J. P. Curley, et al. (2007). "Natural variations in postpartum maternal care in inbred and outbred mice." Physiol Behav **91**(2-3): 325-334.

Cooper, M. A., K. Kobayashi, et al. (2009). "Ephrin-A5 regulates the formation of the ascending midbrain dopaminergic pathways." Dev Neurobiol **69**(1): 36-46.

Cooper, M. A., A. I. Son, et al. (2008). "Loss of ephrin-A5 function disrupts lens fiber cell packing and leads to cataract." Proc Natl Acad Sci U S A **105**(43): 16620-16625.

Craig, I. W. and K. E. Halton (2009). "Genetics of human aggressive behaviour." Hum Genet **126**(1): 101-113.

Crawley, J. N. (2000). Whats Wrong With my Mouse, A. John Wiley & Sons.

Curley, J. P., C. L. Jensen, et al. (2012). "Variation in maternal and anxiety-like behavior associated with discrete patterns of oxytocin and vasopressin 1a receptor density in the lateral septum." Horm Behav **61**(3): 454-461.

Cutforth, T., L. Moring, et al. (2003). "Axonal ephrin-As and odorant receptors: coordinate determination of the olfactory sensory map." Cell **114**(3): 311-322.

Czarnecki, L. A., A. H. Moberly, et al. (2011). "In vivo visualization of olfactory pathophysiology induced by intranasal cadmium instillation in mice." Neurotoxicology **32**(4): 441-449.

Daar, I. O. (2011). "Non-SH2/PDZ reverse signaling by ephrins." Semin Cell Dev Biol.

Dalva, M. B., M. A. Takasu, et al. (2000). "EphB receptors interact with NMDA receptors and regulate excitatory synapse formation." Cell **103**(6): 945-956.

Davy, A., N. W. Gale, et al. (1999). "Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion." Genes Dev **13**(23): 3125-3135.

de Boer, S. F. and J. M. Koolhaas (2005). "5-HT1A and 5-HT1B receptor agonists and aggression: a pharmacological challenge of the serotonin deficiency hypothesis." Eur J Pharmacol **526**(1-3): 125-139.

Deacon, R. (2012). "Assessing burrowing, nest construction, and hoarding in mice." J Vis Exp(59): e2607.

Deacon, R. M. (2006). "Assessing nest building in mice." Nat Protoc **1**(3): 1117-1119.

Delville, Y., K. M. Mansour, et al. (1996). "Testosterone facilitates aggression by modulating vasopressin receptors in the hypothalamus." Physiol Behav **60**(1): 25-29.

Deschamps, C., M. Morel, et al. (2010). "EphrinA5 protein distribution in the developing mouse brain." BMC Neurosci **11**: 105.

Doty, R. L. (1986). "Odor-guided behavior in mammals." Experientia **42**(3): 257-271.

Drescher, U., C. Kremoser, et al. (1995). "In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases." Cell **82**(3): 359-370.

Egea, J. and R. Klein (2007). "Bidirectional Eph-ephrin signaling during axon guidance." Trends Cell Biol **17**(5): 230-238.

Emond, V., C. Joyal, et al. (2009). "[Structural and functional neuroanatomy of attention-deficit hyperactivity disorder (ADHD)]." Encephale **35**(2): 107-114.

Engle, E. C. (2010). "Human genetic disorders of axon guidance." Cold Spring Harb Perspect Biol **2**(3): a001784.

Factor, E. M., A. D. Mayer, et al. (1993). "Peripeduncular nucleus lesions in the rat: I. Effects on maternal aggression, lactation, and maternal behavior during pre- and postpartum periods." Behav Neurosci **107**(1): 166-185.

Falkner, A. L., P. Dollar, et al. (2014). "Decoding ventromedial hypothalamic neural activity during male mouse aggression." J Neurosci **34**(17): 5971-5984.

Faraone, S. V. and E. Mick (2010). "Molecular genetics of attention deficit hyperactivity disorder." Psychiatr Clin North Am **33**(1): 159-180.

Feierstein, C. E., F. Lazarini, et al. (2010). "Disruption of adult neurogenesis in the olfactory bulb affects social interaction but not maternal behavior." Frontiers in Behavioral Neuroscience **4**.

Feldheim, D. A., P. Vanderhaeghen, et al. (1998). "Topographic guidance labels in a sensory projection to the forebrain." Neuron **21**(6): 1303-1313.

Ferguson, J. N., L. J. Young, et al. (2000). "Social amnesia in mice lacking the oxytocin gene." Nat Genet **25**(3): 284-288.

Ferris, C. F., R. H. Melloni, Jr., et al. (1997). "Vasopressin/serotonin interactions in the anterior hypothalamus control aggressive behavior in golden hamsters." J Neurosci **17**(11): 4331-4340.

Ferris, C. F. and M. Potegal (1988). "Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters." Physiol Behav **44**(2): 235-239.

Flenniken, A. M., N. W. Gale, et al. (1996). "Distinct and overlapping expression patterns of ligands for Eph-related receptor tyrosine kinases during mouse embryogenesis." Dev Biol **179**(2): 382-401.

Foo, S. S., C. J. Turner, et al. (2006). "Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly." Cell **124**(1): 161-173.

Franklin, T. B., B. J. Saab, et al. (2012). "Neural Mechanisms of Stress Resilience and Vulnerability." Neuron **75**(5): 747-761.

Fraser, E. J. and N. M. Shah (2014). "Complex chemosensory control of female reproductive behaviors." PLoS One **9**(2): e90368.

Frisen, J., P. A. Yates, et al. (1998). "Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and topographic mapping in the mammalian visual system." Neuron **20**(2): 235-243.

Frye, C. A., M. E. Rhodes, et al. (2002). "Testosterone enhances aggression of wild-type mice but not those deficient in type I 5alpha-reductase." Brain Res **948**(1-2): 165-170.

Gammie, S. C. (2005). "Current models and future directions for understanding the neural circuitries of maternal behaviors in rodents." Behav Cogn Neurosci Rev **4**(2): 119-135.

Gandelman, R., M. X. Zarrow, et al. (1970). "Maternal behavior: differences between mother and virgin mice as a function of the testing procedure." Dev Psychobiol **3**(3): 207-214.

Gao, P. P., Y. Yue, et al. (1998). "Regulation of thalamic neurite outgrowth by the Eph ligand ephrin-A5: implications in the development of thalamocortical projections." Proc Natl Acad Sci U S A **95**(9): 5329-5334.

Gao, W. Q., N. Shinsky, et al. (1998). "Regulation of hippocampal synaptic plasticity by the tyrosine kinase receptor, REK7/EphA5, and its ligand, AL-1/Ephrin-A5." Mol Cell Neurosci **11**(5-6): 247-259.

Gaskill, B. N., C. J. Gordon, et al. (2012). "Heat or insulation: behavioral titration of mouse preference for warmth or access to a nest." PLoS One **7**(3): e32799.

Gerlai, R., N. Shinsky, et al. (1999). "Regulation of learning by EphA receptors: a protein targeting study." J Neurosci **19**(21): 9538-9549.

