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Novel Roles of Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) and its Receptors in Social Behaviors and Stress Responses in the Mouse.

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Matthew Christopher Valdez

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Dissertation Committee:

Dr. Margarita Curras-Collazo, Chairperson
Dr. Michael Adams
Dr. Khaleel Razak

The Dissertation of Matthew Christopher Valdez is approved:

Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Novel Roles of Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) and its Receptors in Social Behaviors and Stress Responses in the Mouse.

by

Matthew Christopher Valdez

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Autism Spectrum Disorder (ASD) is an increasing concern for the world today. The incidence of ASD is steadily increasing every year (currently 1 in 68 U.S. children) but little is known of its etiology (U.S. Center for Disease Control). Research of ASD has centered around the development of mouse models that exhibit all characteristic behaviors of ASD, however those characteristic behaviors are constantly evolving the more we learn about the disorder. It has been proposed that further research of ASD should be directed at single characteristics of ASD in order to find neurobiological treatment targets [340]. We first characterize a social recognition deficit in an mouse lacking the gene for pituitary adenylate cyclase activating polypeptide (PACAP). PACAP is a highly conserved peptide hormone that is required for vasopressin release within the hypothalamus. We then demonstrated the requirement of PACAP in osmotically stimulated vasopressin release. Others have shown that osmotically stimulated vasopressin can improve social recognition by a yet unknown mechanism [68]. Epidemiological studies of ASD have suggested a role for environmental toxins in the etiology of ASD. We demonstrate for the first time that exposure to polybromi-

nated diphenyl ether's (PBDEs), flame retardants that persist in our environment, cause attenuation of social recognition ability. PBDE exposure also induced changes in plasma vasopressin and aberrant gene expression of both vasopressin and PACAP receptors in the brain. Finally, since increase anxiety and stress is comorbid with ASD, we investigated PACAPs role in psychogenic stress. We demonstrate that the PACAP receptor, vasoactive intestinal peptide receptor 2 (VPAC2r), is required for adrenal catecholamine release in response to psychogenic stress. Acute restraint stress decrease adrenal gene expression of VPAC2r concomitant with increase plasma epinephrine. Additionally, VPAC2r gene deletion attenuates plasma epinephrine release induced by acute restraint stress. These effects of PBDE exposure on PACAP signaling may further our understanding of the etiology of social disorders such as autism.

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Chapter 1

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is a member of a peptide hormone superfamily consisting of the vasoactive intestinal peptide (VIP), glucagon, secretin, gastric inhibitory peptide (GIP) and growth hormone-releasing hormone. Via the modulation of G-protein-coupled receptors (GPCR), these peptides play critical roles in a wide range of important biological functions that are strongly linked to hypothalamic function. These include stress responses [313], emotional behavior [248], social behavior [234], circadian rhythms [33], energy balance and glucose metabolism [230, 229, 259, 204, 40, 219], and osmoregulation [105, 278, 279].

Pituitary adenylate cyclase activating polypeptide (PACAP) is is the cleavage product of a 176-amino acid preproprotein that comprises a 24-amino acid signal peptide. At the C-terminal region of this precursor is PACAP-38, the first characterized 38 amino acids sample of PACAP isolated from ovine hypothalamus. It was later discovered that an internal cleavage site could yield a 27 amino acid peptide variant, PACAP-27 [217]. This

27 amino acid cleavage product contains the adenylate cyclase activating region. Upstream from PACAP-38 within the precursor, lies another peptide that is structurally similar to PACAP-27, and is thus referred to as PACAP-related peptide (PRP) [320].

The peptide sequence of PACAP is highly conserved across human, mouse, rat and sheep and near identical in some species of fish [320]. This high level of conservation suggests that PACAP may serve some crucial biological function. PACAP, along with VIP, are members of the secretin family of hormones that all activate G protein coupled receptors. Vasoactive intestinal peptide (VIP) is a 28 amino acid peptide that shares sizable degree, 68%, of sequence homology with PACAP. Thus, it is no surprise that the two peptides have common receptors that both mediate G coupled protein responses. Three major PACAP receptors are classified under two types: Type I receptors (PAC1R), of great importance to the CNS, bind PACAP-27 and PACAP-38 with a much higher affinity than VIP, and Type II receptors found predominantly in the periphery, that bind PACAP and VIP with essentially equivalent affinities. The Type II receptor are further divided based on their affinities for secretin and helodermin, other members of the secretin family of hormones [320, 129]. Each receptor contains a central seven transmembrane domain responsible for transducing extracellular signals as well as an extracellular amino, intracellular carboxyl terminus, and a relatively large extracellular N-terminus [98]. Splice variants have been well characterized for PAC1r, while the functional significance of splice variants of VPAC1 and VPAC2 have yet to be determined.

VPAC1 and VPAC2 are found throughout the CNS. VPAC1 is found in cerebral cortex and hippocampus [129] while VPAC2 is found through thalamus, suprachiasmatic

nucleus, hippocampus, brainstem, spinal cord and dorsal root ganglia [129]. PACAP and PAC1r have various roles throughout the periphery. PACAP and PAC1r have been localized throughout the enteric nervous system [211] and has been implicated in physiological responses to stress. Congruent with the presence of PACAP receptors on adrenaline-containing cells of the adrenal gland [282], PACAP is potent activator of catecholamine release [337, 336].

PACAP signaling is also important in development of the central nervous system. PAC1r is highly expressed in embryonic nervous tissues [277, 352, 335] and throughout brain regions that control emotions, such as olfactory bulb, amygdala, hypothalamus and cortex [129]. Therefore, PACAP signaling has long been thought to be involved in neurodevelopment and consequently the etiology of neuropsychiatric illness. In humans, the PACAP gene resides at 18p11, a chromosomal site that is associated with neurodevelopment disorders. Trisomy of chromosome 18 has been associated with the development of microcephaly and spina bifida and patients with tetrasomy of 18p suffer from microcephaly, mental retardation and congenital hydrocephalus [302]. 18p11 is also associated with an increased susceptibility of schizophrenia [85] and specific autistic phenotypes [235].

Early studies that revealed the abundance of PACAP and PAC1r throughout the developing neural tube suggested a critical role for PACAP signaling in neurodevelopment [285, 277, 352] and indeed further studies have supported this idea. Overexpression of PAC1r leads to decreased proliferation and increased apoptosis of neurons resulting in a reduced thickness of cerebral cortex and the corpus callosum [180]. Reductions in cortical and white matter densities can have severe implications as these markers have been correlated

with disorders such as autism [51, 52]. Furthermore, it has been found that cortical precursors produce and release PACAP in an autocrine fashion to initiate cell cycle withdrawal, allowing for the transition from proliferation to neuronal differentiation [193].

PACAP signaling has furthermore been implicated in behavioral abnormalities associated with disorders such as schizophrenia and autism-spectrum disorder (ASD), which is comorbid with attention deficit hyperactivity disorder (ADHD). PAC1r knockout mice have deficits in processing social olfactory cues and decreased social affiliative behaviors [234]. One of the hallmark behavioral abnormalities resulting from genetic knockout of PACAP is increased hyperactivity and explosive jumping [134, 202, 137]. These behaviors are ameliorated by both serotonin precursor and selective serotonin reuptake inhibitors [280].

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants (BFRs). PBDEs have been widely used in the United States as additives in consumer goods since the 1970's. In products such as high-impact plastics, foams, and various textiles, PBDEs can constitute 5-30% of these products by weight [207]. In the event of a fire, products containing PBDEs release active bromine atoms into the gas phase of the fire and quench the chemical reactions occurring in the flame, reducing the heat generated and slowing or even preventing the burning process.

The chemical formula of all PBDE's is $C_{12}H_{(0-9)}Br_{(1-10)}O$. The total number of hydrogens and bromine molecules inherent in the chemical must always equal 10 and are the foundation of many congeners that arise during their production. There are theoretically 209 possible congeners of PBDEs, which are divided into 10 congener groups based on the

number of bromines (mono- to deca-). Pure single congener formulations are not commonly manufactured for commercial use since the bromination of diphenyl ethers in practice results in mixtures of brominated diphenyl ethers (BDE) congeners. However, few congeners are found in commercial mixtures of PBDEs (e.g. DE-71 and DE-83r). The commercial formulations predominantly consist of penta-BDE (DE-71 and Bromkal 70-5DE), octaBDE (DE-79), and deca-BDE (DE-83r and Saytex 102E). Actually, the penta-BDE formulation is a mixture of tetra- and penta- congeners, and the octaBDE formulation consists of hepta- and octa- congeners. The deca-BDE formulations contain traces of almost all congeners [314].

In the 1994, the European Brominated Flame Retardant Industry Panel (EBFRIP), identified eight manufacturers producing PBDEs: Dead Sea Bromines/Eurobrome (The Netherlands); Atochem (France); Ethyl Corporation (USA); Great Lakes Chemical Corporation (USA); Tosoh (Japan); Matsunaga (Japan); Nippo (Japan); Great Lakes Chemical Ltd (United Kingdom). At that time, the global consumption of PBDEs was 40,000 metric tons. By 2003, the worldwide production of PBDEs had risen to over 55,000 metric tons. North, South and Central America consumed 95% of the global production of penta-BDE, 40% of octaBDE, and 44% of decaBDE.

In the early 2000's, notions of neuroendocrine disruption and neurotoxicity became the focus of investigations concerning PBDEs[29, 166, 324, 207, 73, 74]. Interestingly, around 2001, production began to favor the deca-BDE mixture accounting for 83% of worldwide production, followed by penta-BDE (11%), and octaBDE (6%). Then in 2004 the penta-BDE and octaBDE formulations were voluntarily withdrawn from the U.S. marketplace by

their manufacturers leaving only the most widely produced deca-BDE formulations marketed for use in commercial products in the United States. The penta-BDE and octaBDE formulations were banned throughout Europe, thus leaving the deca-BDE formulation as the only currently used formulation in Europe [314]. Alarmingly there is evidence that these higher brominated decaBDEs can break down in lower brominated products *in vivo* [70, 71].

PBDEs are highly soluble in lipids, and therefore are of concern for fatty tissues such as the brain. By utilizing radioactive PBDEs, it has been shown that PBDEs accumulate in heart, liver and brain following a even single neonatal dose [325]. If that dose is given within a critical window for neurodevelopment (gestation-PND3), the behavioral effects could be long lasting [325]. Mice given a single does of BDE-99 and BDE-47 at postnatal day 10 show persistent irregularities in spontaneous behavior concomitant with decreased ability to habituate in novel environments [74]. These effects on spontaneous behavior appear to result from the presence of the BDE congener during a critical window, as administration of BDE at postnatal day 19 had no effect on spontaneous behavior [73]. PCBs were known to interfere with the cholinergic system and also induce persistent changes in spontaneous behaviors. When exposed to the cholinergic agent, nicotine, mice that were dosed with PBDEs were hypoactive as opposed to controls that increased their activity [324]. The presence of PBDEs during neurodevelopment also influence learning and memory, which may be attributable to reductions in nicotinic acetylcholine receptors in the hippocampus [325]. In utero PBDE exposure has been shown to increase locomotion in adult mice. Interestingly, the same study found similar, although longer lasting effects,

in mice treated with the industrial mixture of PCBs, Arclor 1254 [29].

Most interestingly, PBDE have been shown to interfere with neurohormone systems. Of the most prominent effects is that of DE71, an industrial mixture of predominately pentaBDEs, on the thyroid system [50]. Various studies have reported the reduction in circulating thyroid hormone T4 levels with little changes to thyroid stimulation hormone, TSH [353, 263, 31]. This reduction appears to occur via peripheral actions of PBDEs on liver enzymes that control T4 breakdown [353]. PBDEs, along with PCBs, also affect the vasopressinergic system. Osmotic stimulation of acutely dissected supraoptic nuclei (SON) will respond to bath applied hyperosmotic stimulation by increasing somatodendritic vasopressin (SD-VP). This physiologically stimulated release is attenuated in the presence of both PCBs and PBDEs [44, 43]. This SD-VP release is thought to inversely control systemically released vasopressin via autoinhibition on SON neurons [196, 197]. Since the SON is one of the largest sources of vasopressin in the central nervous system, alteration of this central release can have major consequences for vasopressinergic circuits. Moreover, our lab has also shown that DE71 significantly reduces the expression of major regulator of vasopressin release, pituitary adenylate cyclase activating polypeptide.

Social recognition is a vasopressin/ oxytocin dependent behavior. It is thought that olfactory-based social recognition is processed through the main and accessory olfactory bodies, medial amygdala, bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA), and lateral septum. Indeed these areas increase in cFOS immunoreactivity in WT animals following the brief social exposure [22]. Interestingly, PACAP nerve fibers are found through out all of these areas [127]. What role PACAP has in social recognition is

yet to be revealed. Much of the research regarding social recognition has been centered around the neuropeptides, oxytocin and vasopressin.

Brattleboro rats lack vasopressin and have diminished social recognition ability [68]. However social recognition ability is restored in Brattleboro rats by microinfusion of vasopressin into the lateral septum. Both peripheral injection and intraventricular injection (icv) of vasopressin into rats increase their social recognition ability [186]. Site specific injections of vasopressin into the lateral septum of rats facilitated social recognition by increasing the time in which the memory was held [81, 69]. Additionally, osmotic stimulation of the supraoptic nucleus and paraventricular nucleus of rats increased in intrahypothalamic and intraseptal vasopressin and enhanced social recognition [68, 68].

Although PACAP's role in social memory has not yet been discovered, PACAP's role in mediating vasopressin release has been established. Magnocellular neuroendocrine cells of the SON increase the release of vasopressin from their soma and dendrites in response to osmotic stimulation. This increased intrahypothalamic vasopressin release is accompanied with increased release of PACAP and is blocked by PAC1r antagonists [105]. Additionally, PACAP alone stimulates vasopressin release from mangocellular neuroendocrine cells. Therefore, since osmotically stimulated intrahypothalamic vasopressin release enhances social recognition and this release is mediate by PACAP, we hypothesize that PACAP's role in social recognition is through its function as a vasopressin secretagogue. Whether or not PACAP has the same ability to modulate vasopressin release in other neuroanatomical structures such as the BNST and medial amygdala is unknown.

Hyperosmotic stimulation increases expression of vasopressin and PACAP in the

supraoptic nucleus. In rats perinatally treated with DE71, this increased expression of neuropeptides is greatly diminished. DE71 also reduces basal plasma vasopressin. The DE71 effect on vasopressin may be consequential to its affect on PACAP. Whether or not PACAP or vasopressin is reduced in other areas of the brain in DE71 exposed animals is unknown.

Chapter 2

Intra-adrenal VPAC2 receptors are regulated by psychogenic stress and are required for adrenal epinephrine responses

Introduction

The adrenal gland is critical for the regulation of blood pressure, electrolyte and energy homeostasis and responses to stress. The adrenal gland serves as an important organ for the integration of output from the three major stress systems, the hypothalamo-sympathoadrenal (HSA), hypothalamo–pituitary–adrenocortical (HPA) and the renin-angiotensin-aldosterone system (RAAS). Activation of these axes leads to the biosynthesis, release and/or storage of catecholamines (CA); glucocorticoid and mineralocorticoid hormones from the adrenal medulla and cortex, which have varied systemic and central targets. Pituitary adenylate cyclase-activating polypeptide (PACAP) has emerged as an important master regulator of both the HPA and HSA, and of anxiogenic behavior involved in stress responses [6, 103, 121, 295, 62]. In humans, PACAP and its receptors have recently been linked to acute and chronic stress-related disorders such as sudden infant-death syndrome [55], post-traumatic stress disorder [260], major depression, [135] and schizophrenia [129]. Along with the related peptide, vasoactive intestinal peptide (VIP), PACAP has other important functions in the central nervous system related to the control of circadian rhythms, learning and memory, and responses to injury [129].

Terminals of splanchnic nerve fibers innervating the adrenal gland colocalize PACAP and vesicular acetylcholine transporter (VAChT), a marker of cholinergic neurons, at all mouse adrenomedullary cholinergic synapses [117]. PACAP is also expressed abundantly within the adrenal medulla [282], and intrinsic PACAP immunoreactive fibers and PACAP mRNA in norepinephrine-immunoreactive adrenal chromaffin cells have been identified [158, 206, 46, 218], suggesting a local source of PACAP [95, 64, 145]. Similar observations

have been made for a related peptide, vasoactive intestinal peptide [46, 320]. PACAP has its own receptor, PAC1R, but it also shares PACAP type II receptors with VIP (VPAC1R, VPAC2R). PACAP and PACAP/VIP receptors are distributed across the medulla and zona glomerulosa (but not in zona fasciculata/reticularis). PACAP receptors are coupled to G-protein coupled receptors leading to activation of second messengers which can influence gene expression, CA biosynthesis and release [246, 244, 320]. However, many aspects of PACAP's role in adrenal responses to stress are unclear, including the participation of the PACAP type II receptor, VPAC2R, in the expression of catecholamine biosynthetic genes and responses to stress.

Functional and pharmacological studies have revealed that PACAP (and VIP) is a potent regulator of catecholamine secretion [46]. Using pharmacology, RT- PCR and quantitative autoradiography, Mazzocchi and others (2002) concluded that all three PACAP and PACAP/VIP receptors, PAC1, VPAC1 and VPAC2, are capable of evoking catecholamine release from the adrenal gland. Like VIP, PACAP (as low as 0.01μM) can also prolong ACh-induced catecholamine output responses [97, 336, 114]. It appears that both acetylcholine (ACh) and PACAP, acting within the adrenomedullary synapse, work synergistically to stimulate CA release during splanchnic nerve stimulation [177, 117], with PACAP's actions requiring PAC1-Rs [97]. During conditions of enhanced secretory demand, however, PACAP is the dominant adrenomedullary neurotransmitter since PACAP gene deletion abolishes stimulated (but not basal) CA secretion form adrenal tissue *in vitro* [293]. Not surprisingly, PACAP knockout leads to deficient compensatory catecholamine responses to hypoglycemia and stress-mimicking paradigms studied ex vivo [117, 305, 293].

PACAP is also critical for induction of adrenal mRNA levels of the CA biosynthetic enzymes, TH and PNMT, under some conditions of stress [291, 293]. Pharmacological studies have yielded inconclusive findings on the receptor mechanisms underlying PACAP-mediated responses to stress, in part, because of the lack of selective receptor ligands [206, 320]. One study concluded that regulation of CA biosynthesis in PC12 cultures may be mediated via PAC1 receptors rather than VPAC receptors, although continuous treatment with VIP, triggered a transient increase in TH mRNA content [48]. VIP, possibly acting via VPAC1 and VPAC2 receptors, may also use a paracrine process, involving catecholamines (and ACTH), to regulate adrenal secretion of aldosterone and corticosterone [206, 46, 312, 83]. Recently, Mustafa and others (2015)[228] have shown that the corticosterone elevation seen after a 7 day restraint stress paradigm is severely blunted in PACAP- but not PAC1R-deficient mice, suggesting involvement of other PACAP receptors in stress responses. Studies using VPAC receptor knockouts are needed to clarify the role of these receptors in the regulation of catecholamine responses to stress.

Stress is an important regulator of adrenal processes within the adrenal cortex as well as medulla. Central and peripheral PACAP exert significant regulation over adrenal-level stress hormones, corticosterone and catecholamines, possibly by activating the HPA, hypothalamo-sympatho-adrenal (HAS) axis [295, 295, 305]. Specifically, PACAP knockout mice show blunted stress-induced neuronal activation within the paraventricular nucleus of the hypothalamus, where CRH-producing and pre-autonomic neurons reside [188, 313, 291]. Moreover, PACAP, working through central CRH signaling, may influence anxiogenic behavior [62].

Some research reports indicate that certain adrenal responses may not depend on centrally- mediated HSA or HPA activation. For example, adrenal TH mRNA expression in response to immobilization stress is independent of splanchnic nerve activity and cholinergic antagonists [142]. In addition, NPY responses to stress persist even after hypophysectomy [175]. These findings suggest that adrenal responses to stress enjoy an intrinsic regulatory system. However, the regulation and significance of the intra-adrenal PACAP system during stress is not well understood. In the present study we examined the impact of acute restraint stress on the adrenal mRNA expression of PACAP in association with that of enzymes regulating catecholamine biosynthesis. We also investigated the participation of VPAC2Rs in biosynthetic markers of adrenal catecholamines and catecholamine responses to stress. Our results show that acute stress triggers catecholamine secretion raising plasma epinephrine levels but does not change PACAP, TH or PNMT gene expression. Gene deletion of VPAC2R significantly blunts stimulated epinephrine responses but did not change mRNA levels for catecholamine biosynthetic enzymes. These results suggest that intra-adrenal PACAP signaling via VPAC2R is critical for immediate epinephrine secretion to psychogenic stress. In WT mice, acute stress dramatically lowered VPAC2R transcript levels, possibly indicating the initiation of a compensatory or desensitizing response. Therefore, it appears that VPAC2Rs are critical for secretion of catecholamines during acute responses to stress and that VPAC2R gene expression is also regulated by stress. Finally, we found that gene deletion of VPAC2R increases PAC1R expression in both control and stress conditions. Taken together, these findings point to an intra-adrenal PACAP system that is regulated by psychogenic stress in vivo, and which, through VPAC2R, is critical for CA

secretion during acute psychogenic stress.

Materials and Methods

Animals

Vasoactive intestinal peptide receptor 2 null mice (VPAC2 KO) were obtained at 2-3 months of age. Briefly, the VPAC2 KO mice were used generated in E14/4 embryonic stem cells by replacing the translation start site of the vasoactive intestinal peptide receptor 2 gene with a 132 bp sequence LacZ-Neor cassette. VPAC2 KO mice exhibited no differences from WT littermates in gross morphology or fertility [130, 128]. At one month of age, genotyping was conducted by Transnetyx on tail tissue to verify the identity of homozygous VPAC2R gene deletion.

Confirmed male VPAC2 KO mice and C57BL/6 wild-type (WT) controls were maintained in accordance with the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were group-housed 3-4 per cage in standard polycarbonate plastic cages with heat-treated pine shavings as bedding unless otherwise noted. Food pellets (Purina Lab Diet) and water were provided *ad libitum* except during the experimental period. Temperature was maintained at 21 $\pm$ 2^{o}C and relative humidity at 50 $\pm$ 10% under a 12/12 hour light/dark cycle (lights on from 7:00–19:00 h). All experiments were approved by the IACUC on animal care and use at the University of California Riverside.

Restraint stress

Restraint devices were made from 50 ml clear polystyrene centrifuge tubes which were modified with numerous air holes for ventilation [39]. Restraint devices were placed on a wire cage top to allow for adequate ventilation and cooling of the animals. For acute stress, C57Bl6 mice were placed in the restraint devices for a single 1-hour session and then sacrificed 1 hour after return to their respective home cage. This approach enables examination of early changes in the expression of PACAP and associated gene partners. For chronic stress, C57Bl6 mice were placed in the restraint devices for a 1 hour period daily for two weeks and then sacrificed one hour after the final stress session. Stress sessions were carried out between 11 am and 3 pm. Blood was collected by cardiac puncture under isoflurane anesthesia. This immobilization paradigm was chosen because it can trigger transcriptional activation of genes participating in CA biosynthesis depending on duration of immobilization. Mice were sacrificed by decapitation and adrenals collected on dry ice. All mice used for acute stress were adult males at 2-4 months of age. Those use for chronic stress were adult males between 4-6 months of age.

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative RT-PCR was employed to examine the effect of restraint stress on genes of interest that are involved in regulating catecholamine synthesis in and release from the adrenal gland. We examined adrenomedullary markers, tyrosine hydroxylase (TH), the first synthetic and rate-limiting enzyme in the biosynthesis of catecholamines, and phenylethanolamine N-methyltransferase (PNMT), which methylates norepinephrine

to epinephrine [343]. qPCR was performed using the Bioline SensiFAST^{TM} SYBR No-ROX One-Step Kit and the Bio-Rad CFX 96 Real-Time PCR Detection System. The nucleotide sequences of qPCR primers are listed in **Table**6.2. A temperature gradient from 54-62^{o} C was used on the Bio-Rad CFX 96 station to capture ideal annealing temperatures.

Total RNA was prepared from snap-frozen adrenal glands with the Qiagen RNeasy^{R} Mini Kit. We modified the Qiagen RNA isolation protocol by performing a partial phenol-chloroform extraction instead of using the supplied RLT buffer which we found to be poor at lysing brain tissue. For each experiment, 1 μg of RNA was used per reaction. The reverse transcription was performed prior to the 2-step qPCR cycling protocol on the same plate. In brief, the qPCR plate was ran at 45^{o}C for 10 min. (*reverse transcription, RT*), 95^{o}C for 2 min. (*inactivation of RT enzyme*), then 95^{o}C for 5 sec. (*denaturation*), then 55^{o}C or 61.3^{o}C depending on the primers used for 20sec. (*annealing/extension*). The reactions were run for 40 cycles prior to melt curve analysis. Results are presented as fold expression relative to β-actin, which was determined via the Livak method [191].

Measurement of Plasma Epinephrine

Plasma Epinephrine was measured using a commercially available competitive enzyme-linked immunosorbent assay (ELISA) kit (Eagle Assays, ADU39-K01). Blood was removed via cardiac puncture and plasma was separated via refrigerated centrifugation and frozen at -80^{o}C. Within 10-14 days of plasma collection, 50-250 μl of plasma was removed from each sample and used for the epinephrine assay. The competitive Epinephrine ELISA kit utilizes a microtitre plate wherein epinephrine is bound to the solid phase. Acylated epinephrine from the sample and solid phase compete for finite number of antiserum binding

sites. After removal of excess antigen-antiserum complexes via washing, the resulting antibody bound to the solid phase catecholamine is detected by anti-rabbit IgG/peroxidase. The reaction of the peroxidase and substrate, 3,3',5,5'-Tetramethylbenzidine (TMB), is monitored at 450 nm on a plate reader. This assay is specifically designed for mouse/rat plasma and has a sensitivity of 1.6 pg/ml in a dynamic range of 0.15 - 25 ng/ml. Unknown plasma epinephrine was determined by interpolating absorbance values from a 4-parameter-logarithmic standard curve.

Statistical analysis

Statistical analysis was performed using RStudio [266]. General linear mixed model ANOVA was performed with the *lme4 package* [15] where data met normality and homogeneity of variance assumptions. In some cases, qPCR data was *log-transformed* prior to statistical analysis due to the inherent logarithmic structure of fold-computed data. Two-mean comparisons were determined using Student's t-test with or without equal variances, as needed. Multiple group comparisons were made using the *multcomp package* [146] using only the relevant subset of all-pairwise comparisons *(k matrix)*. Statistical significance was acknowledged at an alpha level of 0.05 or lower.

Results

Acute restraint stress upregulates plasma levels of Epinephrine. To determine the physiological consequence of VPAC2R gene deletion on adrenal medullary hormone output, we measured plasma Epinephrine one hours following acute restraint stress. **Figure**

6.1 shows that our stress paradigm elevated plasma Epinephrine by 72.7% (308.6 ± 45.0 and 532.9 ± 56.1 pg/ml for WT control and WT acute stress, n=7 & 10; respectively). In contrast, the stress-evoked increase in plasma Epinephrine levels was suppressed in VPAC2R KO mice after acute stress (472.6 ± 63.1 and 421.9 ± 46.5 pg/mL for VPAC2R KO control and acute stress, n=7 & n=6; respectively) . Two-way ANOVA revealed a statistically significant interaction between stress and genotype ($F(1,26) = 5.930$; $p=0.022$). Mean ± S.E.M. A post-hoc Tukey's test for multiple comparisons showed a significant elevation in plasma Epinephrine between WT control and WT stress groups ($t=2.996$, $p=0.006$). These results indicate that stress markedly triggered the release of plasma Epinephrine in WT but not VPAC2R knockout mice. We also wanted to investigate long term consequences of VPAC2R gene deletion. We used the chronic stress paradigm, which was the acute stress paradigm carried out for two weeks. While plasma epinephrine was seemingly elevated compared to the acute stress paradigm, there was no difference in between controls (683.9 ± 110.5 and 669.9 ± 136.1 pg/mL for WT control and WT chronic stress, n=5 & n=6; ; respectively) and chronic stress mice (565.6 ± 98.3 and 813.6 ± 114.6 pg/mL for VPAC2R KO control and VPAC2R KO chronic stress, n=5 & n=5; ; respectively). Mean ± S.E.M.

Acute restraint stress downregulates adrenal VPAC2R without affecting TH and PNMT transcript levels. In the present study one hour restraint significantly decreased mean transcript levels for VPAC2R relative to β-actin (**Figure 6.2C**). Means were significantly different using a Student's t- test ($t = 2.6589$, p-value = 0.007536, n=22). Mean ± S.E.M. $\Delta\Delta$Ct values for WT control and WT acute stress were 1.025 ± 0.234, n=10 and 0.543 ± 0.222, n=12, respectively. **Figure 6.2B** shows that gene expression of pituitary

adenylate cyclase activating polypeptide 1 receptor, PAC1R, was generally elevated between WT and VPAC2R KO mice and there was a significant effect of genotype ($F(1,27)= 5.3852$, $p=0.0281$). $\Delta\Delta$Ct values for WT control and WT acute stress were 1.113 ± 0.219, n=8 and 0.960 ± 0.154, n=8, respectively. $\Delta\Delta$Ct values for VPAC2R KO control and VPAC2R KO acute stress were 1.630 ± 0.409, n=8 and 1.573 ± 0.327, n=8, respectively.

No statistically significant differences were seen in mean relative transcript levels for TH, PNMT and PACAP in WT mice. Figure 3 shows that VPAC2R mice showed a similar profile as WT mice under both control and stress conditions. There was no statistically significant effect of stress on TH, PNMT and PACAP transcript levels.

It should be noted that mice showed no signs of adrenal hypertrophy. Adrenal weights were not significantly different amongst the groups, indicating that stress did not cause hypertrophy and, indirectly, that total β-actin is not likely to be different across groups. Mean ($\pm$ s.e.m.) values for WT control, WT $\pm$ stress, VPAC2R KO control, VPAC2R KO + stress were (in mg): 3.2 ± 0.9, 2.6 ± 1.0, 3.8 ± 0.6, 3.0 ± 1.6. Each group had a sample size of 6. A similar lack of effect was evident when Ct values were examined for β-actin transcripts. No differences were seen across treatment and genotype groups for each gene of interest. This indicates that Ct values for our reference gene β-actin were constant across experiment, and that our $\Delta\Delta$Ct method yielded reliable results.

VPAC2R gene deletion affects gene expression responses to chronic stress. Figure 6.3A chronic restraint stress did not affect gene expression of pituitary adenylate cyclase-activating peptide (PACAP) in WT. $\Delta\Delta$Ct values for WT controls and WT chronic stress were 1.042 ± 0.134, n=5, and 1.130 ± 0.149, n=4, respectively. VPAC2

KO mice had elevated transcript levels of PACAP in the no stress condition but not with chronic stress. ΔΔCt values for VPAC2R KO controls and VPAC2R KO chronic stress were 1.830 ± 0.328, n=4, and 0.976 ± 0.206, n=5, respectively. Means ± S.E.M. There was a significant interaction of genotype and treatment in our chronic stress PACAP gene expression ($F(1,13.23)=5.1425$, $p=0.04072$).

Figure 6.3B chronic restraint stress increase expression of pituitary adenylate cyclase-activating peptide receptor type 1 (PAC1r) in WT. ΔΔCt values for WT controls and WT chronic stress were 1.033 ± 0.195, n=3, and 2.477 ± 0.513, n=3,respectively. VPAC2R KO mice did not respond to chronic restraint stress with any change in PAC1R gene expression. ΔΔCt values for VPAC2R KO controls and VPAC2R KO chronic stress were 0.785 ± 0.124, n=4, and 1.132 ± 0.240, n=6, respectively. Means ± S.E.M. Two-factor ANOVA revealed a significant effect of treatment $F(1,10.89)= 9.0592$, $p=0.01200$ and a significant effect of genotype $F(1,10.89)= 7.0637$, $p=0.02245$.

Figure 6.3D chronic restraint stress increased expression of phenylethanolamine N-methyltransferase (PNMT) in a genotype independent fashion. ΔΔCt values for WT controls and WT chronic stress were 1.023 ± 0.155, n=3, and 2.943 ± 0.771, n=3, respectively. ΔΔCt values for VPAC2R KO controls and VPAC2R KO chronic stress were 0.830 ± 0.242, n=4, and 2.280 ± 0.335, n=6, respectively. Means ± S.E.M. We found a significant effect of treatment $F(1,10.74)=17.1769$, $p=0.001718$.

Figure 6.3E chronic restraint stress also increased expression of tyrosine hydroxylase (TH) independent of genotype. ΔΔCt values for WT controls and WT chronic stress were 1.027 ± 0.159, n=3, and 2.817 ± 0.661, n=3, respectively. ΔΔCt values for

VPAC2R KO controls and VPAC2R KO chronic stress were 0.428 ± 0.109, n=4, and 1.592 ± 0.277, n=6, respectively. Means ± S.E.M. Two-factor ANOVA revealed both a significant effect of treatment $F(1,11.42)=18.5164$, $p=0.001148$ and a significant effect of genotype $F(1,11.42)=7.0332$, $p=0.021884$.

Chapter Summary

Our study uses a transgenic mouse model to examine for the first time the role of VPAC2R in catecholamine responses to stress. Other physiological functions associated with VPAC2R are circadian rhythm of serum corticosterone concentration, growth and metabolic rate [83, 8]. In our study we show that VPAC2R-/- mice display compromised epinephrine secretory responses to acute stress. Acute restraint stress for 1 hour (followed by 1 hour rest) significantly increased plasma epinephrine levels in VPAC2R+/+ but not VPAC2R-/- mice, suggesting that VPAC2R is necessary for epinephrine responses to stress. We also assayed catecholamine response to chronic stress. There were no discernible differences in plasma epinephrine levels in response to two weeks of repeated stress. When compared to our acute stress mice, it appears that chronic stress evoked maximum epinephrine release thus establishing a ceiling. Other studies in rats have also shown a ceiling effect in catecholamine responses to chronic stress [329]. Results from previous reports are inconclusive regarding the participation on VPAC2R in catecholamine secretory responses and much less is known if they participate in CA responses to stress. Using pharmacology, RT-PCR and quantitative autoradiography, Mazzocchi and others (2002) concluded that all three PACAP and PACAP/VIP receptors, PAC1, VPAC1 and VPAC2, are capable

of evoking catecholamine release from the adrenal gland. Specifically, a maximally effective concentration of PACAP38 (10-8M) evokes a 3-fold increase in plasma levels of both plasma norepinephrine and epinephrine and this is reduced by antagonists selective for PAC1R/VPAC2R and VPAC1R. However, simultaneous exposure to both antagonists did not abolish the catecholamine response to PACAP, indicating a possible role for VPAC2R. Moreover, VPAC2R selective agonists like Ro25-1553 elicit a 60% increase plasma levels of norepinephrine and epinephrine [206, 320]. Although these pharmacological studies used pharmacological ligands without high selectivity, these data may indicate that VPAC2Rs may also participate in PACAP38-stimulated norepinephrine and epinephrine release [206]. We cannot rule out that VIP also contributes to the VPAC2R-mediated catecholamine secretion under psychogenic stress, although PACAP is a more potent secretagogue that VIP [46]. We now report that VPAC2Rs are critical for epinephrine responses to psychogenic stress.

Our results also warn about compensatory changes in PAC1R gene expression in the VPAC2R-/- mouse model.