Giaginis, C., G. Tsourouflis, et al. (2010). "Clinical significance of ephrin (eph)-A1, -A2, -a4, -a5 and -a7 receptors in pancreatic ductal adenocarcinoma." Pathol Oncol Res **16**(2): 267-276.

Gilbert, D. L., K. M. Isaacs, et al. (2011). "Motor cortex inhibition: a marker of ADHD behavior and motor development in children." Neurology **76**(7): 615-621.

Giros, B., M. Jaber, et al. (1996). "Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter." Nature **379**(6566): 606-612.

Glick, N. R. (1991). "Principles and Practice of Endocrinology and Metabolism - Becker,Kl." Mental Retardation **29**(2): 111-111.

Glinka, M. E., B. A. Samuels, et al. (2012). "Olfactory deficits cause anxiety-like behaviors in mice." J Neurosci **32**(19): 6718-6725.

Gobrogge, K. L., Y. Liu, et al. (2007). "Anterior hypothalamic neural activation and neurochemical associations with aggression in pair-bonded male prairie voles." J Comp Neurol **502**(6): 1109-1122.

Grunwald, I. C., M. Korte, et al. (2004). "Hippocampal plasticity requires postsynaptic ephrinBs." Nat Neurosci **7**(1): 33-40.

Grunwald, I. C., M. Korte, et al. (2001). "Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity." Neuron **32**(6): 1027-1040.

Guellmar, A., J. Rudolph, et al. (2009). "Structural alterations of spiny stellate cells in the somatosensory cortex in ephrin-A5-deficient mice." J Comp Neurol **517**(5): 645-654.

Guillot, P. V. and G. Chapouthier (1996). "Olfaction, GABAergic neurotransmission in the olfactory bulb, and intermale aggression in mice: modulation by steroids." Behav Genet **26**(5): 497-504.

Hansen, S. (1989). "Medial hypothalamic involvement in maternal aggression of rats." Behav Neurosci **103**(5): 1035-1046.

Hattori, M., M. Osterfield, et al. (2000). "Regulated cleavage of a contact-mediated axon repellent." Science **289**(5483): 1360-1365.

Henderson, J. T., J. Georgiou, et al. (2001). "The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function." Neuron **32**(6): 1041-1056.

Henkemeyer, M., O. S. Itkis, et al. (2003). "Multiple EphB receptor tyrosine kinases shape dendritic spines in the hippocampus." J Cell Biol **163**(6): 1313-1326.

Herath, N. I., M. D. Spanevello, et al. (2006). "Over-expression of Eph and ephrin genes in advanced ovarian cancer: ephrin gene expression correlates with shortened survival." BMC Cancer **6**: 144.

Himanen, J. P., M. J. Chumley, et al. (2004). "Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling." Nat Neurosci **7**(5): 501-509.

Hruska, M. and M. B. Dalva (2012). "Ephrin regulation of synapse formation, function and plasticity." Mol Cell Neurosci **50**(1): 35-44.

Insel, T. R. and C. R. Harbaugh (1989). "Lesions of the hypothalamic paraventricular nucleus disrupt the initiation of maternal behavior." Physiol Behav **45**(5): 1033-1041.

Irie, F., M. Okuno, et al. (2005). "EphrinB-EphB signalling regulates clathrin-mediated endocytosis through tyrosine phosphorylation of synaptojanin 1." Nat Cell Biol **7**(5): 501-509.

J, F. K. and T. D. H (1976). "Territorial behavior of laboratory rats under conditions of peripheral anosmia." Animal Learning & Behavior **4**(3): 337-340.

Janes, P. W., N. Saha, et al. (2005). "Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans." Cell **123**(2): 291-304.

Jirkof, P. (2014). "Burrowing and nest building behavior as indicators of well-being in mice." J Neurosci Methods.

Johnson, S. K., K. M. Carlson, et al. (2003). "Effects of nicotine on target biting and resident-intruder attack." Life Sci **73**(3): 311-317.

Kent, P., H. Anisman, et al. (2001). "Central bombesin activates the hypothalamic-pituitary-adrenal axis. Effects on regional levels and release of corticotropin-releasing hormone and arginine-vasopressin." Neuroendocrinology **73**(3): 203-214.

Kessler, M. S., O. J. Bosch, et al. (2011). "Maternal care differs in mice bred for high vs. low trait anxiety: Impact of brain vasopressin and cross-fostering." Social Neuroscience **6**(2): 156-168.

Kim, S. Y., A. Adhikari, et al. (2013). "Diverging neural pathways assemble a behavioural state from separable features in anxiety." Nature **496**(7444): 219-223.

Klein, R. (2009). "Bidirectional modulation of synaptic functions by Eph/ephrin signaling." Nat Neurosci **12**(1): 15-20.

Klomberg, K. F., T. Garland, Jr., et al. (2002). "Dominance, plasma testosterone levels, and testis size in house mice artificially selected for high activity levels." Physiol Behav **77**(1): 27-38.

Knoll, B., K. Zarbalis, et al. (2001). "A role for the EphA family in the topographic targeting of vomeronasal axons." Development **128**(6): 895-906.

Koolhaas, J. M., C. M. Coppens, et al. (2013). "The resident-intruder paradigm: a standardized test for aggression, violence and social stress." J Vis Exp(77): e4367.

Koolhaas, J. M., T. H. C. Vandenbrink, et al. (1990). "Medial Amygdala and Aggressive-Behavior - Interaction between Testosterone and Vasopressin." Aggressive Behavior **16**(3-4): 223-229.

Kozlosky, C. J., T. VandenBos, et al. (1997). "LERK-7: a ligand of the Eph-related kinases is developmentally regulated in the brain." Cytokine **9**(8): 540-549.

Kruk, M. R. (1991). "Ethology and pharmacology of hypothalamic aggression in the rat." Neurosci Biobehav Rev **15**(4): 527-538.

Kruk, M. R., A. M. van der Poel, et al. (1979). "The induction of aggressive behaviour by electrical stimulation in the hypothalamus of male rats." Behaviour **70**(3-4): 292-322.

Kullander, K. and R. Klein (2002). "Mechanisms and functions of Eph and ephrin signalling." Nat Rev Mol Cell Biol **3**(7): 475-486.

Lacombe, A., V. Lelievre, et al. (2007). "Lack of vasoactive intestinal peptide reduces testosterone levels and reproductive aging in mouse testis." J Endocrinol **194**(1): 153-160.

Lesch, K. P., N. Timmesfeld, et al. (2008). "Molecular genetics of adult ADHD: converging evidence from genome-wide association and extended pedigree linkage studies." J Neural Transm **115**(11): 1573-1585.

Levy, F. and M. Keller (2009). "Olfactory mediation of maternal behavior in selected mammalian species." Behav Brain Res **200**(2): 336-345.

Li, J. D., K. J. Burton, et al. (2009). "Vasopressin receptor V1a regulates circadian rhythms of locomotor activity and expression of clock-controlled genes in the suprachiasmatic nuclei." Am J Physiol Regul Integr Comp Physiol **296**(3): R824-830.

Li, Y., H. Wang, et al. (2014). "Differential expression of hippocampal EphA4 and ephrinA3 in anhedonic-like behavior, stress resilience, and antidepressant drug treatment after chronic unpredicted mild stress." Neurosci Lett **566**: 292-297.

Liebl, D. J., C. J. Morris, et al. (2003). "mRNA expression of ephrins and Eph receptor tyrosine kinases in the neonatal and adult mouse central nervous system." J Neurosci Res **71**(1): 7-22.

Lim, Y. S., T. McLaughlin, et al. (2008). "p75(NTR) mediates ephrin-A reverse signaling required for axon repulsion and mapping." Neuron **59**(5): 746-758.

Lin, D., M. P. Boyle, et al. (2011). "Functional identification of an aggression locus in the mouse hypothalamus." Nature **470**(7333): 221-226.

Lisk, R. D., R. A. Pretlow, 3rd, et al. (1969). "Hormonal stimulation necessary for elicitation of maternal nest-building in the mouse (Mus musculus)." Anim Behav **17**(4): 730-737.

Liu, B., R. P. Kwok, et al. (1991). "Colchicine-induced increases in immunoreactive neuropeptide levels in hypothalamus: use as an index of biosynthesis." Life Sci **49**(5): 345-352.

Liu, D., J. Diorio, et al. (1997). "Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress." Science **277**(5332): 1659-1662.

Liu, H. X., O. Lopatina, et al. (2013). "Displays of paternal mouse pup retrieval following communicative interaction with maternal mates." Nat Commun **4**: 1346.

Lofgren, J. L., S. E. Erdman, et al. (2012). "Castration eliminates conspecific aggression in group-housed CD1 male surveillance mice (Mus musculus)." J Am Assoc Lab Anim Sci **51**(5): 594-599.

López-Larrea, C. (2011). Sensing in nature. New York

Austin, Tex., Springer Science+Business Media ;

Landes Bioscience.

Lucas, B. K., C. J. Ormandy, et al. (1998). "Null mutation of the prolactin receptor gene produces a defect in maternal behavior." Endocrinology **139**(10): 4102-4107.

Luttge, W. G. (1972). "Activation and inhibition of isolation induced inter-male fighting behavior in castrate male CD-1 mice treated with steroidal hormones." Horm Behav **3**(1): 71-81.

Maestripieri, D., A. Badiani, et al. (1991). "Prepartal chronic stress increases anxiety and decreases aggression in lactating female mice." Behav Neurosci **105**(5): 663-668.

Maletic, V., M. Robinson, et al. (2007). "Neurobiology of depression: an integrated view of key findings." Int J Clin Pract **61**(12): 2030-2040.

Mamiya, P. C., Z. Hennesy, et al. (2008). "Changes in attack behavior and activity in EphA5 knockout mice." Brain Res **1205**: 91-99.

Mandiyan, V. S., J. K. Coats, et al. (2005). "Deficits in sexual and aggressive behaviors in Cnga2 mutant mice." Nat Neurosci **8**(12): 1660-1662.

Manning, M., S. Stoev, et al. (2008). "Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V1a, V1b, V2 and OT receptors: research tools and potential therapeutic agents." Prog Brain Res **170**: 473-512.

Marcus, R. C., N. W. Gale, et al. (1996). "Eph family receptors and their ligands distribute in opposing gradients in the developing mouse retina." Dev Biol **180**(2): 786-789.

Marler, K. J., E. Becker-Barroso, et al. (2008). "A TrkB/EphrinA interaction controls retinal axon branching and synaptogenesis." J Neurosci **28**(48): 12700-12712.

Marquardt, T., R. Shirasaki, et al. (2005). "Coexpressed EphA receptors and ephrin-A ligands mediate opposing actions on growth cone navigation from distinct membrane domains." Cell **121**(1): 127-139.

McBride, K., B. Slotnick, et al. (2003). "Does intranasal application of zinc sulfate produce anosmia in the mouse? An olfactometric and anatomical study." Chem Senses **28**(8): 659-670.

McClelland, A. C., M. Hruska, et al. (2010). "Trans-synaptic EphB2-ephrin-B3 interaction regulates excitatory synapse density by inhibition of postsynaptic MAPK signaling." Proc Natl Acad Sci U S A **107**(19): 8830-8835.

McGinnis, M. Y., A. R. Lumia, et al. (2002). "Physical provocation potentiates aggression in male rats receiving anabolic androgenic steroids." Horm Behav **41**(1): 101-110.

Meaney, M. J. (2001). "Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations." Annu Rev Neurosci **24**: 1161-1192.

Meijer, W. M., A. Faber, et al. (2009). "Current issues around the pharmacotherapy of ADHD in children and adults." Pharm World Sci **31**(5): 509-516.

Melo, A. I., V. Lovic, et al. (2006). "Maternal and littermate deprivation disrupts maternal behavior and social-learning of food preference in adulthood: tactile stimulation, nest odor, and social rearing prevent these effects." Dev Psychobiol **48**(3): 209-219.

Miller, D. B. and J. P. O'Callaghan (2002). "Neuroendocrine aspects of the response to stress." Metabolism **51**(6 Suppl 1): 5-10.

Miyakawa, T., T. Yagi, et al. (2001). "Differential effect of Fyn tyrosine kinase deletion on offensive and defensive aggression." Behav Brain Res **122**(1): 51-56.

Mori, T., A. Wanaka, et al. (1995). "Differential expressions of the eph family of receptor tyrosine kinase genes (sek, elk, eck) in the developing nervous system of the mouse." Brain Res Mol Brain Res **29**(2): 325-335.

Mugford, R. A. and N. W. Nowell (1970). "Pheromones and their effect on aggression in mice." Nature **226**(5249): 967-968.

Murai, K. K., L. N. Nguyen, et al. (2003). "Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling." Nat Neurosci **6**(2): 153-160.

Nelson, R. J. (2006). Biology of aggression. Oxford ; New York, Oxford University Press.

Nelson, R. J. and S. Chiavegatto (2000). "Aggression in knockout mice." ILAR J **41**(3): 153-162.

Nelson, R. J. and B. C. Trainor (2007). "Neural mechanisms of aggression." Nature Reviews Neuroscience **8**(7): 536-546.

Nolt, M. J., Y. Lin, et al. (2011). "EphB controls NMDA receptor function and synaptic targeting in a subunit-specific manner." J Neurosci **31**(14): 5353-5364.

Numan, M. (2014). Neurobiology of social behavior: toward understanding of the prosocial and antisocial brain, elsevier.

Numan, M. and T. R. Insel (2003). The neurobiology of parental behavior. New York, Springer.

Numan, M. and D. S. Stolzenberg (2009). "Medial preoptic area interactions with dopamine neural systems in the control of the onset and maintenance of maternal behavior in rats." Front Neuroendocrinol **30**(1): 46-64.

Palmer, A. and R. Klein (2003). "Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function." Genes Dev **17**(12): 1429-1450.