There is limited information on the role of PACAP in mediating catecholaminergic responses to stress. PACAP may be required for adrenal epinephrine homeostasis necessary for survival during prolonged hypoglycemia [117]. Moreover, adrenomedullary CA responses to electrical stimulation mimicking stress ex vivo, also require PACAP [293]. Because PACAP appears to be critical for CA secretion triggered by metabolic and psychogenic stressors, Stroth and others (2013) have suggested that PACAP is the dominant adrenomedullary neurotransmitter during conditions of enhanced secretory demand. Rel-

evant to this, PACAP (and VIP) predominantly stimulate epinephrine relative to norepinephrine [114]. Our results showing that VPAC2R is required for stimulated epinephrine release indicates that VPAC2R may mediate the important role of adrenal PACAP during stress. The requirement of VPAC2Rs may reflect a deficient stimulus- secretion coupling or abnormal innervation/morphology of the adrenal medulla, processes associated with PACAP (and VIP) signaling [95, 46]. However, we found that adrenal weights from VPAC2R+/+ and VPAC2R-/- mice were no different. The drastic functional changes observed in VPAC2R (-/-) mice may also represent participation of central VPAC2Rs, associated with PACAPergic sympathetic functions [150, 64, 295, 305]. Indeed, VPAC2Rs have been localized on POMC-expressing hypothalamic neurons that may be associated with CA responses to stress [222]. However, the role of central VPAC2R is unknown.

It should be noted that intra-adrenal CA secretion may serve as an indirect way for local PACAP signaling to evoke corticosterone and aldosterone secretion from adrenal gland [129, 232, 242, 32]. This process is blocked by the antagonist PACAP6-38, but it remains to be seen if VPAC2R participate under these conditions [46]. Related to this, VPAC2R-dependent CA secretion may impact VPAC2R's regulation of circadian rhythmicity and activity- dependent secretion of adrenal mineralocorticoid and glucocorticoid hormones [206, 312, 83]. Using real time quantitative PCR we have shown that the mouse adrenal gland expresses PACAP, PAC1R and VPAC2R mRNA transcripts. Previous reports have shown mRNA for all PACAP receptors in adrenomedullary chromaffin cells and/or ganglion cells, all likely targets of extrinsic and intrinsic PACAP [218, 206]. Intra-adrenal PACAP mRNA has been demonstrated in adrenal medulla lacking splanchnic nerve fiber innervation and

intrinsic PACAPergic nerve fibers and cell bodies surrounding chromaffin cells have been reported [299, 95, 145, 218, 158, 206]. Interestingly, PACAP may be produced by TH- but not PNMT-expressing chromaffin cells [283]. Since sympathetic PACAPergic fibers in the splanchnic nerve innervating the adrenal are not likely to contain PACAP mRNA, our mRNA transcripts likely represent intra-adrenal sources. We also report 100% expression of VPAC2R in mouse whole adrenal glands. Previous autoradiography and PCR studies of rat adrenal glands and cultures of human adrenal medulla and zona glomerulosa cells have also shown gene expression of VPAC2R [206].

This study shows for the first time that psychogenic stress can alter adrenal expression of VPAC2R, indicating a possible role of VPAC2R in catecholamine secretory capacity. Under the specific conditions of acute challenge, VPAC2R transcription levels were decreased relative to the housekeeping gene β-actin. In contrast, the other genes of interest showed no changes due to stress. Other types of stress (maternal separation) do not appear to alter transcription of adrenal Vipr2 or Adcyap1 in neonatal rats possibly due to the age of the subject, and/or the more intense and prolonged nature of the stress [221]. Centrally, PACAPergic gene markers do respond to stressors, depending on the type and duration [122, 126, 72]. These changes may have functional consequences on stress responses, since PACAP signaling is required for neuronal activation of PVN neurons after restraint or defeat stress [188, 313]. Unfortunately, little is known about the regulation of VPAC2R gene. In combination with functional consequences of VPAC2R gene deletion, these results suggest that psychogenic stress triggers early changes in intra-adrenal VPAC2R gene expression. The significance of this may be linked to stimulus-secretion coupling in-

volving Ca2+. Alternatively, VPAC2R downregulation may represent a compensatory or desensitization mechanism. Future work will examine the role of VPAC2R on CA responses under chronic stress conditions.

In contrast to VPAC2R downregulation, parallel changes in gene markers for CA biosynthesis (i.e., TH and PNMT mRNA) were not seen probably because of the brief stress period. However, we did see such changes in our chronic stress mice. In support of this, Stroth and Eiden (2010) showed a significant increase in mouse adrenal TH and PNMT mRNA following 6 hours of unrelieved restraint, but not after a 1 hour period of restraint. A slightly longer stress paradigm (single immobilization for 2 h) can significantly increase adrenomedullary TH, and PNMT mRNA levels compared to unstressed mouse controls [172]. Our findings show that adrenals of VPAC2R knockout mice also lacked the stress-induced rise in mRNA transcripts for the catecholamine biosynthetic enzymes, TH and PNMT. Further support that VPAC2R may not regulate TH and PNMT genes is shown by the lack of effect of the VPAC2 antagonist N-AC-Tyr(1)-D-Phe(2)-GRF-(1-29)-NH(2) on PACAP-evoked transient (¡ 3hour) induction of TH transcription. In contrast, PACAP6-38 was effective in blocking PACAP actions suggesting PAC1R involvement [48, 320]. Nevertheless, our findings indicate that immediate regulation of intra-adrenal VPAC2R mRNA may be secondary to an acute secretory demand for epinephrine and may not impact short-term gene transcription of CA biosynthetic enzymes.

Taken together, our findings indicate a epinephrine secretory responses to psychogenic stress. The fact that acute stress can dramatically regulate local VPAC2R mRNA may represent an intra-adrenal PACAPergic system that is regulated by psychogenic stress

in vivo, and which, through VPAC2R, is critical for CA secretion during acute psychogenic stress. Additional study is necessary to determine the mechanisms underlying gene regulation of VPAC2R and to further elucidate the roles played by intra-adrenal PACAP signaling in the orchestration of sympathoadrenal responses.

Chapter 3

Pituitary adenylate cyclase activating polypeptide gene deletion impairs PVN vasopressin content and plasma vasopressin responses during chronic osmotic challenge

Introduction

PACAP's role in osmoregulation has been suspected since discovery of its presence in the osmosensitive and neuroendocrine regions of the hypothalamus [303, 171, 162]. The ability of intracerebroventricularly injected PACAP to elevate systemic vasopressin was first identified in the rat over 20 years ago [226]. Shortly thereafter, the dipsogenic effect of hypothalamically injected PACAP was characterized, adding to a growing body of evidence that PACAP plays a role in osmoregulation [254]. Further investigation revealed PACAP's ability to excite and trigger somatodendritic vasopressin release from magnocellular neuroendocrine cells (MNCs) of the supraoptic nucleus (SON) [278, 278]. Previous findings from our lab demonstrate that PACAP signaling via PAC1r is not only required but sufficient for stimulated vasopressin release from SON MNCs in response to hyperosmotic challenge [105]. Importantly, these findings were generated under conditions of endogenous PACAP release, which increases dramatically within the SON during acute hyperosmotic challenge. That the PACAPergic system participates in osmoregulation is further supported by our findings that PACAP-27, PACAP-38 and PAC1r immunoreactivity are significantly upregulated in acute hyperosmotic stimulated rats [105].

Therefore, PACAP seems to be a vital component of the osmoregulatory system within the hypothalamus. However, little is known of the physiological consequences of PACAP gene deletion on the dual release of vasopressin from MNCs during chronic osmotic challenge. In this study we also explore whether PACAP participates in ingestive behavior associated with the vasopressinergic system in the hypothalamus. Using a PACAP knockout (KO) model we test for deficits in hypothalamic content of vasopressin, both central and

systemic secretion of vasopressin, food intake, drinking, and plasma osmolality.

Materials and Methods

PACAP knockout mice

PACAP KO animals (backcrossed to C57BL/6 for greater than ten generations) were created and maintained at University of California at Los Angeles (UCLA) by the Waschek group as described previously [45]. At one month of age, PCR was conducted on tail tissue taken from animals at UCLA to verify they were homozygous PACAP knockout. Confirmed male PACAP KO mice and C57BL/6 wild-type (WT) controls from the same colony were transferred from UCLA to the University of California at Riverside (UCR) when they were around 2–3 month old. Animals were housed under standard vivarium condition with *ad libitum* access to food and water unless otherwise specified (see exceptions below). All procedures were approved by UCLA and UCR Animal Care and Use Committee and conducted in accordance with the guidelines in National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Osmotic Stimulation

For acute osmotic stimulation, on the day of sacrifice mice were injected with either 3.5 M NaCl (to produce hyperosmotic stimulation) or 0.9% NaCl (normosmotic) intraperitoneally (0.4-0.7 cc/100g body weight). 45 minutes later, animals were put under isoflurane anesthesia (5%:95% O2 induction and 2.5%:97.5% O2 maintenance). For salt-loading experiments, normosmotic mice were maintained on *ad libitum* access to standard

mouse chow pellets and water until the day of sacrifice. Hyperosmotic mice were provided 2g% saline drinking water for seven days and *ad libitum* access to standard mice chow pellets. For experiments involving basal release of vasopressin, mice were immediately put under anesthesia. While under anesthesia, blood was collected via cardiac punctures (0.3-1.0 ml) and centrifuged at 13,000rpm for 10-20 min at 4^oC to separate out the plasma. Plasma was then delipidized using acetone-petroleum ether treatment vacuum evaporated and resuspended in assay buffer for quantification of vasopressin.

Immunohistochemistry and Densitometry

Immunohistochemistry: Brain sections of perinatal A1254 exposed male WT and PACAP KO mice were transcardially perfused with 4% paraformaldehyde in phosphate buffer (PB) under pentobarbital anesthesia (150mg/kg body weight). Brains were removed, post-fixed overnight and sectioned into 60 μm coronal sections using a vibratome. Sections of the hypothalamus were then washed in cold PB plus 0.9g% NaCl (PBS) and incubated in a block/permeabilization solution containing 0.3% Triton-X and 1-3% bovine serum albumin for 30-45 min at room temperature according to a procedure modified from Decavel and Curras, 1997 [58]. Tissue sections were washed in PBS and incubated with a rabbit anti-VP-neurophysin (VP-NP) antibody (gift of G. Hoffman, University of Maryland; Roberts et al, 1993) at 1:150 dilution for 48 hr at 4^oC. After washes, sections were incubated with a polyclonal anti-rabbit IgG conjugated to horseradish peroxidase (Dako) for 30 min at room temperature followed by diaminobenzidine (Sigma). In each experiment methodological control sections received all solutions except the primary antibody. Sections were washed in PBS and mounted on gelatin-subbed glass slides and air-dried overnight. Sections were

dehydrated in an alcohol series and cover-slipped using DPX mounting medium (Electron Microscopy Sciences).

Densitometric Analysis: Bright-field microscope images were taken of SON, PVN and SCN (3-7 images per nucleus per mouse) using a digital camera (Spot Insight). Images were converted to a gray scale (0-255) after background subtraction using methodological controls. Images were then converted to gray scale, formatted as tif files and these were analyzed using computer-assisted densitometry (Image Pro Plus 4.5, Media Cybernetics). The SON, PVN and SCN were manually outlined in 3-6 sections per mouse. VP-NP immunoreactive densities for each area of interest were expressed as integrated optical density (IOD) calculated as density x area outlined and reported as arbitrary units. Mean IOD values for VP-NP were obtained for each experimental group by pooling IOD values from each region of interest.

In vitro experiments

Three to five month-old PACAP male knockout (KO) mice [108] and their C57BL/6 wild type (+/+) counterparts were maintained on *ad libitum* access to standard mouse chow pellets and water until the day of sacrifice. One week prior to the experiment, KO and WT mice were subdivided into two groups: tap-water and salt-water. The salt-water group was given *ad libitum* access to 2g% NaCl water while tap water group remained on standard vivarium tap water. During this period, the animals body weight, food intake (g) and water intake (g) were monitored daily between 1200-1500 hours. On the day of the experiment animals were sacrificed by decapitation, trunk blood was collected, plasma separated and osmolality was verified via osmometer (Wescor 5500). Brains were removed to cold,

oxygenated (100% O_2) aCSF (Locke's solution) and SON was dissected bilaterally from 1 mm coronal brain sections. Each bilateral SON sample was transferred into a micro-well containing oxygenated (100% O_2), Locke's solution (pH 7.4, 37^{o}C) matching to their respective osmolality to which bacitracin was added to retard tissue degradation. SON tissue was dissected from male KO and WT mice. Immediately after dissection, each bilateral SON sample was placed in a trans-well equipped with a semi-permeable basal membrane through which liquid could distribute into a slightly larger primary well containing aCSF (500 l of osmotically matched Locke's solution, pH 7.4, 37^{o}C) as described [105]. Following a 30-minute equilibration period, the analysate was replaced with 500 l of fresh Locke's solution and the SON punches were incubated for an additional 10-min experimental period, after which the analysate samples were collected. At the conclusion of the experimental period 200 l of analysate was collected and immediately frozen at –20^{o}C for subsequent VP analysis.

Quantification of VP

VP content in samples was measured using an enzyme-immunoassay (arg^8-vasopressin correlate enzyme-immunoassay kit, Assay Designs) and a 4-parameter curve fitting computer program (STATLIA; Brendan).

Statistics

Statistical analysis was performed using RStudio [266]. General linear mixed model ANOVA was performed with the *lme4 package* [15] where data met normality and homogeneity of variance assumptions. Two-mean comparisons were determined using Student's

t-test with or without equal variances, as needed. Multiple group comparisons were made using the *multcomp package* [146] using only the relevant subset of all-pairwise comparisons *(k matrix)*. Statistical significance was acknowledged at an alpha level of 0.05 or lower.

Results

Vasopressin immunoreactivity was identified in the supraoptic, paraventricular and suprachiasmatic nuclei of the hypothalamus in both wild type and PACAP knockout mice. In PACAP knockout mice vasopressin immunoreactivity decreased in the magnocellular region of the paraventricular nucleus (PVN) but not in the supraoptic nor suprachiasmatic nuclei. Reduced VP-NP immunoreactivity was observed in both the cell bodies and in axons projecting to the posterior pituitary. Mean optical density values were lower in PACAP KO as compared to WT in the PVN (1903.0 $\pm$ 230.7 vs. 2675.0 $\pm$ 60.94, respectively), but not the SON (3290.0 $\pm$ 109.6 vs. 3667.0 $\pm$ 140.5, respectively) nor the SCN (2439.0 $\pm$ 166.4 vs. 2516.0 $\pm$ 183.7, respectively) **Figure 6.7**. Unpaired Student's t-test revealed a significant difference between WT and KO vasopressin immunoreactivity in magnocellular cells of the PVN (t=2.485, df=6, p=0.0475).

The lower expression of vasopressin staining observed in some sections of the supraoptic nucleus (SON) was not statistically significant when comparing the mean optical density between WT and KO groups **Figure 6.7**.

Previously, PACAP has been shown to excite magnocellular neurons of the supraoptic nucleus and stimulate somatodendritic and plasma vasopressin responses [105, 278, 279]. Here we measured the effect of gene deletion on the PACAP's secretagogue influence on

somatodendritic vasopressin release by comparing basal and stimulated vasopressin concentration in tap water and salt water drinking groups, respectively. **Figure 6.5** demonstrates that, in gene-deleted mice, stimulated elevations of somatodendritic vasopressin was abolished in both SON. Mean vasopressin concentration values for WT mice treated with tap water and salt water drinking were 45.8 ± 5.4 pg/mL and 66.6 ± 9.7 pg/mL, respectively. Corresponding values for PACAP KO mice were 58.3 ± 13.0 and 51.9 ± 8.5 pg/mL, respectively. An unpaired Student's T test showed a significant difference in the WT group only (t=1.948,, df=33, p=0.03). No significant difference was found in the PACAP KO group.

To determine whether the reduction in stimulated vasopressin release was due to reduced stimulation we evaluated plasma osmolality values in stimulated and control groups of both genotype mice. Salt water drinking induced elevated plasma osmolality in both WT and PACAP KO mice after one week of dehydration (**Figure 6.6**). Mean plasma osmolality values were (313.5 ± 2.607 vs. 323.7 ± 2.148 for basal and stimulated WT mice, respectively (n=16-19). Mean plasma osmolality values were 306.2 ± 4.235 vs. 321.5 ± 4.438 in PACAP KO mice (n=9-11). Student t tests revealed significant increases in plasma osmolalities in both WT (t=3.042, df=33, p-value=0.0046) and PACAP KO (t=2.452, df=18, p-value=0.0246) confirming that dehydration has the same effect on plasma osmolality across genotype.

Although the stimulated condition shows similar elevations in plasma osmolality, the groups maintained on tap water, which are representative of a basal condition, did appear different. A comparison of only the tap water groups indicates a potential effect of PACAP gene deletion on basal plasma osmolality: t(24)= 1.676, p=0.0533.

To further investigate the consequences of diminished somatodendritic vasopressin release from SON magnocellular neurons, we next looked at changes in systemically released vasopressin. Although the specific details regarding the relationship between somatodendritic vasopressin release and the release of vasopressin from axon terminals in the posterior pituitary are unclear, they are intimately related [196, 195]. Given the proposed regulatory role of PACAP on somatodendritic vasopressin release, we sought to look for aberrations in stimulated systemic release of the neuropeptide. Mice were osmotically challenged with normosmotic and hyperosmotic saline administered via i.p. injection. As reported by others [195], hyperosmotic saline will evoke a peak plasma vasopressin response 30-60 minutes post injection. This was indeed the case for both male and female controls (**Figure 6.4A**: Mean plasma vasopressin concentrations for WT hyperosmotic vs normosmotic male mice were 746.3 $\pm$ 223.9 vs. 262.2 $\pm$ 29.2 pg/mL, respectively. in contrast, the robust increase in plasma vasopressin levels was obliterated in gene deleted mice. Mean values for hyperosmotic and normosmotic PACAP KO male mice were 185.6 $\pm$ 12.8 vs. 133.8 $\pm$ 13.7 pg/mL, respectively. Similar differences were measured under hyperosmotic and normosmotic conditions for female WT mice: 325.6 $\pm$ 34.2 vs. 131.2 $\pm$ 16.0 pg/mL and female PACAP KO mice: 106.3 $\pm$ 26.0 vs. 70.3 $\pm$ 25.7 pg/mL, respectively (**Figure 6.4B**).