Parkitna, J. R., A. Bilbao, et al. (2010). "Loss of the serum response factor in the dopamine system leads to hyperactivity." FASEB J **24**(7): 2427-2435.

Pasquale, E. B. (2004). "Eph-ephrin promiscuity is now crystal clear." Nat Neurosci **7**(5): 417-418.

Pasquale, E. B. (2005). "Eph receptor signalling casts a wide net on cell behaviour." Nat Rev Mol Cell Biol **6**(6): 462-475.

Passante, L., N. Gaspard, et al. (2008). "Temporal regulation of ephrin/Eph signalling is required for the spatial patterning of the mammalian striatum." Development **135**(19): 3281-3290.

Patel, A., A. Siegel, et al. (2010). "Lack of aggression and anxiolytic-like behavior in TNF receptor (TNF-R1 and TNF-R2) deficient mice." Brain Behav Immun **24**(8): 1276-1280.

Pedersen, C. A., J. A. Ascher, et al. (1982). "Oxytocin induces maternal behavior in virgin female rats." Science **216**(4546): 648-650.

Pedersen, C. A., J. D. Caldwell, et al. (1985). "Oxytocin Antiserum Delays Onset of Ovarian Steroid-Induced Maternal-Behavior." Neuropeptides **6**(2): 175-182.

Pedersen, C. A. and A. J. Prange, Jr. (1979). "Induction of maternal behavior in virgin rats after intracerebroventricular administration of oxytocin." Proc Natl Acad Sci U S A **76**(12): 6661-6665.

Pereira, M. and J. I. Morrell (2011). "Functional mapping of the neural circuitry of rat maternal motivation: effects of site-specific transient neural inactivation." J Neuroendocrinol **23**(11): 1020-1035.

Pitulescu, M. E. and R. H. Adams (2010). "Eph/ephrin molecules--a hub for signaling and endocytosis." Genes Dev **24**(22): 2480-2492.

Poelmans, G., D. L. Pauls, et al. (2011). "Integrated genome-wide association study findings: identification of a neurodevelopmental network for attention deficit hyperactivity disorder." Am J Psychiatry **168**(4): 365-377.

Raskin, K., K. de Gendt, et al. (2009). "Conditional inactivation of androgen receptor gene in the nervous system: effects on male behavioral and neuroendocrine responses." J Neurosci **29**(14): 4461-4470.

Reber, M., R. Hindges, et al. (2007). "Eph receptors and ephrin ligands in axon guidance." Adv Exp Med Biol **621**: 32-49.

Rich, M. E., E. J. deCardenas, et al. (2014). "Impairments in the initiation of maternal behavior in oxytocin receptor knockout mice." PLoS One **9**(6): e98839.

Rilling, J. K. and L. J. Young (2014). "The biology of mammalian parenting and its effect on offspring social development." Science **345**(6198): 771-776.

Robinson, D. L., D. L. Zitzman, et al. (2011). "Mesolimbic dopamine transients in motivated behaviors: focus on maternal behavior." Front Psychiatry **2**: 23.

Robinson, S., C. A. Penatti, et al. (2012). "The role of the androgen receptor in anabolic androgenic steroid-induced aggressive behavior in C57BL/6J and Tfm mice." Horm Behav **61**(1): 67-75.

Rodenas-Ruano, A., M. A. Perez-Pinzon, et al. (2006). "Distinct roles for ephrinB3 in the formation and function of hippocampal synapses." Dev Biol **292**(1): 34-45.

Rood, B. D., R. T. Stott, et al. (2012). "Site of origin of and sex differences in the vasopressin innervation of the mouse (Mus musculus) brain." J Comp Neurol.

Rood, B. D., R. T. Stott, et al. (2013). "Site of origin of and sex differences in the vasopressin innervation of the mouse (Mus musculus) brain." J Comp Neurol **521**(10): 2321-2358.

Ropartz, P. (1968). "The relation between olfactory stimulation and aggressive behaviour in mice." Anim Behav **16**(1): 97-100.

Rossi-George, A., F. LeBlanc, et al. (2004). "Effects of bacterial superantigens on behavior of mice in the elevated plus maze and light-dark box." Brain Behav Immun **18**(1): 46-54.

Sahin, M., P. L. Greer, et al. (2005). "Eph-dependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse." Neuron **46**(2): 191-204.

Sandau, U. S., Z. Alderman, et al. (2012). "Astrocyte-specific disruption of SynCAM1 signaling results in ADHD-like behavioral manifestations." PLoS One **7**(4): e36424.

Savelieva, K. V., I. Rajan, et al. (2008). "Learning and memory impairment in Eph receptor A6 knockout mice." Neurosci Lett **438**(2): 205-209.

Schuh, R. A., J. R. Richardson, et al. (2009). "Effects of the organochlorine pesticide methoxychlor on dopamine metabolites and transporters in the mouse brain." Neurotoxicology **30**(2): 274-280.

Schulz, K. M., T. A. Menard, et al. (2006). "Testicular hormone exposure during adolescence organizes flank-marking behavior and vasopressin receptor binding in the lateral septum." Horm Behav **50**(3): 477-483.

Sheleg, M., C. L. Yochum, et al. (2013). "Ephrin-A5 deficiency alters sensorimotor and monoaminergic development." Behav Brain Res **236**(1): 139-147.

Sherwin, C. M. (1997). "Observations on the prevalence of nest-building in non-breeding TO strain mice and their use of two nesting materials." Lab Anim **31**(2): 125-132.

Shih, J. C., M. J. Ridd, et al. (1999). "Ketanserin and tetrabenazine abolish aggression in mice lacking monoamine oxidase A." Brain Res **835**(2): 104-112.

Shingo, T., C. Gregg, et al. (2003). "Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin." Science **299**(5603): 117-120.

Shoji, H. and K. Kato (2009). "Maternal care affects the development of maternal behavior in inbred mice." Dev Psychobiol **51**(4): 345-357.

Shrenker, P., S. C. Maxson, et al. (1985). "The role of postnatal testosterone in the development of sexually dimorphic behaviors in DBA/1Bg mice." Physiol Behav **35**(5): 757-762.

Siegel, A. (2005). The neurobiology of aggression and rage. Boca Raton, CRC Press.

Siegel, A., T. A. Roeling, et al. (1999). "Neuropharmacology of brain-stimulation-evoked aggression." Neurosci Biobehav Rev **23**(3): 359-389.

Simon, N. G., A. Cologer-Clifford, et al. (1998). "Testosterone and its metabolites modulate 5HT1A and 5HT1B agonist effects on intermale aggression." Neurosci Biobehav Rev **23**(2): 325-336.

Singewald, N., P. Salchner, et al. (2003). "Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs." Biol Psychiatry **53**(4): 275-283.

Slotnick, B., D. Restrepo, et al. (2010). "Accessory olfactory bulb function is modulated by input from the main olfactory epithelium." Eur J Neurosci **31**(6): 1108-1116.

Snyder, J. A., M. W. Rogers, et al. (2004). "The impact of hemolysis on Ortho-Clinical Diagnostic's ECi and Roche's elecsys immunoassay systems." Clin Chim Acta **348**(1-2): 181-187.

Son, A. I., M. Sheleg, et al. (2014). "Formation of persistent hyperplastic primary vitreous in ephrin-a5-/- mice." Invest Ophthalmol Vis Sci **55**(3): 1594-1606.

St John, J. A., E. B. Pasquale, et al. (2002). "EphA receptors and ephrin-A ligands exhibit highly regulated spatial and temporal expression patterns in the developing olfactory system." Brain Res Dev Brain Res **138**(1): 1-14.

Stolzenberg, D. S. and M. Numan (2011). "Hypothalamic interaction with the mesolimbic DA system in the control of the maternal and sexual behaviors in rats." Neurosci Biobehav Rev **35**(3): 826-847.

Stolzenberg, D. S. and E. F. Rissman (2011). "Oestrogen-independent, experience-induced maternal behaviour in female mice." J Neuroendocrinol **23**(4): 345-354.

Stowers, L., P. Cameron, et al. (2013). "Ominous odors: olfactory control of instinctive fear and aggression in mice." Curr Opin Neurobiol **23**(3): 339-345.

Stowers, L., T. E. Holy, et al. (2002). "Loss of sex discrimination and male-male aggression in mice deficient for TRP2." Science **295**(5559): 1493-1500.

Svare, B., C. Betteridge, et al. (1981). "Some situational and experiential determinants of maternal aggression in mice." Physiol Behav **26**(2): 253-258.

Tachikawa, K. S., Y. Yoshihara, et al. (2013). "Behavioral transition from attack to parenting in male mice: a crucial role of the vomeronasal system." J Neurosci **33**(12): 5120-5126.

Takahashi, N., L. L. Miner, et al. (1997). "VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity." Proc Natl Acad Sci U S A **94**(18): 9938-9943.

Taniguchi, Y., M. Yoshida, et al. (1988). "The distribution of vasopressin- or oxytocin-neurons projecting to the posterior pituitary as revealed by a combination of retrograde transport of horseradish peroxidase and immunohistochemistry." Arch Histol Cytol **51**(1): 83-89.

Taylor, T. N., W. M. Caudle, et al. (2009). "Nonmotor symptoms of Parkinson's disease revealed in an animal model with reduced monoamine storage capacity." J Neurosci **29**(25): 8103-8113.

Tessier-Lavigne, M. and C. S. Goodman (1996). "The molecular biology of axon guidance." Science **274**(5290): 1123-1133.

Thomas, S. A. and R. D. Palmiter (1997). "Impaired maternal behavior in mice lacking norepinephrine and epinephrine." Cell **91**(5): 583-592.

Toda, K., T. Saibara, et al. (2001). "A loss of aggressive behaviour and its reinstatement by oestrogen in mice lacking the aromatase gene (Cyp19)." J Endocrinol **168**(2): 217-220.

Toren, P., M. Rehavi, et al. (2005). "Decreased platelet vesicular monoamine transporter density in children and adolescents with attention deficit/hyperactivity disorder." Eur Neuropsychopharmacol **15**(2): 159-162.

Trinh, K. and D. R. Storm (2003). "Vomeronasal organ detects odorants in absence of signaling through main olfactory epithelium." Nat Neurosci **6**(5): 519-525.

Tsuneoka, Y., T. Maruyama, et al. (2013). "Functional, anatomical, and neurochemical differentiation of medial preoptic area subregions in relation to maternal behavior in the mouse." J Comp Neurol **521**(7): 1633-1663.

Vahl, T. P., Y. M. Ulrich-Lai, et al. (2005). "Comparative analysis of ACTH and corticosterone sampling methods in rats." Am J Physiol Endocrinol Metab **289**(5): E823-828.

van Erp, A. M. and K. A. Miczek (2000). "Aggressive behavior, increased accumbal dopamine, and decreased cortical serotonin in rats." J Neurosci **20**(24): 9320-9325.

van Eyll, J. M., L. Passante, et al. (2006). "Eph receptors and their ephrin ligands are expressed in developing mouse pancreas." Gene Expr Patterns **6**(4): 353-359.

Veenema, A. H. (2009). "Early life stress, the development of aggression and neuroendocrine and neurobiological correlates: what can we learn from animal models?" Front Neuroendocrinol **30**(4): 497-518.

Veenema, A. H., A. Blume, et al. (2006). "Effects of early life stress on adult male aggression and hypothalamic vasopressin and serotonin." Eur J Neurosci **24**(6): 1711-1720.

Volkow, N. D., G. J. Wang, et al. (2007). "Brain dopamine transporter levels in treatment and drug naive adults with ADHD." Neuroimage **34**(3): 1182-1190.

vom Saal, F. S. and L. S. Howard (1982). "The regulation of infanticide and parental behavior: implications for reproductive success in male mice." Science **215**(4537): 1270-1272.

Wagner, G. C., D. R. Nabert, et al. (1983). "The Effects of Tail Shock on Target-Biting Behavior of Confined Mice." Aggressive Behavior **9**(4): 309-313.

Wang, T. H., J. L. Chang, et al. (2012). "EphrinA5 suppresses colon cancer development by negatively regulating epidermal growth factor receptor stability." FEBS J **279**(2): 251-263.

Wang, Z. S. and D. R. Storm (2011). "Maternal Behavior is Impaired in Female Mice Lacking Type 3 Adenylyl Cyclase." Neuropsychopharmacology **36**(4): 772-781.

Weber, E. M. and I. A. S. Olsson (2008). "Maternal behaviour in Mus musculus sp.: An ethological review." Applied Animal Behaviour Science **114**(1-2): 1-22.

Wersinger, S. R., H. K. Caldwell, et al. (2007). "Disruption of the vasopressin 1b receptor gene impairs the attack component of aggressive behavior in mice." Genes Brain Behav **6**(7): 653-660.

Wersinger, S. R., H. K. Caldwell, et al. (2007). "Vasopressin 1a receptor knockout mice have a subtle olfactory deficit but normal aggression." Genes Brain Behav **6**(6): 540-551.

Wersinger, S. R., E. I. Ginns, et al. (2002). "Vasopressin V1b receptor knockout reduces aggressive behavior in male mice." Mol Psychiatry **7**(9): 975-984.

Wersinger, S. R., J. L. Temple, et al. (2008). "Inactivation of the oxytocin and the vasopressin (Avp) 1b receptor genes, but not the Avp 1a receptor gene, differentially impairs the Bruce effect in laboratory mice (Mus musculus)." Endocrinology **149**(1): 116-121.

Wettschureck, N., A. Moers, et al. (2004). "Heterotrimeric G proteins of the Gq/11 family are crucial for the induction of maternal behavior in mice." Mol Cell Biol **24**(18): 8048-8054.

Wilkinson, D. G. (2001). "Multiple roles of EPH receptors and ephrins in neural development." Nat Rev Neurosci **2**(3): 155-164.

Willi, R., C. Winter, et al. (2012). "Loss of EphA4 impairs short-term spatial recognition memory performance and locomotor habituation." Genes Brain Behav.