To evaluate other homeostatic functions of the hypothalamus related to osmoregulation after PACAP gene deletion we monitored average daily food and water intake for 1 week. PACAP KO animals displayed significantly lower food intake (**Figure 6.8A**). A Student t test revealed that PACAP KO mice consumed significantly less food than WT ($t=4.921$, $df=38$, p-value<0.0001). Mean food intake values were 0.29 $\pm$ 0.01 vs 0.22 $\pm$ 0.01

g/$g_{b.w.}$/day for WT and PACAP KO mice, respectively. Additionally, when provided with *ad libitum* access to water, PACAP KO animals drank significantly less (**Figure 6.8B**). A Student t test revealed that PACAP KO mice consumed significantly less water compared to WT (t=4.297, df=37, p-value=0.0001). Mean food intake values were 0.36 ± 0.02 vs. 0.24 ± 0.02 mL/$g_{b.w.}$/day for WT and PACAP KO mice, respectively. The reduced food and water intake in PACAP KO animals may have contributed to a significantly reduced weight gain over a 6 day period (**Figure 6.8C**). A Student t test revealed that PACAP KO mice gain significantly less weight over 6 days (t=3.166, df=30, p-value=0.0035). Mean body weight gain values were 2.83 ± 0.63 vs. 0.36 ± 0.47 % g weight gain in 6 days for WT and PACAP KO mice, respectively.

Chapter Summary

We have previously shown that the endogenous PACAP system is regulated by osmotic state and that PACAP signaling is required for stimulated release of vasopressin within the SON [105]. Our current findings show that PACAP gene deletion suppresses stimulated vasopressin responses within the SON and PVN in mice stimulated osmotically in vivo. These results confirm our previous findings and extend them to PVN. In vivo stimulation via i.p. injection of hyperosmotic aCSF is a robust stimulus in activating the osmoregulatory mechanisms underlying vasopressin release into the circulation 45 min after challenge [105, 195, 65]. PACAP knockout mice also show abrogated plasma VP responses to osmotic challenge. The marked dysregulation seen in both somatodendritic and axonal VP responses may represent a general deficiency in the release machinery or in the sensitivity

of MNCs or of osmosensors feeding into vasopressinergic MNCs to osmotic signals. Another possibility is that VP-producing MNCs produce less VP in PACAP knockout mice. To examine this possibility we used immunohistochemistry to compare vasopressin content between wild type and PACAP KO mice. PACAP gene deletion did not downregulate VP-NP immunoreactivity in the SON, but did reduce content in the PVN. The reduced VP-NP content in the PVN seems to be due to both lower density of vasopressin-producing neurons and reduced immunoreactivity. Both magnocellular and parvocellular populations appear to be affected. VP content is a result of balance between input (production), output (release) and degradation.

The PACAP receptor, VPAC2R, has been reported to positively regulate gene expression of VP within the suprachiasmatic nucleus [130]. A similar action may occur in the SON and PVN which, if disturbed by PACAP gene deletion, may lead to reduced VP transcription and translation. A notable finding reported for PVN is that PACAP is colocalized in noradrenergic terminals terminating onto vasopressin parvocellular neurons, likely to be preautonomic. These neurons originate in the A1 group of the medulla and are implicated in autonomic control of the PVN [281]. The lack of this source of PACAP in KO mice may figure prominently in the reduction of vasopressin content in the PVN specifically. PACAP can regulate the release of vasopressin from SON neuroendocrine cells by increasing intracellular calcium [278, 278]. Therefore one reason for reduced vasopressin release during osmotic stimulation may pertain to calcium dysregulation. We speculate that a number of other processes impacted by PACAP may also contribute. PACAP increases NMDA receptor currents in CA1 neurons [200, 347], making it possible that synaptic glutamatergic

transmission is reduced in PACAP KO mice. In the SON, others and we have shown that hyperosmotic stimulation increases extracellular levels of glutamate and that glutamate receptors participate in hyperosmotic induced vasopressin release [287, 104]. These possible mechanisms potentially utilized by PACAP to regulate vasopressin release do not preclude a normal maintenance of vasopressin content. This is consistent with our finding of normal vasopressin immunoreactivity in the SON of PACAP KO mice.

Of great interest are the concomitant decreases in both somatodendritic and axonal vasopressin release in PACAP KO animals. It is known that peripheral osmotic stimulation can increase both somatodendritic and plasma vasopressin, but it does so sequentially [195, 195, 196]. The peak in plasma peptide content occurs within 30 min of osmotic stimulation, while that of the somatodendritic release takes an additional 2 hours when plasma vasopressin begins to normalize [195]. Somatodendritic vasopressin may act to restrain axonal release of vasopressin. For example, local application of a vasopressin within the SON dramatically reduces phasic bursting of MNCs [196]. In vivo microdialysis of V1Ar antagonist prolongs phasic bursting of SON MNCs, suggesting that somatodendtrically released VP acts to inhibit the activity of SON MNCs [33]. If the local release of vasopressin is presumably to reduce MNC activity and thereby reduce peripheral release, then how does loss of PACAP affect this auto regulation? In the present study, we measure the accumulation of somatodendritic vasopressin within a 10-minute period following a week of salt loading and the peripheral release of vasopressin 45-minutes after acute stimulation. Reductions in both forms of vasopressin release could be a direct result of the nature of the stimulation. Further investigation of the time profiles of vasopressin release from both SON and plasma

in a PACAP knockout animal may clarify the role of PACAP in the auto-regulation of the SON MNCs.

Since PACAP gene deletion reduced plasma VP responses to osmotic change, we tested for potential differences in the osmotic state of WT and KO after chronic dehydration. Our results show similar responsiveness of plasma osmolality values in both groups and maximal stimulated responses were similar. This suggests that the deficient plasma VP response is not likely due to differential osmotic activation. Another contributing factor to plasma osmolality is water intake. We found that PACAP KO mice drank significantly less water under basal conditions, a finding that could potentially aggravate the effects of chronic osmotic challenge the elevated. PACAP KO mice showed reduced plasma osmolality, a result that further confirms that PACAP is important for osmoregulation. At first, this result seems to be counterintuitive since these mice also show decreased basal plasma vasopressin and drink significantly less. This peculiar profile could be partly explained by reduced food intake, which itself is associated with water intake . Alternatively the reduced drinking in PACAP KO mice may be independent of plasma vasopressin content and osmolality. PACAP gene deletion may directly reduced water in take since intrahypothalamic administration of PACAP produces dipsogenic responses in rats [254].

Another possible reason is that in spite of reduced circulating vasopressin, the expression of vasopressin receptors in the kidney maybe unregulated. This possibility needs to be confirmed through future studies.

The role of PACAP in energy balance has been well established, but that precise nature of its role is still indefinable. Others have demonstrated that injection of

PACAP either intracerebroventricular [219, 224] or intrahypothalamically [40, 259] produces an anorectic effect immediately after injection lasting only a few hours. Interestingly, this study supports previous findings [229] that PACAP KO also show decreased food intake. In both studies, lower food consumption appears to be chronic, being that basal consumption was randomly monitored for days to weeks.

Chapter 4

Pituitary adenylate cyclase activating polypeptide gene deletion impacts social recognition ability independent of vasoactive intestinal peptide receptor 2

Introduction

Vasopressin, along with oxytocin, have been implicated in social behaviors to varying degrees and in varied circumstances [42, 22]. Early studies in the Brattleboro rat, a rat model that has a severely attenuated vasopressin system and impaired social recognition ability, found that microdialysis of vasopressin into the septum of these rats improved their ability to recognition social cues [67, 68]. Building from this early work in rats almost a decade later, researchers used a vasopressin receptor, V1Ar, knockout mouse to establish the requirement of vasopressin in social recognition [21]. Bielsky *et. al* demonstrated that mice lacking the V1Ar gene lacked social recognition ability and that they could restore the behavior by re-expressing the V1Ar receptor with a viral vector targeting the lateral septum [21]. Osmotic state was also found to influence social recognition ability as endogenous vasopressin release from within the hypothalamus is regulated by plasma osmolality [68]. One question left unanswered is the location of the source of vasopressin within the brain that mediates social behavior. Vasopressin is primarily released from magnocellular neurons residing within the supraoptic nucleus, both within the hypothalamus and from axon projections to the posterior pituitary [68, 196, 104]. There are other populations of vasopressin containing neurons throughout the brain including the suprachiasmatic nucleus, the medial amygdala and the paraventricular nucleus. However, the majority of immunoreactivity of vasopressin is within the supraoptic nucleus [104]. The distribution of vasopressin receptors is much more disperse than the distribution of cell bodies containing vasopressin. Presently, we know that vasopressin is critical for social interactions and but we are not clear what regions mediate its release. Within the supraoptic nucleus, PACAP and its recep-

tors are required for vasopressin-mediated osmoregulation [104]. Recently, we have shown that PACAP-deficient mice show aberrant vasopressin release from magnocellular neuroendocrine cells (MNCs) in supraoptic tissue punches challenged hyperosmotically *Chapter 3*. Since PACAP has a permissive role over vasopressin release from within the hypothalamus, and vasopressin is critical for social recognition, we investigated the role of PACAP in social recognition. Others have suggested a role for PACAP in social behaviors before [275]. PAC1 receptor knockout show abnormal response to social olfactory cues, indicating a role for PACAP in social recognition [234]. There are a dose-dependent disruptions in motivation, social interaction, and attention were produced by PACAP intracerebroventricular infusion [60]. There is even a PACAP-like gene in the fruit fly that mediates social memory [331]. In this study, we utilized the PACAP knockout mouse to investigate social recognition in addition to other social and anxiety related behaviors. We first corroborated previous studies that have established a behavioral profile for PACAP KO mice run on the elevated plus maze and open field test to validated our model. Near the completion of our study, we obtained a PACAP receptor knockout mouse, vasoactive intestinal peptide receptor (VPAC2R), as a gift and used these mice to follow up with results of the social recognition task in the PACAP KO mouse.

Methods

PACAP knockout mice

PACAP KO animals were obtained as described in **Chapter 3**. Briefly, animals were backcrossed to C57BL/6 for greater than ten generations and created and maintained

at University of California at Los Angeles (UCLA) by the Waschek group as described previously [45]. At one month of age, PCR was conducted on tail tissue taken from animals at UCLA to verify they were homozygous PACAP knockout. Confirmed male PACAP KO mice and C57BL/6 wild-type (WT) controls from the same colony were transferred from UCLA to the University of California at Riverside (UCR) when they were around 2–3 month old. Animals were housed under standard vivarium condition with *ad libitum* access to food and water. All procedures were approved by UCLA and UCR Animal Care and Use Committee and conducted in accordance with the guidelines in National Institutes of Health Guide for the Care and Use of Laboratory Animals.

VPAC2R knockout mice

Vasoactive intestinal peptide receptor 2 null mice (VPAC2 KO) were obtained as described in **Chapter 2**. Briefly, the VPAC2 KO mice were used generated in E14/4 embryonic stem cells by replacing the translation start site of the vasoactive intestinal peptide receptor 2 gene with a 132 bp sequence LacZ-Neor cassette. VPAC2 KO mice exhibited no differences from WT littermates in gross morphology or fertility [130, 128]. At one month of age, genotyping was conducted by Transnetyx on tail tissue to verify the identity of homozygous VPAC2R gene deletion. All procedures were approved by UCLA and UCR Animal Care and Use Committee and conducted in accordance with the guidelines in National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Elevated Plus Maze

We used the elevated plus maze to assess effects of gene deletion on anxiety [109]. The arms of the elevated plus maze were constructed out of black acrylic plastic measuring 3.5 inches wide by 44 inches long and stood 3 feet off the ground. The two arms that were closed had 8.5 inch black plastic walls. The entire maze was kept behind a black curtain with low light. Experimenters stood at opposite ends and observed the mice each mouse for 5 min. trials. At the beginning of each trial mice were placed in the middle of the maze facing a closed arm. Time and frequency was collected with stopwatches and tally counters, respectively.

Ultrasonic Vocalization

Male PACAP KO mouse was placed in an enclosure containing a petri dish with 10 grams of soiled bedding collected from female mouse cages. Ultrasonic vocalizations were recorded for 5 min using a Peterson Elektronic AB Ultrasonic Detector maintained 10cm from the edge of the container. Vocalizations were later visualized using Batsound software. Call characteristics were assessed using customized MATLAB software provided by the Razak lab at UC Riverside.

Open Field

To test for hyperactivity we used the Open Field test [109]. The apparatus consists of a chamber made from plexiglass and covered with white vinyl to increase the contrast to better visualize the dark mice. Each mouse was placed in the center of the apparatus and videotaped for one hour. Analysis was performed in 5 minute blocks using Ethovision

7.1 software donated by the Carde lab at UC Riverside (Nodlus). Within each time bin, distances traveled (inches) and average velocity (inches/second) were measured.

Social Recognition Test

We used a paradigm adapted from Macbeth et al 2009 [199] (refer to schematic for details on construction of the setup). Single housed male mice were transferred from the vivarium to the test room the morning of the trials. Male mice were allowed to habituate to test cage for 30 min and then to wire-mesh corrals introduced into cage for 30 min. Following the hour of habituation, one corral was removed from the cage and a gonadally intact female mouse was placed into the remaining corral to begin a 5 min Trial 1. The female mouse was removed and the subject was again left alone in the cage with two empty corrals for 30 min. In trial 2, both corrals remained in the cage and the initial (familiar) female and a novel female were placed in the corrals for 5 min. Digital video records were later analyzed using JWatcher in a double-blind manner. Videos were watched at half speed and the time of olfactory investigation (defined as a directed nose poke within 1cm of the corralled mouse) was scored. Later the times of investigation were summed and dived by 2 to reflect actual time.

Sociability

Sociability was performed in a three-chamber apparatus using methods described previously by Yang et al 2011. The apparatus was a rectangular, three-chamber box made from clear polycarbonate. Retractable doorways within the two dividing walls allowed access from the center to the side chambers. The day of the experiment the subject mouse

(male) was allowed to acclimate to the apparatus for 20 min before the sociability test – 10 min in the central chamber with the doors closed, followed by 10 min in the entire empty arena with the doors open. The subject was then briefly confined to the center chamber while empty inverted wire cages (the novel object) were placed in the side chambers. A female mouse used as the initial stimulus target for measurement of sociability was placed inside only one of the wire cages in the side chambers. After both stimuli were positioned, the doors were simultaneously reopened and the mouse allowed access all three chambers for 10 mins to assess sociability. Number of entries and time spent in the chambers were manually determined from recorded video using the event-recorder software JWatcher

Olfactory Preference Test

These experiments were adapted from a protocol made by Witt *et. al* 2009 [341]. Mice were placed in a series of 4 large cages for 15 min to habituate. After habituation in the fourth cage, 2 x 2 inch squares of blotting paper saturated with 10 % peanut butter, 1 % butyric acid or water were presented in separate trials to mice for 3 min with 1 min breaks between trials. The trials were recorded on a digital video camcorder and the videos were analyzed using JWatcher in a double- blind manner.

Quantification of VP

VP content in samples was measured using an enzyme-immunoassay (arg^8-vasopressin correlate enzyme-immunoassay kit, Assay Designs) and a 4-parameter curve fitting computer program (STATLIA; Brendan).

Statistical analysis

Statistical analysis was performed using RStudio [266]. General linear mixed model ANOVA was performed with the *lme4 package* [15] where data met normality and homogeneity of variance assumptions. Two-mean comparisons were determined using Student's t-test with or without equal variances, as needed. Multiple group comparisons were made using the *multcomp package* [146] using only the relevant subset of all-pairwise comparisons *(k matrix)*. Statistical significance was acknowledged at an alpha level of 0.05 or lower.

Results

Elevated Plus Maze

It has been reported that PACAP KO mice have deficits in anxiety when run on an elevated plus maze [134]. In **Figure 6.9** we found that WT mice prefer to spend more time in the closed arms of the elevated plus maze compared the open arms (150.040 $\pm$ 7.414 vs. 81.286 $\pm$ 7.310, respectively, n=45; Paired t-test: t =-5.1582, df= 44, p-value =5.709e^{-06}). Mean $\pm$ S.E.M. PACAP KO mice also spent more time in the closed arms of the maze compared to the open arms (134.252 $\pm$ 7.076 vs. 103.100 $\pm$ 8.670, respectively, n=38; Paired t-test: t =-2.0077, df= 37, p-value =0.05202), however the difference was not as great as in the WT.

Ultrasonic Vocalization

We sought to determine if PACAP gene deletion affected any form of communication in the mouse. Mice predominantly communicate via olfactory cues, however other

forms of communication have been proposed [342, 297]. Mice emit ultrasonic vocalizations (U.S.V.s) and it has been suspected that this may be one form of communication. To assess effects of PACAP gene deletion on communication male mice were presented with soiled bedding from female cages in a novel environment for 5 minutes. U.S.V.s were detected on an ultrasonic detector. Recordings were processed in *MATLAB* and several characteristics were analyzed. First we analyzed the calls per second and **Figure 6.11A** shows no discernible difference (PACAP KO: 2.98 ± 0.58 calls/second vs. WT: 3.59 ± 0.52 calls/second; Approximative Two-Sample Fisher-Pitman Permutation Test: Z = -0.78623, p-value = 0.4391). We also looked at the inverse, seconds without calls, and in like manner **Figure 6.11B** no major difference across genotype (PACAP KO: 199.22 ± 26.81 seconds vs. WT: 162.25 ± 25.25 seconds; Welch Two Sample t-test: t = -1.0039, df = 14.999, p-value = 0.3313). **Figure 6.11C** depicts the average call duration (PACAP KO: 30.27 ± 1.99 seconds vs. WT: 31.05 ± 1.31 seconds; Welch Two Sample t-test: t = 0.32555, df = 13.544, p-value = 0.7497). **Figure 6.11D** shows that there was no relevant difference in total call duration (PACAP KO: 14884.89 ± 4778.94 seconds vs. WT: 18169.26 ± 3468.40 seconds; Welch Two Sample t-test: t = 0.55621, df = 14.158, p-value = 0.5868). We found a consistent average call frequency (kHz) across genotypes (**Figure 6.11E**: PACAP KO: 68.71 ± 1.00 seconds vs. WT: 70.86 ± 1.51 seconds; Welch Two Sample t-test: t = 1.1861, df = 12.386, p-value = 0.2578). Finally, **Figure 6.11F** show no difference in dynamic range (PACAP KO: 23111.18 ± 2486.27 decibels vs. WT: 24175.42 ± 2265.48 decibels; Welch Two Sample t-test: t = 0.3164, df = 14.99, p-value = 0.7561).

Open Field

Others have reported that PACAP gene deletion results in increased and sustained locomotion [134]. To corroborated these findings in our mice we placed them in an open field maze for an hour while video recording. Videos were analyzed with Ethovision^{TM} track tracing software (Nodlus) and distance traveled (*inches*) and average velocity (*inches/second*) was record in 5 minute time bins. Our WT mice traveled longer distances in the first few time bins then gradually decreased distance traveled as the hour long trial progressed (**Figure 6.10A**: *00-05min*: 857.26 ± 47.47 in.; *05-10min*: 771.54 ± 61.45 in.; *10-15min*: 684.25 ± 67.00 in.; *15-20min*: 672.08 ± 81.92 in.; *20-25min*: 545.33 ± 72.84 in.; *25-30min*: 504.94 ± 73.01 in.; *30-35min*: 453.95 ± 70.69 in.; *35-40min*: 458.55 ± 58.92 in.; *40-45min*: 379.93 ± 64.48 in.; *45-50min*: 300.61 ± 66.04 in.; *50-55min*: 348.36 ± 67.83 in.; *55-60min*: 398.49 ± 57.83 in.; n=10; Mean ± S.E.M.). In stark contrast, the PACAP KO mice traveled a comparable distance in the first time bin and maintained that distance traveled per time bin for the duration of the hour long trial (**Figure 6.10A**:*00-05min*: 1098.66 ± 93.16 in.; *05-10min*: 1142.33 ± 137.36 in.; *10-15min*: 1075.81 ± 114.41 in.; *15-20min*: 1095.54 ± 165.03 in.; *20-25min*: 1164.07 ± 174.61 in.; *25-30min*: 1094.41 ± 185.90 in.; *30-35min*: 1152.75 ± 168.64 in.; *35-40min*: 1022.11 ± 110.55 in.; *40-45min*: 1151.91 ± 159.64 in.; *45-50min*: 1020.11 ± 190.05 in.; *50-55min*: 1050.82 ± 146.04 in.; *55-60min*: 779.89 ± 104.16 in.; n=5; Mean ± S.E.M.). A repeated measures two-factor ANOVA revealed a significant effect of genotype ($F(1,13)=18.44$, p-value=0.000873), time bin ($F(11,143)=20.428$, p-value$<2e^{-16}$) and their interaction ($F(11,143)=7.445$, p-value$=5.17e^{-10}$). The velocity profiles complemented the profile of distance traveled. The WT mice moved faster in the first few time

bins, then decreased in speed as the hour proceeded (**Figure 6.10B**: *00-05min*: 2.97 ± 0.11 in./sec.; *05-10min*: 2.75 ± 0.18 in./sec.; *10-15min*: 2.47 ± 0.21 in./sec.; *15-20min*: 2.43 ± 0.24 in./sec.; *20-25min*: 1.93 ± 0.23 in./sec.; *25-30min*: 1.76 ± 0.21 in./sec.; *30-35min*: 1.65 ± 0.19 in./sec.; *35-40min*: 1.62 ± 0.18 in./sec.; *40-45min*: 1.37 ± 0.19 in./sec.; *45-50min*: 1.15 ± 0.19 in./sec.; *50-55min*: 1.29 ± 0.23 in./sec.; *55-60min*: 1.31 ± 0.19 in./sec.; n=10; Mean ± S.E.M). In contrast, the PACAP KO mice maintained a fast speed of travel through the entirety of the hour long trail (**Figure 6.10B**: *00-05min*: 3.66 ± 0.31 in./sec.; *05-10min*: 3.82 ± 0.46 in./sec.; *10-15min*: 3.59 ± 0.38 in./sec.; *15-20min*: 3.65 ± 0.55 in./sec.; *20-25min*: 3.88 ± 0.58 in./sec.; *25-30min*: 3.65 ± 0.62 in./sec.; *30-35min*: 3.84 ± 0.56 in./sec.; *35-40min*: 3.40 ± 0.37 in./sec.; *40-45min*: 3.84 ± 0.53 in./sec.; *45-50min*: 3.43 ± 0.65 in./sec.; *50-55min*: 3.50 ± 0.48 in./sec.; *55-60min*: 2.59 ± 0.34 in./sec.; n=5; Mean ± S.E.M). A repeated measures two-factor ANOVA revealed a significant effect of genotype ($F(1,13)=17.55$, p-value=0.00106), time bin ($F(11,143)=21.587$, p-value$<2e^{-16}$) and their interaction ($F(11,143)=7.322$, p-value$=7.59e^{-10}$).

Social Recognition

In addition to U.S.V., mice communicate predominantly via olfactory ques [42]. Mice identify one another via olfactory investigation of mouse urinary proteins (M.U.P.s) which are unique to each individual mouse [42]. The social recognition task relies on the ability of a mouse to recall a familiar mouse based on social memory of olfactory cues. When presented with a familiar animal and a novel animal, mice will spend more time investigating the animal that is unfamiliar. In this study, we compared WT mice to PACAP KO mice and the PACAP receptor (VPAC2r) KO mouse to further investigate the role of PACAP in

social interactions. **Figure 6.12** shows that WT mice did spend more investigating novel mice in the presence of a familiar mouse (Familiar: 20.99 ± 2.16 sec., n=22 vs. WT Novel 37.24 ± 3.59 sec., n=22). The PACAP KO mice however, spent nearly equal amount of time investigating both familiar and novel mice during the test (Familiar 28.61 ± 3.69 sec., n=22 vs. PACAP KO Novel 33.96 ± 2.63 sec., n=22). Interestingly, the VPAC2r KO did not show the deficit displayed in the PACAP KO mice (Familiar: 57.46 ± 6.91 sec., n=18 vs. Novel: 87.86 ± 6.53 sec., n=18).

An ANOVA of type III with Satterthwaite approximation for degrees of freedom revealed a significant effect of genotype ($F(2,3.157)=15.022$, p-value=0.024) and stimulus animals $F(1,116.22)=28.164$, p-value=$5.408e^{-07}$). A *post-hoc* multiple comparisons shows that within groups, only the WT and VPAC2r KO mice spent significantly more time investigating novel mice over familiar ones while the PACAP KO mice spent equivalent amounts of time investigating each.

Sociability

In order to further explore the social ramifications resulting from PACAP gene deletion we ran all three sets of mice (WT, PACAP KO and VPAC2r) through the three-chambered sociability test [349]. The three chambered apparatus is interconnected via two sliding doors the each separate the middle chamber from the an other chamber. In each of the outer chambers, there is either an empty metal corral (novel object chamber) or a corral containing a mouse (social chamber). A preference towards sociability is indicated by more time spent in the social chamber compared to the novel object chamber. **Figure 6.13** shows that WT mice spent more time in the social chamber compared to the middle

chamber and novel object chamber (346.07 ± 29.17 sec.; 86.76 ± 14.78 sec.; 167.17 ± 19.64 sec., respectively, n=19). PACAP KO mice also spent more time in the social chamber compared to the middle and novel object chamber (460.58 ± 22.44 sec.; 36.89 ± 4.86 sec.; 102.53 ± 19.12 sec., respectively, n=13). Additionally, VPAC2r KO mice also spent more time in the social chamber compared to the middle chamber and novel object chamber (336.61 ± 21.82 sec.; 54.79 ± 5.55 sec.; 175.91 ± 12.76 sec., respectively, n=18). An ANOVA of type III with Satterthwaite approximation for degrees of freedom revealed a significant interaction of genotype and chamber preference ($F(2,93.972)=10.694$, p-value=$6.541e^{-05}$). Multiple Comparisons revealed that each genotype spent significantly more time in the social chamber compared to the novel object chamber (WT p-value= $2.23e^{-09}$; VPAC2r KO p-value= $2.20e^{-07}$; PACAP KO p-value $<2e^{-16}$).

Olfactory Preference

Since the social recognition task, and to a lesser degree the sociability test, are dependent on olfactory cues, we performed a cursory olfactory preference test. Mice were presented with absorbent paper saturated with 4 odors: peanut butter, butyric acid, vanilla and water. The saturated papers were randomly presented to the mice and times of investigation were recorded (**Figure 6.14**). WT mice preferred the peanut butter odor over all other odors (*butyric acid*: 7.84 ± 1.31 sec., n=43; *peanut butter*: 27.76 ± 5.04 sec., n=43; *vanilla*: 9.38 ± 1.41 sec., n=43; *water*: 9.45 ± 1.76 sec., n=35). PACAP KO mice also preferred peanut butter odor over other odors (*butyric acid*: 5.78 ± 1.69 sec., n=31; *peanut butter*: 29.26 ± 6.10 sec., n=31; *vanilla*: 6.97 ± 1.39 sec., n=31; *water*: 7.22 ± 1.60 sec., n=24). Despite the normal olfactory preference profile in the PACAP KO mice, the

VPAC2r KO mice did not show a preference for any odor (*butyric acid*: 19.49 ± 3.00 sec., n=19; *peanut butter*: 18.02 ± 2.06 sec., n=19; *vanilla*: 16.36 ± 3.31 sec., n=19; *water*: 19.12 ± 3.95 sec., n=19). An ANOVA of type III with Satterthwaite approximation for degrees of freedom revealed a significant interaction of genotype and odor ($F(6,339.88)=2.6102$, p-value=0.01736).

Plasma Vasopression

It has been demonstrated that peripherally administered vasopressin can influence social recognition ability [22]. It is not known whether or not peripheral vasopressin plays a role in social interaction or if it is merely a consequence of central release. Regardless, plasma vasopressin represents a good proxy of vasopressinergic signaling that is easy to assay. **Figure 6.15A** shows that PACAP KO mice have reduce plasma vasopressin content as compared to WT (110.32 ± 4.50, n=14 vs. 163.67 ± 13.81, n=18, respectively). It should be noted, that due to limited availability of PACAP KO mice, data presented here are from a female cohort of mice. However, we suspect there are similar reductions in plasma vasopressin in males. **Figure 6.15B** shows that plasma vasopressin in VPAC2r KO mice is no different than that of WT mice (79.45 ± 12.22, n=13 vs. 74.13 ± 7.80, n=10, respectively).

Chapter Summary

Others have reported robust behavioral analysis of the PACAP KO mouse [138, 134], however those studies have been conducted on mixed genetic backgrounds due the

inherent prevalence of postnatal mortality resulting from PACAP gene deletion. Although there is degree of consistency of behavioral profiles across mouse strains, there are distinct differences that can be attributed to strains of mice [53]. In this study we used PACAP KO mice of a pure C57Bl/6 background that were provided from the Wazchek lab at University of California, Los Angeles. We sought to corroborate previous findings in PACAP KO mice of mixed backgrounds with our recessive C57Bl/6 KO mice. PACAP gene deletion has been shown in previous studies to reduce anxiety and our findings support these conclusions [101, 137, 134]. We ran PACAP KO mice through the elevated plus maze (EPM) and compared them to WT controls to evaluate the reduced anxiety as reported by others. We performed within group comparisons and defined baseline anxiety as significantly *less* time spent in the open arms of the maze. Our WT mice spent significantly less time in the open arms whereas the PACAP KO mice did not. Evidence has been accumulating for a pivotal role of PACAP in anxiety disorders ranging from emotional aspects of chronic pain to post-traumatic stress disorder (PTSD) [261, 119]. For instance, circulating levels of PACAP in humans is positively associated with increased PTSD symptoms [261]. Additionally, Hammack *et. al* 2010 noticed anxiolytic effects of a PAC1 receptor antagonist in elevated plus maze [120]. It would appear that increased anxiety is coincident with increased circulating PACAP. Therefore, reduced anxiety in animals devoid of the PACAP gene is not surprising.

It has been shown that rodents emit ultrasonic vocalizations (USVs) and that these vocalizations have structure, however the reason they emit USVs is still unknown. It has been proposed that deficits in frequency modulation of USVs in FMR1 KO mice (Fragile X Syndrome model) may be analogous to the communication deficits inherent in

patients afflicted with Fragile X Syndrome and even autism [16]. Impairments of USVs in mice lacking the SHANK1 gene have been detected and SHANK1 deletions are commonly found in human males with Autism [342, 271]. Also, in support of the theory that USVs are form of communication, a study has found that the structure of USVs are different across mouse strains and that they may be used by female mice to choose mates [297]. In this study we sought to assess any alterations in mouse USVs in response to female odor deposits. We looked at calls emitted per second, seconds without calls, average and total call duration and average call frequency. PACAP gene deletion has no obvious impact in USVs in male mice compared to the WT. Our assessment of USVs in the PACAP KO mouse was a very general analysis of sounds emitted, and leaves room for the possibility of syllable or structure abnormalities resulting from deletion of the PACAP gene. One of the most prominent behavioral abnormalities in the PACAP KO mouse is hyperactivity [134, 152, 137]. Our PACAP KO mice, on the C57bl/6 background, displayed elevated locomotion in the open field as compared to WT mice as they maintained the same high-level of distance traveled and velocity for the duration of the 1-hour trial. Early studies that reported the hallmark excessive locomotion in PACAP KO mice, did so via interpretation of scores in the open field maze just as we did here [134]. It has been proposed that perhaps the increased locomotion in PACAP KO mice may be dependent on the mouse's familiarity with the environment. Hattori *et.al* 2012, measured locomotion in both the open field maze and in the animal's home-cage and only found increased locomotion in the open field maze, as the test trial was the animals first time in the maze [137]. Although we did not measure locomotion in the home-cages of animals in this study, we did spend a significant amount

of time with these animals in order to complete the battery of tests and we can attest that the increased locomotion was not apparent in any other environment expect the open field. Because of these findings we propose that the effect of PACAP gene deletion is not merely a driver of locomotion but that there is a contextual element based on environment that drives the behavior, as it is not seen in all environments.

Perhaps the most novel finding in this study is the effect of PACAP gene deletion on social recognition.

Social recognition in rodents has been considerably investigated [22]. Neuropeptides play a critical role in social recognition throughout multiple brain regions. Androgens and estrogens control social information processing by regulating oxytocin and vasopressin and this control is finely tuned for different forms of social information processing [42]. There is evidence that an intrinsic vasopressin system in olfactory bulbs of rats is involved in social recognition [311]. Work done in vasopressin receptor knockout mice shows that V1Ar in the lateral septum is required for social recognition in mice and does not affect other memory tasks [21]. Furthermore, osmotic stimulation of hypothalamic regions, the SON and PVN, in rats produces an increase in intra-hypothalamic and intra-septal vasopressin and these increases in central vasopressin improves social recognition ability [68]. Previous work form our lab has implicated the modulation of PACAP in the SON as a critical component of intra-hypothalamic vasopressin release [104, 105]. Therefore, we proposed that given the permissive relationship between PACAP and vasopressin release in the SON, that PACAP may be critical for social recognition in mice. **Figure 6.12** shows that PACAP KO mice lack the ability to recognize a novel from a familiar conspecific. This

social recognition paradigm is based on the natural propensity of mice towards olfactory investigation of novel mice. Presented with a novel and familiar mouse our WT mice are able to make the distinction by spending more time during the trial investigating the novel mouse. We hypothesize that this inability of PACAP KO mice to recognize familiar animals is in part due to attenuated intra-hypothalamic vasopressin signaling resulting from PACAP gene deletion. PACAP KO mice have been shown in the past to have impairments in the novel object recognition; a condition which is alleviated by a selective metabotropic glutamate 2/3 (mGlu2/3) receptor agonist, MGS0028 [5]. To follow up with this finding, we obtained a PACAP receptor, VPAC2, knockout mouse from the Wazchek lab at University of California, Los Angeles. Interestingly we found that while the VPAC2 KO mouse did have social recognition ability, they spent significantly more time investigating both animals during the trial. This can be interpreted two ways, either the deletion of the PACAP receptor impaired the animals ability to ascertain social information from olfactory cues, thus indicating a social memory processing deficit, or the gene deletion may be interfering with the animals ability to sense olfactory cues. To determine which possibility was likely at play we performed two follow up tests to clarify the results of the social recognition task, a three-chambered sociability test and an olfactory preference test. The sociability test is used to measure direct social approach towards other conspecifics when given a choice [349]. We found no discernible differences in the sociability across all genotypes. Therefore, we conclude that the social recognition deficit in the PACAP KO mice was not influenced by any aversions toward social interactions nor any shortcomings in general sociability. The olfactory preference test we employed was a cursory assessment of basic olfactory ability

in mice. We randomized the order which we presented four odors: water, vanilla, peanut butter and butyric acid (a particularly fowl smelling compound). We expected mice to have a preference towards the peanut butter odor and a dislike of the butyric acid, while preferring the water and vanilla somewhere in between. Wild type mice showed a significant preference for the peanut butter and almost equal interest on all other odors. PACAP KO mice also displayed this profile in odor preference (**Figure 6.14**). Surprisingly, VPAC2 KO mice did not exhibit a preference for any odor and actually spent more time investigating all odors during the trial compared to both WT and PACAP KO. PACAP mRNA expression appears to be distributed throughout the olfactory epithelium of E13 mice and localized to basal cells, neuronal cells and olfactory ensheathing cells in olfactory epithelium. Although the release mechanism of PACAP within the olfactory system is unknown, there is evidence that functional receptors are present [139, 124]. Olfactory discrimination tests conducted on PAC1 receptor knockout mice have indicated that type 1 PACAP receptors may not be involved in olfaction ability as PAC1 receptor knockout mice do not have attenuated olfaction [234]. Furthermore, other have found that VPAC2 receptors are expressed in mitral and external tufted cells in the olfactory bulb and are critical for odor detection performance [212]. Considering all three tests, we conclude that PACAP and its receptor, VPAC2, play significant, albeit different roles in olfactory processing.