Winslow, J. W., P. Moran, et al. (1995). "Cloning of AL-1, a ligand for an Eph-related tyrosine kinase receptor involved in axon bundle formation." Neuron **14**(5): 973-981.

Wu, Z., A. E. Autry, et al. (2014). "Galanin neurons in the medial preoptic area govern parental behaviour." Nature **509**(7500): 325-330.

Xu, N. J. and M. Henkemeyer (2011). "Ephrin reverse signaling in axon guidance and synaptogenesis." Semin Cell Dev Biol.

Xu, N. J., S. Sun, et al. (2011). "A dual shaping mechanism for postsynaptic ephrin-B3 as a receptor that sculpts dendrites and synapses." Nat Neurosci **14**(11): 1421-1429.

Yang, M. and J. N. Crawley (2009). "Simple behavioral assessment of mouse olfaction." Curr Protoc Neurosci **Chapter 8**: Unit 8 24.

Yoo, S., J. Shin, et al. (2010). "EphA8-ephrinA5 signaling and clathrin-mediated endocytosis is regulated by Tiam-1, a Rac-specific guanine nucleotide exchange factor." Mol Cells **29**(6): 603-609.

Young, W. S., J. Li, et al. (2006). "The vasopressin 1b receptor is prominent in the hippocampal area CA2 where it is unaffected by restraint stress or adrenalectomy." Neuroscience **143**(4): 1031-1039.

Yu, X., G. Wang, et al. (2013). "Accelerated experience-dependent pruning of cortical synapses in ephrin-A2 knockout mice." Neuron **80**(1): 64-71.

Yue, Y., J. Su, et al. (1999). "Selective inhibition of spinal cord neurite outgrowth and cell survival by the Eph family ligand ephrin-A5." J Neurosci **19**(22): 10026-10035.

Zametkin, A. J. and J. L. Rapoport (1987). "Neurobiology of attention deficit disorder with hyperactivity: where have we come in 50 years?" J Am Acad Child Adolesc Psychiatry **26**(5): 676-686.

Zarbalis, K. and W. Wurst (2000). "Expression domains of murine ephrin-A5 in the pituitary and hypothalamus." Mech Dev **93**(1-2): 165-168.

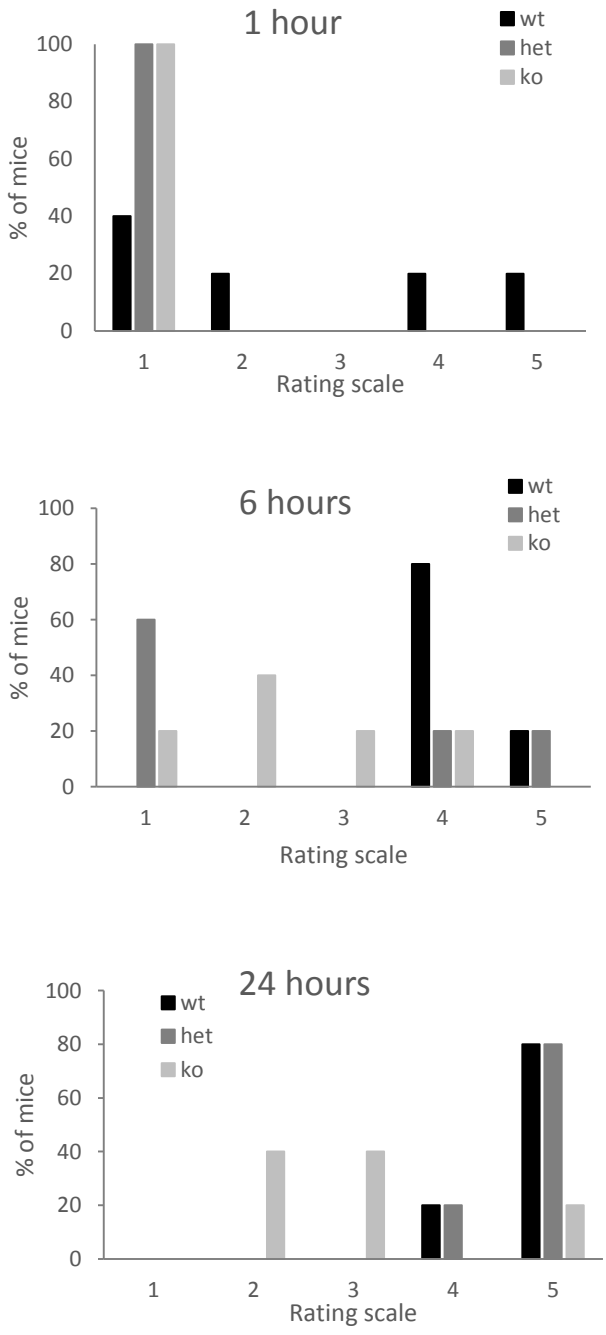
Zhang, J. H., D. P. Cerretti, et al. (1996). "Detection of ligands in regions anatomically connected to neurons expressing the Eph receptor Bsk: potential roles in neuron-target interaction." J Neurosci **16**(22): 7182-7192.

Zhou, Q. Y., C. J. Quaife, et al. (1995). "Targeted disruption of the tyrosine hydroxylase gene reveals that catecholamines are required for mouse fetal development." Nature **374**(6523): 640-643.

APPENDIX:

<u>Appendix-1:</u> Impaired nesting behavior in pregnant ephrin-A5$^{-/-}$ mice

Ephrin-A5$^{-/-}$ pregnant females had lower nesting score compared to heterozygous and wild-type controls.