Our data supports the hypothesis that PACAP signaling is require for social recognition, possibly via interactions with neuropeptide systems. **Figure 6.15** supports this hypothesis, since plasma vasopressin is only affected in PACAP KO mice and not VPAC2r KO mice. While we do not suspect peripheral vasopressin plays a role in social interactions,

it does allude to a deficit in the vasopressinergic systems that underline social recognition. This PACAP signaling does not appear to occur via activation of VPAC2 receptors, as deletion of VPAC2 gene did not impair the social recognition. However, deletion of the VPAC2 gene did appear to affect the olfactory ability of mice in this study. What is perplexing is that while mice were not able to process olfactory cues of foods, they were able to process social olfactory cues although it took a longer amount of time to do so. There may exist a role for VPAC2 in social recognition in regards to the time of acquisition of the cue, but not the processing of social information. Others have shown that PACAP receptor knockouts (PAC1r) have attenuated social odor recognition with no deficits in non-social odor discrimination (strawberry or vanilla) [234]. Along with our data, these studies provide evidence that PACAP signaling via type 1 receptors may be more involved in social odor processing, whereas signaling via type 2 receptors may be involved in processing of non-social odors. A recently study has also found a role for secretin, a peptide closely related to PACAP, in social recognition [301]. Researchers found that application of secretin into the supraoptic nucleus facilitated social recognition, and this facilitation was blocked by applying an oxytocin receptor antagonist into the medial amygdala. They also produced a secretin-receptor KO mouse, which had a deficit in social recognition just as our PACAP KO mice did. Social recognition ability was restored after oxytocin treatment in these secretin-receptor KO mice. Together with our findings, this recent study suggests a role for PACAP, and other members of the secretin family in social recognition. What role they play, to what extent and more importantly where specifically in the brain are they carry out their role is left to be determined.

Chapter 5

Developmental Exposure to Brominated Flame Retardants Act as Environmental Risk Factors for Autistic Phenotype: The Role of PACAP and Vasopressin

Introduction

Autism Spectrum Disorder (ASD) is an increasing concern for the health of children today. The incidence of ASD has reached epidemic proportions (1 in 68 U.S. children) but little is known of its etiology (U.S. Center for Disease Control). In California, there has been a 7-to-8-fold increase in the number children born with autism since 1990 [315]. We know very little of autism's prevalence in Mexico but the number of reported cases has been steadily increasing by 10-14 % since 1980 and efforts are underway to assess and prepare for a high incidence of undiagnosed autism cases. The dramatic escalation in the incidence over just a few years may be due to yet unclear environmental risk factors.

Today in U.S., the cost of supporting an individual with ASD is between 1.4-2.4 million for the duration of their lifespan [34], a cost greater than the entire Gross Domestic Product (GDP) of 139 countries around the world. For children autism poses severe challenges on their developmental benchmarks. Autism is among the most disabling forms of developmental disabilities, in part, because of three core symptom domains: 1) deficits in communication 2) impaired social interactions and 3) restricted, repetitive patterns of behavior, interests, or activities. As a result of their disorder, autistic children suffer from learning disabilities resulting in poor academic progress and harder times adjusting the social settings. These behaviors can be challenging to manage and can negatively impact quality of life for the whole family [187].

Little is known of the etiology of ASD. Contributors likely include diet, maternal medications and environmental pollutants in addition to genetic factors. Importantly, behavioral deficits can be produced after developmental exposure to organohalogens such as

polybrominated diphenyl ethers (PBDEs), a class of brominated flame retardants (BFRs) used widely throughout the US and Mexico as additives in indoor consumer goods since the 1970's. In products such as high-impact plastics, foams, and various textiles, PBDEs can constitute up to 30% of these products by weight. PBDEs and other BFRs have been added superficially so they are released easily into the indoor environment and bioaccumulate easily, especially in toddlers as a California study of family members describes [270].

PBDEs are highly soluble in lipids, and can accumulate in brain, fat, blood and breast-milk following a even single neonatal dose given within a critical window for neurodevelopment (gestation- PND3). Behavioral effects are long lasting [29, 73, 324, 325, 326, 327, 328], including persistent irregularities in spontaneous behavior and decreased ability to habituate in novel environments [73]. PBDEs during neurodevelopment also influence social affiliation [328, 105]. PBDEs, along with PCBs, may act by altering vasopressin responses within the supraoptic nucleus of the hypothalamus which has been demonstrated before [43, 44]. We do know that osmoregulation and cardiovascular control, both of which are vasopressin–related functions, are impaired [274]. Social recognition is a vasopressin/oxytocin dependent behavior mediated in part by the olfactory bulb, piriform cortex, lateral septum, medial preoptic area (MPOA), medial amygdala and hippocampus. Oxytocin knockout mice (OTKO) lack social recognition ability while maintaining olfactory discrimination ability [185, 42]. Central injection of oxytocin in OTKO mice will rescue the missing social recognition ability even after a single injection. Brattleboro rats, which lack vasopressin, and have diminished social recognition ability [68]. However social recognition ability is restored in Brattleboro rats by microinfusion of vasopressin into the lateral

septum. Both peripheral injection and intraventricular injection (icv) of vasopressin into rats increase their social recognition ability [67]. Site specific injections of vasopressin into lateral septum facilitates social recognition by increasing memory duration [81, 344, 325].

PACAP is another important player since it regulates vasopressin responses and may also participate in social functions. PAC1r is highly expressed throughout brain regions that control emotions, such as olfactory bulb, amygdala, hypothalamus and cortex [129] and reduced expression produces deficits in processing social olfactory cues and decreased social affiliative behaviors [234]. In collaboration with Dr. Leon-Olea, Instituto Nacional de Mexico, our lab has shown that PBDE-71 significantly reduces the expression of PACAP. Moreover, mice lacking the PACAP gene have significant defects in social recognition (**Figure 6.12**) vasopressin responses (data not shown). In humans, the PACAP gene resides at 18p11, a chromosomal site that is associated with increased susceptibility of schizophrenia and specific autistic phenotypes. Epidemiological studies have demonstrated a significant inverse linear relationship between global DNA methylation and plasma concentrations of dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), β-hexachlorocyclohexane (β-HCH), oxychlordane, α-chlordane and mirex [268]. In elderly Swedish population body levels of dioxin and polychlorinated diphenyl ethers (PCBs) were positively associated with global hypermethylation [190]. An analysis of postmortem human brains of people with ASD has also demonstrated a positive correlation between the level of PCB and PBDE congeners and DNA methylation [215]. Behavioral assays in animals exposed to PBDEs have strengthened the hypothesis that PBDEs can influence ASD phenotypes via epigenetics. By utilizing a mouse with mutations in the epigenetic factor

methyl- CpG binding protein 2 (Mecp2), Woods et al was able to rescue PBDE induced social and memory deficits [344].The authors speculate that perhaps the methylation induced by PBDE exposure utilizes Mecp2 to influence the heritable adverse effect on sociability. In this study we sought to profile autistic behaviors in mice exposed to an industrial mixture of PBDEs (DE71) and to amounts that are consistent with human exposure.

Methods

In Utero Exposure to DE-71

To model human exposure, we adapted the dosing paradigm from Woods *et. al* 2012 [344]. Mouse mothers were orally dosed with DE-71, an industrial mixture of PBDEs, for 10 weeks. This 10 week regime includes a 4 week pre-dose period, gestation (3 weeks), and up through weaning (3 weeks). Female mice were allocated to three groups: Corn oil control, DE-71 low dose (0.1 mg/Kg/day), and DE-71 high dose (0.4 mg/Kg/day). This dosing paradigm provided us with stress-free means to developmentally expose embryos/fetuses. DE71 was a gift from our collaborator at the Environmental Protection Agency, Dr. Prasada Kodavanti.

Order of Behavioral Tests

Juvenile Play (PND 20), Sociability (PND21), Open Field (PND 30-40), Marble Burying (PND 40-50), Olfactory Preference (PND 50-55), Social Recognition (PND 55-60), and Elevated Plus (PND 60-65). Plasma, brains, and other tissues were collected and snap frozen on the day of sacrifice for assays and PCR analysis.

Elevated Plus Maze

We used the elevated plus maze to assess effects of gene deletion on anxiety [109]. The arms of the elevated plus maze (EPM) were constructed out of black acrylic plastic measuring 3.5 inches wide by 44 inches long and standing 3 feet off the ground. The two arms that were closed had 8.5 inch black plastic walls. The entire maze was kept behind a black curtain with low light. Experimenters stood at opposite ends and each mouse for 5 min. trials. At the beginning of each trial mice were placed in the middle of the maze facing a closed arm. Overhead video was recorded. Videos were later analyzed for time spent in each arm of the maze.

Open Field

To test for hyperactivity we used the Open Field test [109]. The apparatus consists of a chamber made from plexiglass and covered with white vinyl to increase the contrast to better visualize the dark mice. Each mouse was placed in the center of the apparatus and videotaped for one hour. Analysis was performed in 5 minute blocks using Ethovision 7.1 software donated by the Carde lab at UC Riverside (Nodlus). Within each time bin, distances traveled (inches) and average velocity (inches/second) were measured.

Social Recognition Test

We used a paradigm adapted from Macbeth *et. al* 2009 [199]. Single housed male mice were transferred from the vivarium to the test room the morning of the trials. Male mice were allowed to habituate to test cage for 30 min and then to wire-mesh corrals introduced into cage for 30 min. Following the hour of habituation, one corral was removed

from the cage and an age, weight and sex matched mouse was placed into the remaining corral to begin a 5 min Trial 1. The stimulus mouse was removed and the subject was again left alone in the cage with two empty corrals for 30 min. In trial 2, both corrals remained in the cage and the initial (familiar) mouse and a novel age, weight and sex matched mouse were placed in the corrals for 5 min. All trials were video recorded with a high definition web video camera. Videos were then analyzed with JWatcher at 0.5X speed for olfactory investigation which was defined as directed nose pokes into each of the corrals.

Three-Chamber Sociability

Sociability was performed in a three-chamber apparatus using methods described previously [349]. The apparatus was a rectangular, three-chamber box made from clear polycarbonate. Retractable doorways within the two dividing walls allowed access from the center to the side chambers. The day of the experiment the subject mouse (male) was allowed to acclimate to the apparatus for 20 min before the sociability test – 10 min in the central chamber with the doors closed, followed by 10 min in the entire empty arena with the doors open. The subject was then briefly confined to the center chamber while empty inverted wire cages (the novel object) were placed in the side chambers. An age, weight and sex matched mouse used as the stimulus target for measurement of sociability was placed inside only one of the wire cages in the side chambers. After both stimuli were positioned, the doors were simultaneously reopened and the mouse allowed access all three chambers for 10 mins to assess sociability. Number of entries and time spent in the chambers were manually determined from recorded video using a high definition web video camera. More time spent in the chamber with the corral and animal is interpreted as a

preference to sociability. More time spent in the chamber with the corral only is interpreted as a preference to novelty but not sociability. To keep times spent in the outer chamber mutually exclusive, the middle chamber is omitted from statistical analysis.

Olfactory Preference Test

These experiments were adapted from a protocol made by Witt *et. al* 2009 [341]. Mice were placed in a series of 4 large cages for 15 mins to habituate to the novel environment. After habituation in the fourth cage, 2x2 inch squares of blotting paper saturated with one of four odors (10 % peanut butter, 1 % vanilla, 1 % butyric acid and water) were randomly presented to the mice for 3 minutes with 1 minute breaks in between trials. All trials were video recorded with a high definition web video camera. The time the animal spent with its nose 1cm from the 2x2 saturated paper was recorded with JWatcher.

Marble Burying

To measure compulsory and repetitive behaviors in mice we measured the amount of marbles buried within 30 minutes of presentation. When under stress, compulsive behaviors in mice will manifest as excessive self-grooming or excessive digging. Therefore, to measure compulsive digging in mice, a grid (5X4) of dark marbles are carefully placed inside a mouse cage with 5cm of extra corncob bedding. If the test subject is inclined to dig excessively, their digging will result in more marbles dropping below the bedding. The cage preparation involved filling clean cages (27x16.5x12.5 cm) with 4.5-cm corncob bedding, followed by gently overlaying 20 black glass marbles (15 mm diameter) equidistant in a 4x5 arrangement. Testing consisted of a 30-min exploration period. The number of

marbles buried (>50 % marble covered by bedding material) was recorded. Marbles buried was counted by 3-5 researchers and counts were averaged to curtail inherent subjectivity.

Video Analysis

All video were scored blind using the event analysis software JWatcher [25]. After weaning mice were randomly assigned to cages with alphanumeric cage IDs. The gender and treatment were unknown to the researchers performing the behaviors tests and also the researchers performing the video analysis. Sex and treatment were re-matched to each animal once raw data was collected.

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative reverse-transcriptase PCR was employed to examine the effect of DE71 exposure on genes of interest that are involved in sociability in the brain. We examined pituitary adenylate cyclase-activating polypeptide (*ADCYAP1*), pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*), arginine vasopressin (*Avp*), arginine vasopressin receptor 1A (*Avpr1a*), oxytocin receptor (*Oxtr*) and β-actin (*Actb*).

qPCR was performed using the Bioline SensiFAST^{TM} SYBR No-ROX One-Step Kit and the Bio-Rad CFX 96 Real-Time PCR Detection System. The nucleotide sequences, accession numbers and efficiencies of qPCR primers are listed in **Table**6.2. A temperature gradient from $54-62^{o}$C was used on the Bio-Rad CFX 96 station to capture ideal annealing temperatures.

Total RNA was prepared from bilateral micropunched brain samples with the Qiagen RNeasy^{R} Micro Kit. We modified the Qiagen RNA isolation protocol by performing

a partial phenol-chloroform extraction instead of using the supplied RLT buffer which we found to be poor at lysing brain tissue. For each experiment, 1 ng of RNA was used per reaction. The reverse transcription was performed prior to the 2-step qPCR cycling protocol on the same plate. In brief, the qPCR plate was ran at 45^{o}C for 10 min. (*reverse transcription, RT*), 95^{o}C for 2 min. (*inactivation of RT enzyme*), then 95^{o}C for 5 sec. (*denaturation*), then 55^{o}C for 20sec. (*annealing/extension*). The reactions were run for 40 cycles prior to melt curve analysis. Results are presented as fold expression relative to β-actin, which was determined via the Pfaffl method [252].

Quantification of VP

VP content in samples was measured using an enzyme-immunoassay (arg8-vasopressin correlate enzyme-immunoassay kit, Assay Designs) and a 4-parameter curve fitting computer program (STATLIA; Brendan).

Statistical analysis

Statistical analysis was performed using RStudio [266]. General linear mixed model ANOVA was performed with the *lme4 package* [15] where data met normality and homogeneity of variance assumptions. Two-mean comparisons were determined using Student's t-test with or without equal variances, as needed. Multiple group comparisons were made using the *multcomp package* [146] using only the relevant subset of all-pairwise comparisons *(k matrix)*. Statistical significance was acknowledged at an alpha level of 0.05 or lower.

Results

Elevated Plus Maze

To assess any effects of DE71 exposure on male anxiety, we employed the elevated plus maze (EPM). Within five minute trials, typical mice spend nearly twice as much time in the closed arms relative to the open arms of the EPM, thus establishing a baseline from which to compare. **Figure 6.16A** shows that our male oil control mice displayed typical mouse anxiety (closed vs. open arm: 188.11 ± 20.52 sec. vs. 68.45 ± 22.93 sec., respectively n=7). Furthermore, both our male low dose (closed vs. open arm: 192.18 ± 17.75 sec. vs. 48.40 ± 16.04 sec., respectively n=4), and male high dose (closed vs. open arm: 179.23 ± 14.87 sec. vs. 60.60 ± 12.19 sec., respectively n=4) displayed normal anxiety compared to the oil controls. Mean ± S.E.M. A two factorial ANOVA, with DE71 treatment and arm of the EPM being the main factors revealed a significant effect of EPM arm ($F(1,24)= 55.8045$, p-value=$1.038e^{-07}$).

We also evaluated anxiety in the female DE71 exposed mice. Similar to the males, the female mice also displayed the typical mouse behavior on the EPM (**Figure 6.16B**. The female oil controls mice preferred the closed arm of the maze (closed vs. open arm: 191.45 ± 7.52 vs. 51.18 ± 8.19 sec. n=8, respectively). The female low dose mice preferred the closed arm of the maze (closed vs. open arm: 164.39 ± 9.22 vs. 85.29 ± 7.99 sec. n=6, respectively). The female high dose mice preferred the closed arm of the maze (closed vs. open arm: 192.93 ± 16.51 vs. 52.31 ± 13.79 sec. n=4, respectively). Mean ± S.E.M. A two factorial ANOVA, with DE71 treatment and arm of the EPM being the main factors revealed a significant interaction between DE71 treatment and arm of the EPM ($F(2,30)=$

6.6513, p-value= 0.004065).