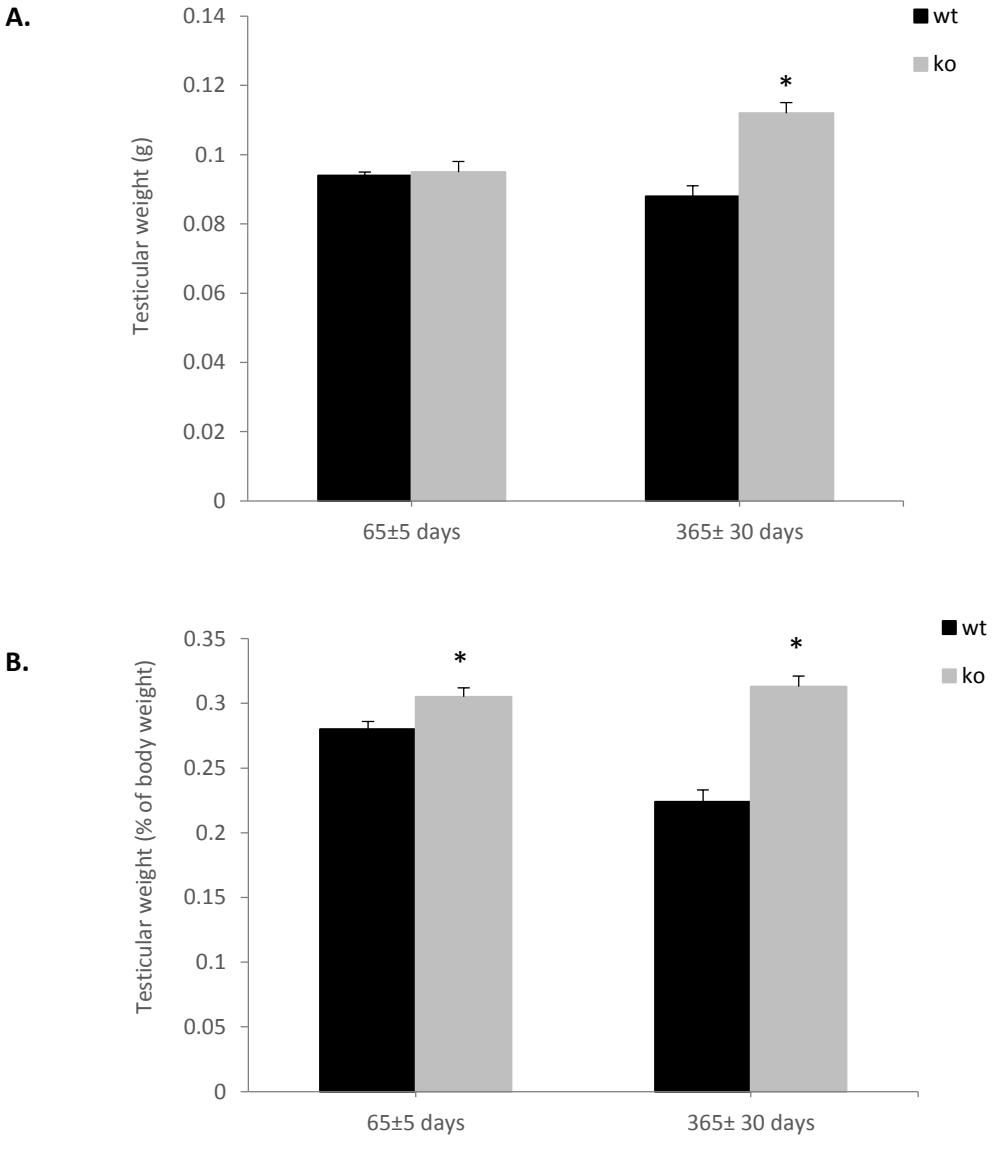
<u>Appendix-2:</u> Increased testicular weight in ephrin-A5$^{-/-}$ mice

Testicular weights were assessed in ephrin-A5$^{-/-}$ and wild-type male mice on 65±5 and 365±5 days after birth (n=11 for 65±5, per genotype and n=13 ko, 15 wt for 365±5).

(A) Testicular weight was significantly higher in null mice compared to wild-type mice [ANOVA, $F(1,46)=23.82$, $p<0.0001$)]. *Post hoc* test revealed that at 365±5 days after birth null mice had significantly higher weight ($p<0.0001$), this difference was not observed at 65±5 days after birth ($p=0.8174$).

(B) The percentage of testis weight was significantly different between the genotypes [ANOVA, $F(1,46)=53.01$, $p<0.0001$)]. Ephrin-A5$^{-/-}$ mice had higher percentage on both 65±5 ($p=0.013$) and 365±5 ($p<0.0001$) days after birth

* indicates significantly different from wild-type mice; $p<0.05$.



<u>Appendix-3:</u> Total AVP levels in the SCN

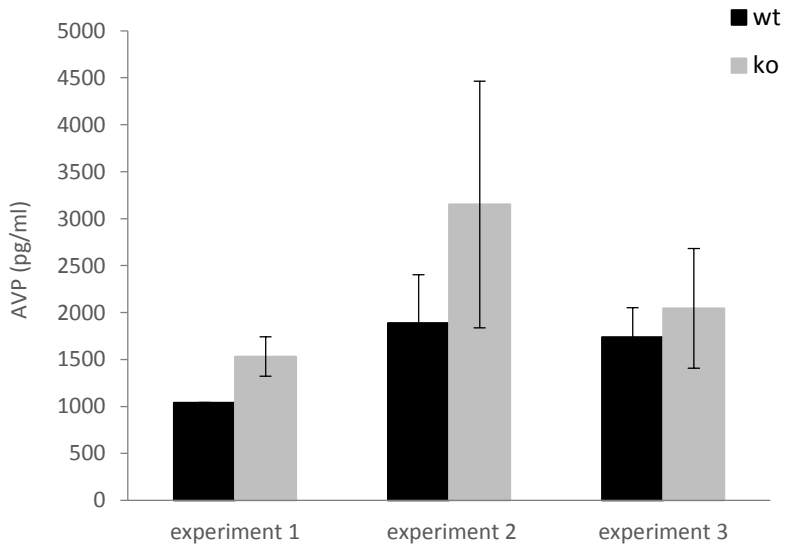
AVP levels were measured in the SCN of adult (p>60) ephrin-A5$^{-/-}$ and wild-type male mice (n=3 per genotype / experiment).

The entire SCN (400 µM) and part of the cortex (400µM) were dissected out, sonicated and boiled in 500 µl of 0.1N acetic acid containing 0.02N HCl for 10 minutes. Samples were then chilled on ice, centrifuged and the supernatant dried in spin vac and reconstituted in buffer.

AVP levels were measured using an AVP EIA kit (Cayman cat# 583951).

AVP levels in the cortex were used as negative controls and were subtracted from the AVP levels in the SCN.

No significant differences were found between the genotypes.



<u>Appendix-4:</u> SCN-AVP release in organotypic culture

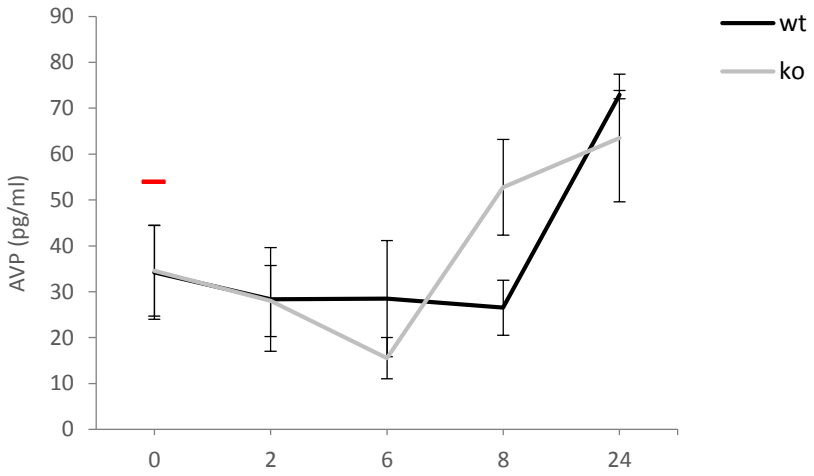
Organotypic SCN culture were made from adults (p>60 days) ephrin-A5$^{-/-}$ and wild-type mice (n=3 per genotype/ experiment). After 3 days, medium samples were taken at the following time points 0, 2, 6, 8 and 24 hours, and measured for AVP release using AVP EIA kit (Cayman cat# 583951).

- represent negative control.

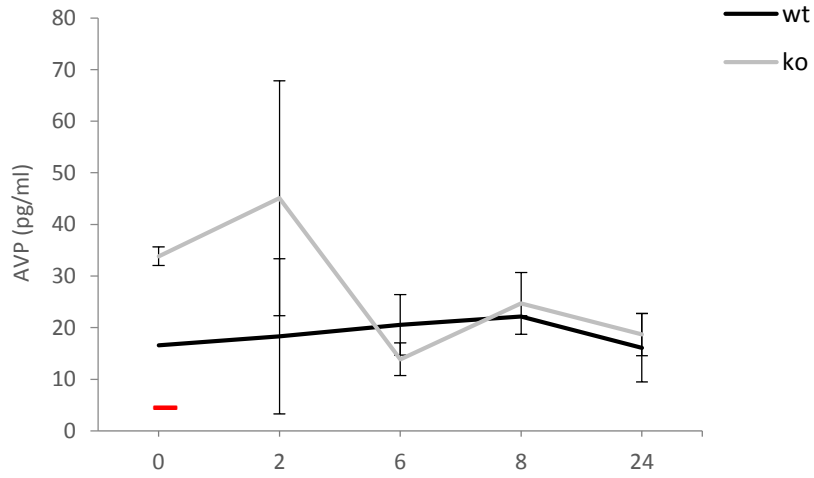
(A) Experiment 1- there were no significant differences between the genotype

(B) Experiment 2- there were no significant differences between the genotype

A.



B.



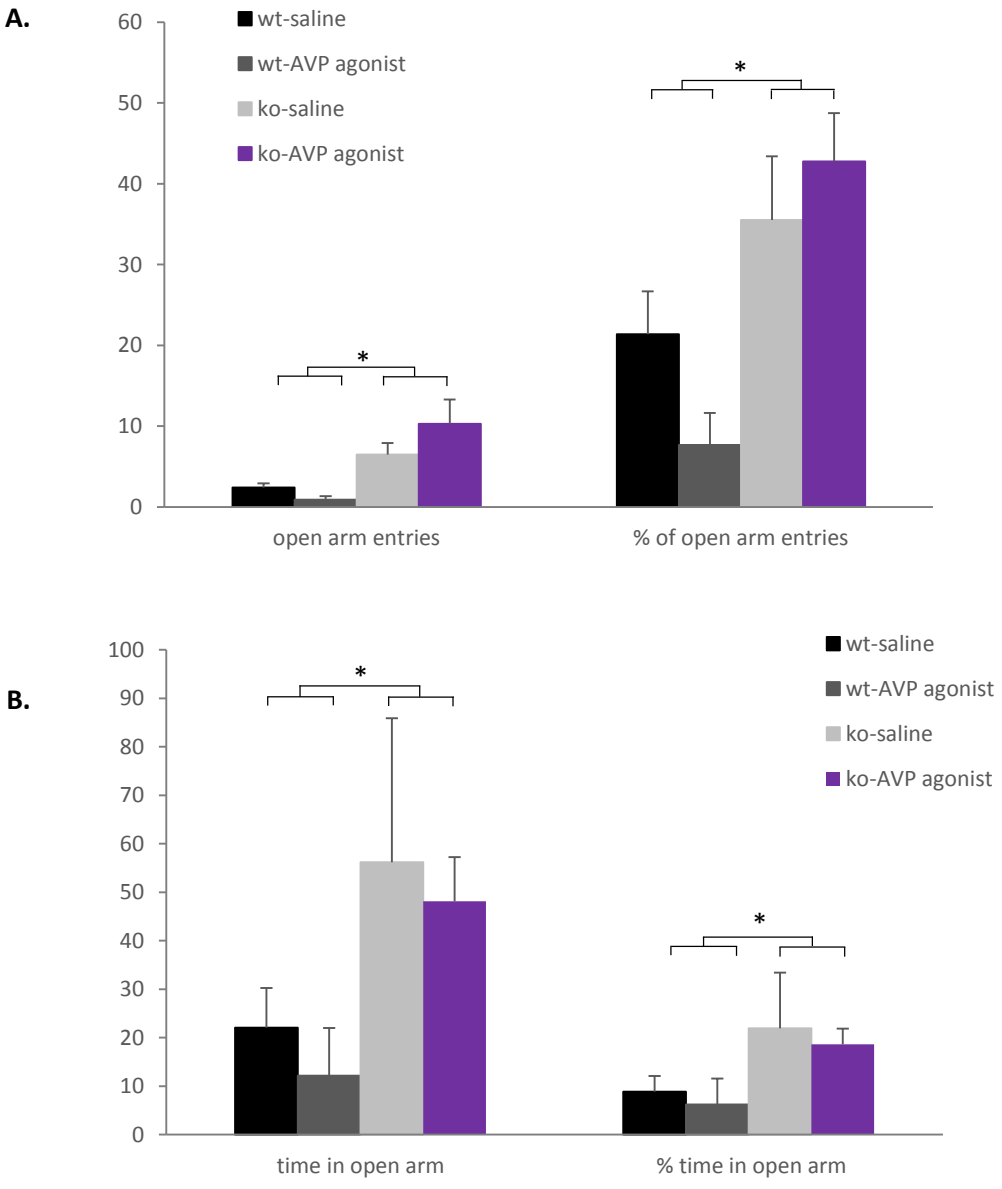
<u>Appendix-5:</u> Effect of intravenous injection of Desamino-[D-Arg8] vasopressin (DDAVP), an AVP agonist, on anxiety-like behavior in the elevated plus maze (EPM)

Adults (p>60 days) male ephrin-A5$^{-/-}$ and wild-type mice were injected with DDAVP (4 mg/kg) or saline (n=5 per genotype/ treatment) via the intravenous route (retro-orbital injection). Animals were tested on the EPM 20 minutes after injections

(A) DDAVP treatment did not change the number as well as the percentage of open arm entries in wild-type and ephrin-A5$^{-/-}$ mice. However, ephrin-A5$^{-/-}$ mice had increase in the number as well as percentage of open arm entries compared to wild-type mice.

(B) DDAVP treatment did not change the number as well as the percentage of time spent in the open arm in wild-type and ephrin-A5$^{-/-}$ mice. However, ephrin-A5$^{-/-}$ mice had increase in the time spent in the open arm compared to wild-type mice.

* indicates significantly different from wild-type mice; p<0.05.



<u>Appendix-6:</u> Impaired motor learning on the rotorod in ephrin-A5$^{-/-}$ mice

Motor skills and learning was measured on the rotorod in male and female ephrin-A5$^{-/-}$ (n=10 per sex) and wild-type (n=9 male and 10 females) mice. The latency to fall was significantly lower in ephrin-A5$^{-/-}$ females compared to wild-type females during the last 3 days of testing (Right panel). In addition both male and female wild-type mice showed increase motor learning on the rotating rod manifest by increased latency to fall between the first and last day of testing. This increased duration on the rotating rod over the test days was not observed for the null mice suggesting impairment in motor learning.

Data represent as mean latency to fall (sec) ±SEM.

* indicates significantly different from wild-type; $p<0.05$.