Open Field

We analyzed the distance traveled and velocity of mice in an open field maze to determine any effects of DE71 exposure on locomotion. **Figure 6.18A** summarizes the distance traveled by male mice in the open field. We found that all three treatment groups traveled similar distances per 5 minute time bin and that there was a general decrease in distance traveled throughout the 1 hour trial. Male Oil Control:*Start-05 min.*: 787.37 ± 48.26 in.; *05-10 min.*: 707.99 ± 30.47 in.; *10-15 min.*: 630.59 ± 49.61 in.; *15-20 min.*: 577.15 ± 49.74 in.; *20-25 min.*: 669.46 ± 48.99 in.; *25-30 min.*: 566.61 ± 51.05 in.; *30-35 min.*: 537.25 ± 61.15 in.; *35-40 min.*: 484.89 ± 85.49 in.; *40-45 min.*: 554.60 ± 32.24 in.; *45-50 min.*: 490.64 ± 47.94 in.; *50-55 min.*: 442.97 ± 61.19 in.; *55-60 min.*: 435.79 ± 54.65 in., n=8. Mean ± S.E.M. Male Low Dose: *Start-0:05 min.*: 842.38 ± 47.19 in.; *05-10 min.*: 668.45 ± 50.02 in.; *10-15 min.*: 599.55 ± 44.87 in.; *15-20 min.*: 530.09 ± 59.93 in.; *20-25 min.*: 527.45 ± 47.09 in.; *25-30 min.*: 523.03 ± 47.80 in.; *30-35 min.*: 489.06 ± 79.61 in.; *35-40 min.*: 518.20 ± 50.06 in.; *40-45 min.*: 465.30 ± 44.59 in.; *45-50 min.*: 500.13 ± 71.00 in.; *50-55 min.*: 405.75 ± 61.42 in.; *55-60 min.*: 412.82 ± 38.67 in., n=8. Mean ± S.E.M. Male High Dose: *Start-05 min.*: 820.00 ± 74.36 in.; *05-10 min.*: 688.41 ± 42.26 in.; *10-15 min.*: 722.51 ± 35.82 in.; *15-20 min.*: 662.68 ± 46.65 in.; *20-25 min.*: 633.87 ± 43.53 in.; *25-30 min.*: 695.03 ± 44.98 in.; *30-35 min.*: 628.26 ± 46.89 in.; *35-40 min.*: 641.86 ± 39.92 in.; *40-45 min.*: 536.24 ± 60.71 in.; *45-50 min.*: 638.89 ± 64.49 in.; *50-55 min.*: 532.19 ± 55.41 in.; *55-60 min.*: 477.17 ± 67.73 in., n=9. Mean ± S.E.M. A repeated measures two factor ANOVA was performed with DE71 treatment group and time bin as factors. A

significant effect of time bin was found (F(11,242)=19.725, p-value=$<2e^{-16}$). *Control-High Dose:00-05min* p-value= 1.000; *Control-Low Dose:00-05min* p-value= 1.000; *Control-High Dose:05-10min* p-value= 1.000; *Control-Low Dose:05-10min* p-value= 1.000; *Control-High Dose:10-15min* p-value= 1.000; *Control-Low Dose:10-15min* p-value= 0.995; *Control-High Dose:15-20min* p-value= 1.000; *Control-Low Dose:15-20min* p-value= 0.998; *Control-High Dose:20-25min* p-value= 0.784; *Control-Low Dose:20-25min* p-value= 1.000; *Control-High Dose:25-30min* p-value= 1.000; *Control-Low Dose:25-30min* p-value= 0.868; *Control-High Dose:30-35min* p-value= 1.000; *Control-Low Dose:30-35min* p-value= 0.996; *Control-High Dose:35-40min* p-value= 1.000; *Control-Low Dose:35-40min* p-value= 0.578; *Control-High Dose:40-45min* p-value= 0.998; *Control-Low Dose:40-45min* p-value= 1.000; *Control-High Dose:45-50min* p-value= 1.000; *Control-Low Dose:45-50min* p-value= 0.677; *Control-High Dose:50-55min* p-value= 1.000; *Control-Low Dose:50-55min* p-value= 0.997; *Control-High Dose:50-55min* p-value= 1.000; *Control-Low Dose:50-55min* p-value= 1.000.

As seen in **Figure 6.18B** the velocity at which mice traveled was also evaluated. A three treatment groups of male mice maintained similar speeds through out the duration of the test. Just as in the distance traveled, there was also a steady decrease in speed as the trial progressed. Male Oil Control: *Start-05 min.*: 2.63 ± 0.16 in./sec.; *05-10 min.*: 2.36 ± 0.10 in./sec.; *10-15 min.*: 2.10 ± 0.17 in./sec.; *15-20 min.*: 1.92 ± 0.17 in./sec.; *20-25 min.*: 2.23 ± 0.16 in./sec.; *25-30 min.*: 1.89 ± 0.17 in./sec.; *30-35 min.*: 1.79 ± 0.20 in./sec.; *35-40 min.*: 1.62 ± 0.29 in./sec.; *40-45 min.*: 1.85 ± 0.11 in./sec.; *45-50 min.*: 1.64 ± 0.16 in./sec.; *50-55 min.*: 1.48 ± 0.20 in./sec.; *55-60 min.*: 1.45 ± 0.18 in./sec., n=8. Mean ± S.E.M. Male Low Dose: *Start-05 min.*: 2.81 ± 0.16 in./sec.; *05-10 min.*: 2.23 ±

0.17 in./sec.; *10-15 min.*: 2.00 ± 0.15 in./sec.; *15-20 min.*: 1.77 ± 0.20 in./sec.; *20-25 min.*: 1.75 ± 0.16 in./sec.; *25-30 min.*: 1.74 ± 0.16 in./sec.; *30-35 min.*: 1.63 ± 0.27 in./sec.; *35-40 min.*: 1.73 ± 0.17 in./sec.; *40-45 min.*: 1.55 ± 0.15 in./sec.; *45-50 min.*: 1.67 ± 0.24 in./sec.; *50-55 min.*: 1.35 ± 0.21 in./sec.; *55-60 min.*: 1.38 ± 0.13 in./sec., n=8. Mean ± S.E.M. Male High Dose: *Start-05 min.*: 2.73 ± 0.25 in./sec.; *05-10 min.*: 2.30 ± 0.14 in./sec.; *10-15 min.*: 2.41 ± 0.12 in./sec.; *15-20 min.*: 2.21 ± 0.15 in./sec.; *20-25 min.*: 2.11 ± 0.14 in./sec.; *25-30 min.*: 2.32 ± 0.15 in./sec.; *30-35 min.*: 2.09 ± 0.16 in./sec.; *35-40 min.*: 2.14 ± 0.13 in./sec.; *40-45 min.*: 1.78 ± 0.20 in./sec.; *45-50 min.*: 2.13 ± 0.23 in./sec.; *50-55 min.*: 1.77 ± 0.19 in./sec.; *55-60 min.*: 1.59 ± 0.23 in./sec., n=9. Mean ± S.E.M. A repeated measures two factor ANOVA was performed with DE71 treatment group and time bin as factors. As with distance traveled, a significant effect of time bin was found ($F(11, 242) = 19.733$ p-value=$< 2^{-16}$). The multiple comparisons of means is as follows. *Control-High Dose:00-05min* p-value= 1.000; *Control-Low Dose:00-05min* p-value= 1.000; *Control-High Dose:05-10min* p-value= 1.000; *Control-Low Dose:05-10min* p-value= 1.000; *Control-High Dose:10-15min* p-value= 1.000; *Control-Low Dose:10-15min* p-value= 0.995; *Control-High Dose:15-20min* p-value= 1.000; *Control-Low Dose:15-20min* p-value= 0.998; *Control-High Dose:20-25min* p-value= 0.784; *Control-Low Dose:20-25min* p-value= 1.000; *Control-High Dose:25-30min* p-value= 1.000; *Control-Low Dose:25-30min* p-value= 0.868; *Control-High Dose:30-35min* p-value= 1.000; *Control-Low Dose:30-35min* p-value= 0.996; *Control-High Dose:35-40min* p-value= 1.000; *Control-Low Dose:35-40min* p-value= 0.578; *Control-High Dose:40-45min* p-value= 0.998; *Control-Low Dose:40-45min* p-value= 1.000; *Control-High Dose:45-50min* p-value= 1.000; *Control-Low Dose:45-50min* p-value= 0.677;

Control-High Dose:50-55min p-value= 1.000; *Control-Low Dose:50-55min* p-value= 0.997; *Control-High Dose:50-55min* p-value= 1.000; *Control-Low Dose:50-55min* p-value= 1.000.

We performed the same analysis of distance for the female mice as well. **Figure 6.18C** shows the distance traveled of female mice in the open field. Distance traveled by all female treatment groups was not different per 5 minute time bin. Female Oil Control: *Start-05 min.*: 818.913 */pm* 54.04 in.; *05-10 min.*: 644.031 */pm* 25.27 in.; *10-15 min.*: 647.127 */pm* 35.50 in.; *15-20 min.*: 553.414 */pm* 39.14 in.; *20-25 min.*: 482.450 */pm* 45.80 in.; *25-30 min.*: 498.483 */pm* 35.98 in.; *30-35 min.*: 468.310 */pm* 51.97 in.; *35-40 min.*: 403.738 */pm* 41.52 in.; *40-45 min.*: 419.274 */pm* 49.83 in.; *45-50 min.*: 420.078 */pm* 41.77 in.; *50-55 min.*: 352.959 */pm* 60.68 in.; *55-60 min.*: 334.210 */pm* 50.91 in., n=9. Mean ± S.E.M. Female Low Dose: *Start-05 min.*: 789.87 */pm* 52.40 in.; *05-10 min.*: 615.72 */pm* 50.57 in.; *10-15 min.*: 591.04 */pm* 49.15 in.; *15-20 min.*: 506.18 */pm* 40.14 in.; *20-25 min.*: 516.29 */pm* 55.22 in.; *25-30 min.*: 535.62 */pm* 35.60 in.; *30-35 min.*: 515.94 */pm* 54.62 in.; *35-40 min.*: 419.93 */pm* 50.76 in.; *40-45 min.*: 400.69 */pm* 38.86 in.; *45-50 min.*: 378.03 */pm* 51.14 in.; *50-55 min.*: 337.00 */pm* 63.05 in.; *55-60 min.*: 351.61 */pm* 71.59 in., n=9. Mean ± S.E.M. Female High Dose: *Start-05 min.*: 648.99 */pm* 12.92 in.; *05-10 min.*: 542.95 */pm* 56.23 in.; *10-15 min.*: 541.79 */pm* 70.53 in.; *15-20 min.*: 559.22 */pm* 81.28 in.; *20-25 min.*: 408.39 */pm* 100.80 in.; *25-30 min.*: 429.81 */pm* 14.48 in.; *30-35 min.*: 439.23 */pm* 74.68 in.; *35-40 min.*: 429.04 */pm* 17.79 in.; *40-45 min.*: 363.50 */pm* 47.04 in.; *45-50 min.*: 325.81 */pm* 34.68 in.; *50-55 min.*: 332.56 */pm* 80.58 in.; *55-60 min.*: 345.41 */pm* 78.80 in., n=3. Mean ± S.E.M. A repeated measures ANOVA was performed just as it was with the males. A significant effect of time was found ($F(11,198)=26.923$, p-value=$2e^{-16}$). The

multiple comparisons of means is as follows. *Control-High Dose:00-05min* p-value= 1.000; *Control-Low Dose:00-05min* p-value= 0.838; *Control-High Dose:05-10min* p-value= 1.000; *Control-Low Dose:05-10min* p-value= 1.000; *Control-High Dose:10-15min* p-value= 1.000; *Control-Low Dose:10-15min* p-value= 0.999; *Control-High Dose:15-20min* p-value= 1.000; *Control-Low Dose:15-20min* p-value= 1.000; *Control-High Dose:20-25min* p-value= 1.000; *Control-Low Dose:20-25min* p-value= 1.000; *Control-High Dose:25-30min* p-value= 1.000; *Control-Low Dose:25-30min* p-value= 1.000; *Control-High Dose:30-35min* p-value= 1.000; *Control-Low Dose:30-35min* p-value= 1.000; *Control-High Dose:35-40min* p-value= 1.000; *Control-Low Dose:35-40min* p-value= 1.000; *Control-High Dose:40-45min* p-value= 1.000; *Control-Low Dose:40-45min* p-value= 1.000; *Control-High Dose:45-50min* p-value= 1.000; *Control-Low Dose:45-50min* p-value= 1.000; *Control-High Dose:50-55min* p-value= 1.000; *Control-Low Dose:50-55min* p-value= 1.000; *Control-High Dose:50-55min* p-value= 1.000; *Control-Low Dose:50-55min* p-value= 1.000.

Figure 6.18D reports the velocity of female mice in each five minute time bin. We also found that as the 1 hour trial progressed the female mice decreased their speed. Female Oil Control: *Start-05 min.*: 2.73 ± 0.18 in./sec.; *05-10 min.*: 2.15 ± 0.08 in./sec.; *10-15 min.*: 2.16 ± 0.12 in./sec.; *15-20 min.*: 1.84 ± 0.13 in./sec.; *20-25 min.*: 1.61 ± 0.19 in./sec.; *25-30 min.*: 1.66 ± 0.12 in./sec.; *30-35 min.*: 1.56 ± 0.17 in./sec.; *35-40 min.*: 1.35 ± 0.14 in./sec.; *40-45 min.*: 1.40 ± 0.17 in./sec.; *45-50 min.*: 1.40 ± 0.14 in./sec.; *50-55 min.*: 1.18 ± 0.20 in./sec.; *55-60 min.*: 1.11 ± 0.17 in./sec., n=9. Mean ± S.E.M. Female Low Dose: *Start-0:05 min.*: 2.63 ± 0.17 in./sec.; *05-10 min.*: 2.05 ± 0.17 in./sec.; *10-15 min.*: 1.97 ± 0.16 in./sec.; *15-20 min.*: 1.69 ± 0.13 in./sec.; *20-25 min.*: 1.72 ± 0.18

in./sec.; *25-30 min.*: 1.79 ± 0.12 in./sec.; *30-35 min.*: 1.72 ± 0.18 in./sec.; *35-40 min.*: 1.40 ± 0.17 in./sec.; *40-45 min.*: 1.34 ± 0.13 in./sec.; *45-50 min.*: 1.26 ± 0.17 in./sec.; *50-55 min.*: 1.12 ± 0.21 in./sec.; *55-60 min.*: 1.17 ± 0.24 in./sec., n=9. Mean ± S.E.M. Female High Dose: *Start-05 min.*: 2.16 ± 0.04 in./sec.; *05-10 min.*: 1.81 ± 0.19 in./sec.; *10-15 min.*: 1.81 ± 0.25 in./sec.; *15-20 min.*: 1.86 ± 0.27 in./sec.; *20-25 min.*: 1.36 ± 0.34 in./sec.; *25-30 min.*: 1.43 ± 0.05 in./sec.; *30-35 min.*: 1.46 ± 0.25 in./sec.; *35-40 min.*: 1.43 ± 0.06 in./sec.; *40-45 min.*: 1.21 ± 0.16 in./sec.; *45-50 min.*: 1.09 ± 0.12 in./sec.; *50-55 min.*: 1.11 ± 0.27 in./sec.; *55-60 min.*: 1.15 ± 0.26 in./sec., n=3. Mean ± S.E.M. A repeated measures two factor ANOVA was performed with DE71 treatment group and time bin as factors. As with distance traveled, a significant effect of time bin was found $F(11,198)=26.931$ p-value$<2e^{-16}$. The multiple comparisons of means is as follows. *Control-High Dose:00-05min* p-value= 1.000; *Control-Low Dose:00-05min* p-value= 0.838; *Control-High Dose:05-10min* p-value= 1.000; *Control-Low Dose:05-10min* p-value= 1.000; *Control-High Dose:10-15min* p-value= 1.000; *Control-Low Dose:10-15min* p-value= 0.999; *Control-High Dose:15-20min* p-value= 1.000; *Control-Low Dose:15-20min* p-value= 1.000; *Control-High Dose:20-25min* p-value= 1.000; *Control-Low Dose:20-25min* p-value= 1.000; *Control-High Dose:25-30min* p-value= 1.000; *Control-Low Dose:25-30min* p-value= 1.000; *Control-High Dose:30-35min* p-value= 1.000; *Control-Low Dose:30-35min* p-value= 1.000; *Control-High Dose:35-40min* p-value= 1.000; *Control-Low Dose:35-40min* p-value= 1.000; *Control-High Dose:40-45min* p-value= 1.000; *Control-Low Dose:40-45min* p-value= 1.000; *Control-High Dose:45-50min* p-value= 1.000; *Control-Low Dose:45-50min* p-value= 1.000; *Control-High Dose:50-55min* p-value= 1.000; *Control-Low Dose:50-55min* p-value= 1.000;

Control-High Dose:50-55min p-value= 1.000; *Control-Low Dose:50-55min* p-value= 1.000.

Marble Burying

It is thought that increased anxiety may underlie the tendency of mice to compulsively dig [237]. Our male oil control mice buried about a quarter of the marbles within the 30 minute trial (**Figure 6.17A**: 5.00 ± 1.19 marbles, n=12). The male low dose mice buried around the same amount of marbles as did the oil controls (5.59 ± 1.82 marbles, n=8). The male mice exposed to a high dose of DE71 buried less marbles than both oil controls and low dose (2.00 ± 0.46 marbles, n=11) however a one-factor ANOVA with DE71 treatment representing the only factor did not meet significance ($F(2,28)=1.5382$, p-value=0.2324).

Female oil control mice and female low dose mice buried roughly similar amounts of marbles as their male counterparts (**Figure 6.17B**: 4.17 ± 0.62 marbles, n=12; 4.640 ± 1.27 marbles, n=10, respectively). The high dose of DE71 again resulted in fewer marbles buried (1.09 ± 0.675 marbles, n=7), however for the female group a one-factor ANOVA did reveal a significant effect of DE71 treatment ($F(2,26)=4.9972$, p-value=0.01458).

Social Recognition

We sought determine if DE71 treatment has any impact on an animals ability to recognize conspecifics. When presented with a two conspecifics, a familiar animal and a novel animal, a mouse will recognize the familiar animal and spend more time investigating the novel animal. We found that male oil control mice spent more time investigating novel mice over familiar mice (**Figure 6.19A**: 35.42 ± 3.52 sec. vs. 23.70 ± 2.99 sec., respectively, n=11). Male high dose mice also spent more time investigating novel animals compared to

familiar (36.71 ± 4.79 sec. vs. 21.70 ± 4.31 sec., respectively, n=9).

Interestingly, male low dose mice spent an equivalent amount of time investigating both novel and familiar mice (38.13 ± 3.55 sec. vs. 30.21 ± 3.05 sec., respectively, n=15). A two-factor ANOVA, with DE71 treatment and stimulus animals being the main factors, found a significant effect of stimulus animal ($F(1,61.968)=17.2464$, p-value=0.0001).

This effect on social recognition ability discovered in the male treatment groups was mimicked in the females. Female oil control mice spent more time investigating novel over familiar mice (**Figure 6.19B**: 34.05 ± 4.09 sec. vs. 18.43 ± 2.87 sec., n=14, respectively). Female high dose mice also spent more time investigating novel over familiar mice 26.48 ± 4.33 sec. vs. 14.54 ± 4.18 sec., n=9, respectively). However, female low dose mice failed to spend more time investigating novel over familiar mice (33.75 ± 2.36 sec. vs. 28.62 ± 3.71 sec., n=15, respectively). A two-factor ANOVA interestingly revealed significant main effects of both DE71 treatment and stimulus animal ($F(2,68.354)=6.3820$, p-value=0.0029; $F(1,67.042)=26.0752$, p-value=$2.992e^{-6}$, respectively).

Sociability

To further investigate the effects of DE71 treatment on social interactions, we employed the three-chambered sociability test [349]. This test places a mouse in an apparatus with three chambers connect in series by sliding doors. The two outer chambers each contain a corral and only one corral contains an animal. During a ten minute trial the time spent in each outer chamber is measured. All three male treatment groups spent more time in the social chamber compared to the novel object chamber (**Figure 6.20A**: oil control: 281.94 ± 34.19 sec. vs. 188.40 ± 13.02 sec., respectively, n=11; low dose: 221.64 ± 28.31

sec. vs. 136.26 ± 8.19 sec., respectively, n=24; high dose: 392.83 ± 37.89 sec. vs. 125.48 ± 10.59 sec., respectively, n=12). An ANOVA revealed a significant main effect of chamber ($F(1,84.240)=87.155$, p-value=$1.221e^{-14}$).

All three female treatments also displayed a preference for sociability by spending more time in the social chamber compared to the novel object chamber (**Figure 6.20B**: oil control: 312.87 ± 53.391 sec. vs. 169.33 ± 31.05 sec., respectively, n=9; low dose: 288.22 ± 40.38 sec. vs. 133.93 ± 11.85 sec., respectively, n=9; high dose: 304.96 ± 52.95 sec. vs. 155.80 ± 31.06 sec., respectively, n=9). An ANOVA revealed a significant main effect of chamber ($F(2,71.08)=25.432$, p-value=$3.372e^{-6}$).

Olfactory Preference

The social recognition task relies heavily on olfaction, since olfactory investigation is measured during the task. Therefore, to account for the differences in treatment in the social recognition task performed a rudimentary olfactory preference test [199]. In order to establish an olfactory preference profile , we exposed male oil control mice four 2 inch squares of absorbent paper saturated with the following odors: water, peanut butter, butyric acid and vanilla (**Figure 6.21A**). Male oil control mice showed a significant preference for peanut butter odor (*butyric acid*: 11.08 ± 2.79 sec.; *peanut butter*: 29.42 ± 8.58 sec.; *vanilla*: 16.51 ± 2.69 sec.; *water*: 10.01 ± 2.06 sec., n=8). The male low dose group did not have the same olfactory preference profile (*butyric acid*: 24.88 ± 3.23 sec.; *peanut butter*: 22.49 ± 2.99 sec.; *vanilla*: 18.47 ± 2.76 sec.; *water*: 16.26 ± 2.47 sec., n=19). The male high dose group also failed to show a preference for the peanut butter odor (*butyric acid*: 15.34 ± 3.95 sec.; *peanut butter*: 23.63 ± 6.55 sec.; *vanilla*: 14.53 ± 4.44 sec.; *water*: 17.97

± 5.19 sec., n=6). A two-factor ANOVA, with DE71 treatment and odor being the main factors, revealed a significant main effect of odor F(3,115)=2.73757, p-value=0.0467.

The female oil controls showed the same preference for peanut butter odor as did the male oil controls (**Figure 6.21B**: *butyric acid*: 8.14 ± 4.32 sec.; *peanut butter*: 17.19 ± 3.24 sec.; *vanilla*: 7.78 ± 3.21 sec.; *water*: 8.16 ± 2.89 sec., n=8). The female low dose group did not display a preference for any of the odors (*butyric acid*: 19.47 ± 4.19 sec.; *peanut butter*: 21.94 ± 3.30 sec.; *vanilla*: 11.73 ± 2.83 sec.; *water*: 14.19 ± 3.21 sec., n=9). The female high dose group also no preference for any odor (*butyric acid*: 7.91 ± 4.45 sec.; *peanut butter*: 14.35 ± 3.02 sec.; *vanilla*: 9.33 ± 3.56 sec.; *water*: 4.64 ± 2.77 sec., n=5). A two-factor ANOVA, with DE71 treatment and odor being the main factors, revealed a significant main effect of odor F(3,70.00)=5.708, p-value=0.001488.

Plasma Vasopression

The neuropeptide vasopressin has been extensively studied in the context of sociability [93]. Most of these studies looked at the role of centrally released vasopressin within the brain, but evidence exists for a prosocial role of peripherally released vasopressin [257]. We assayed cardiac blood from male (**Figure 6.22A**) and female (**Figure 6.22B**) mice of all treatment groups. Male oil control mice had 199.55 ± 42.00 pg/mL, n=8 of plasma vasopressin. We found that male low dose mice had 148.366 ± 42.14 pg/mL, n=6 plasma vasopressin, and although this amount was less than that of oil controls it was not significantly less. Interestingly, male high dose mice had higher basal plasma vasopressin than both oil controls and low dose mice (341.77 ± 31.78 pg/mL, n=6). An ANOVA, with DE71 treatment being the sole factor, revealed a significant effect of treatment (F(2,16.01)=7.315,

p-value=0.0055). The female oil controls, low dose and high dose all had roughly equivalent amount of basal vasopressin (244.71 ± 88.60 pg/mL, n=7; 300.64 ± 88.14 pg/mL, n=5; 255.592 ± 76.62 pg/mL, n=5, respectively). There was no significant difference amongst female treatment groups (F(2,13.12)=0.026, p-value=0.975).

Quantitative PCR- Piriform Cortex

In order to probe the underpinnings of the social recognition deficit in the male low dose group, we looked along the central social recognition pathway as described previously by others [22]. We analyzed genes encoding for neuropeptides and their receptors in the piriform cortex, lateral septum, paraventricular nucleus, supraoptic nucleus and medial amygdala. Given the cooperative relationship between neuropeptides and pituitary adenylate cyclase-activating polypeptide (*ADCYAP1*), we also assessed transcript levels of PACAP and its receptors where appropriate.

Within the piriform cortex (**Figure 6.23**) we evaluated gene expression of arginine vasopressin receptor 1A (*Avpr1a*). Relative to β-actin, male oil controls expressed an equivalent transcript level of *Avpr1a* compared to both male low dose and high dose (Oil Control: 1.04 ± 0.16 fold-expression, n=4; Low Dose: 1.04 ± 0.10 fold-expression, n=3; High Dose: 0.99 ± 0.12 fold-expression, n=8). A one-way ANOVA revealed no significant difference in *Avpr1a* gene expression across all three groups F(2,12)=0.0388, p-value=0.0962.

Quantitative PCR- Cingulate Cortex

Within the cingulate cortex (**Figure 6.24**), we explored two genes of interest: pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) and pi-

tuitary adenylate cyclase-activating polypeptide (*ADCYAP1*). We found that within the cingulate cortex, *ADCYAP1r1* gene expressed was blunted in the male high dose as compared to male oil controls and male low dose Oil Control: 1.01 ± 0.078 fold-expression, n=4; Low Dose: 0.72 ± 0.079 fold-expression, n=4; High Dose: 0.14 ± 0.014 fold-expression, n=8). A one-way ANOVA revealed a significant difference in *ADCYAP1r1* gene expression F(2,13)=91.857, p-value=$2.141e^{-08}$. Gene expression of that receptors ligand, *ADCYAP1*, was also significantly decreased in the male high dose compared to the oil controls and low dose (Oil Control 1.04 ± 0.17 fold-expression, n= 4; Low Dose 0.98 ± 0.03 fold-expression, n= 4; High Dose 0.39 ± 0.05 fold-expression, n= 8). A one-way ANOVA revealed a significant difference in *ADCYAP1* gene expression F(2,13)=19.919, p-value=0.00011.

Quantitative PCR- Lateral Septum

We looked gene expression of both vasopressin and PACAP receptors in the lateral septum (**Figure 6.25**). We found that gene expression of pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) was significantly decreased in the male high dose (Oil Control: 1.30 ± 0.32 fold-expression, n=8; Low Dose: 1.40 ± 0.30 fold-expression, n=11; High Dose: 0.16 ± 0.01 fold-expression, n=8). A one-way ANOVA revealed a significant difference in *ADCYAP1r1* gene expression F(2,24)=6.1268, p-value=0.007085. Gene expression of arginine vasopressin receptor 1A (*Avpr1a*) had an apparent dose dependent increase (Oil Control: 1.30 ± 0.34 fold-expression, n=7; Low Dose: 1.99 ± 0.30 fold-expression, n=10; High Dose: 2.37 ± 0.39 fold-expression, n=7), however this trend was not significant F(2,21)=2.2909, p-value=0.1259.

Quantitative PCR- Paraventricular Nucleus

We analyzed PACAP and its receptor in the paraventricular nucleus (**Figure 6.26**). We found that gene expression of pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) was significantly decreased in male high dose compared to oil control mice and low dose (Oil Control: 1.08 ± 0.16 fold-expression, n=7; Low Dose: 1.22 ± 0.15 fold-expression, n=11; High Dose: 0.50 ± 0.04 fold-expression, n=9). A one-way ANOVA found a significant difference across treatment F(2,24)=9.9092, p-value=0.001277.

Additionally we found no difference in gene expression of pituitary adenylate cyclase-activating polypeptide (*ADCYAP1*) between male high dose, low dose and oil controls (Oil Control: 1.32 ± 0.40 fold-expression, n=7; Low Dose: 1.67 ± 0.49 fold-expression, n=11; High Dose: 2.07 ± 0.51 fold-expression, n=9). A one-way ANOVA revealed no difference across treatment F(2,24)=0.9904, p-value=0.3861.

Quantitative PCR- Supraoptic Nucleus

We found that gene expression of arginine vasopressin (*Avp*) within the supraoptic nucleus (**Figure 6.27**) was higher in male low dose compared to the oil controls and high dose (Oil Control: 1.32 ± 0.40 fold-expression, n=3; Low Dose: 1.67 ± 0.49 fold-expression, n=11; High Dose: 2.07 ± 0.51 fold-expression, n=9). This difference was not significant as indicated by a one-way ANOVA F(2,18)=0.5162, p-value=0.6053. We found that gene expression of pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) within the supraoptic nucleus was decreased in male high dose compared to low dose and oil controls (Oil Control: 0.85 ± 0.23 fold-expression, n=5; Low Dose: 0.88

± 0.14 fold-expression, n=7; High Dose: 0.31 ± 0.08 fold-expression, n=8). A one-way ANOVA revealed a significant effect of treatment F(2,17)=5.4091, p-value=0.01521. Additionally, within the supraoptic nucleus, we found that gene expression of pituitary adenylate cyclase-activating polypeptide (*ADCYAP1*) had an apparent increase in both low and high dose compared to oil controls (Oil Control: 1.23 ± 0.42 fold-expression, n=5; Low Dose: 2.51 ± 0.49 fold-expression, n=6; High Dose: 2.36 ± 0.65 fold-expression, n=7). However, the difference was not significant F(2,15)=1.3807, p-value=0.2816.

Quantitative PCR- Medial Amygdala

Figure 6.28 We found that gene expression of arginine vasopressin (*Avp*) was considerably elevated in male high and low dose compared to oil controls (Oil Control: 1.45 ± 0.75 fold-expression, n=4; Low Dose: 11.12 ± 4.69 fold-expression, n=7; High Dose: 7.91 ± 6.30 fold-expression, n=4). In spite of this increase, a one-way ANOVA revealed no significant difference across treatment F(2,12)1.6589, p-value=0.2312. We found that expression of oxytocin receptor (*Oxtr*) was lower in male high dose compared to low dose and oil controls (Oil Control: 0.94 ± 0.11 fold-expression, n=6; Low Dose: 0.91 ± 0.08 fold-expression, n=10; High Dose: 0.39 ± 0.07 fold-expression, n=5). A one-way ANOVA revealed a significant difference in treatment F(2,18)=8.483, p-value=0.002539. Gene expression of pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) in male high dose was lower than both low dose and oil controls (Oil Control: 1.14 ± 0.23 fold-expression, n=7; Low Dose: 1.27 ± 0.25 fold-expression, n=11; High Dose: 0.27 ± 0.05 fold-expression, n=7). This decreased gene expression was significantly different F(2,22)=13.788, p-value=0.0001314. Additionally, we found an increased expression of pi-

tuitary adenylate cyclase-activating polypeptide (*ADCYAP1*) in male high dose compared to male oil controls (Oil Control: 1.25 ± 0.44 fold-expression, n=4; Low Dose: 2.39 ± 0.99 fold-expression, n=7; High Dose: 4.62 ± 0.60 fold-expression, n=5). A one-way ANOVA revealed a significant difference $F(2,13)=3.5242$, p-value=0.0500.

Chapter Summary

Autism spectrum disorder (ASD) is a complex, multifaceted disorder with a classification that is continually in flux the more we learn of it [131]. As autisms definition evolves, there are a few key tenants of the disorder that persist. Autism exists on a spectrum. This has made modeling the disorder very difficult. The core behavior deficits (sociability and communication) in autism are uniquely human, and finding an animal model and tests that maintains a high degree of face-validity to complex human behaviors has been challenging [54, 116, 284]. There is certainly a genetic component to autism, however this genetic factor is not solely responsible for the disorder [182, 183]. There is strong evidence that an *environmental factor* is involved in autism and perhaps this environmental factor may act upon an underlying genetic susceptibility to produce autistic phenotypes. There are also common comorbidities in autism such as attention-deficit disorder, increased anxiety and varying degrees of mental retardation. In this study we sought to investigate the involvement, if any, of polybrominated flame retardants (PBDEs) in autism spectrum disorders. Although there have been studies of behavioral consequence of exposure to specific PBDE congeners, there has not been a complete behavioral assessment of industrial mixtures of PBDEs, e.g. DE71 [35, 262, 59]. PBDEs are not applied as single pure congener mixtures

in industry but as mixtures of related congeners as it is more practical and cost effective. In order to model human exposure to PBDEs we decided to use the penta-congener mixture of PBDEs, DE71, and expose mice in utero and via lactation [344]. Anxiety is a common comorbidity of autism [9]. We used the elevated plus maze to assess any affects on anxiety produce by DE71 exposure. There were no differences in preference of open/closed arms across treatment or sex. We also wanted to assess locomotion deficits in mice treated with DE71. PACAP deficient mice display a hallmark increased locomotion in an open field maze [137]. We tested both male and female mice in the open field maze for 1-hour and found no significant differences across treatment groups in both distance traveled and velocity. One of the core symptom domains of autism is repetitive/ritualistic behaviors [9]. These repetitive behaviors manifest in a mouse with increased self grooming or excessive digging. We measured digging behaviors in mice with the marble burying test. Interestingly, we found that the high dose DE71 treatment decreased digging in mice, but only statistically significant in the female mice. Unfortunately, this test was not video recorded so we are not able to account for what these high dose mice preoccupied themselves with during the 30-minute trial. Therefore, we cannot rule out the possibility that these mice were involved in any other form of stereotypy during their trial. Most high dose animals did not so much as partially bury even one marble. This could be a result of either the mice staying completely immobile during the 30-minute trial or being engaged in another behavior that we did not account for. Immobility seems unlikely as high dose animals were not significantly different from other treatment groups in the open field test. We intend to follow up with a more in depth analysis of particular stereotypies in high dose DE71 mice in the future. Previous

work in our lab has revealed a significant role for PACAP in social recognition ability. We hypothesized that the deficit in social recognition in the PACAP knockout mouse was due to the permissive function of PACAP in intra-hypothalamic vasopressin release [105]. Based on the aforementioned decrease of intra-hypothalamic PACAP resulting from DE71 treatment, we hypothesized there would be a corresponding effect on social recognition ability and indeed we found it in our low dose mice. In both male and female (**Figure 6.19**) mice we found that our low dose treatment impaired the animals ability to recognize a familiar mouse in the presence of a novel mouse. We followed up with this finding by performing a sociability task aimed at measuring baseline social approach behavior. We found no effect of DE71 treatment on sociability across sex and treatment levels. Therefore, the lack of social recognition ability in our low dose mice cannot be accounted for by any abnormal sociability issue. The social recognition task we employed was based on the olfactory investigation of conspecific mice. Having ruled out any influence of social approach shortcomings to explain the social recognition deficit we next sought to rule out olfactory impairments. We used a cursory olfactory preference test to profile olfaction in DE71 treated mice. We found normal olfaction profiles only in oil control mice in both female and male mice. Low and high dose mice did not show the typical preference for the peanut butter odor as mice generally do. The could possibly have swayed the results of the social recognition task in our low doses but we are doubtful. If the sole reason for the our social recognition deficit was olfaction, one would expect the same deficit in the high dose mice as they performed arguably worse on the olfactory preference test. This was not the case, so we suspect a more probable explanation could be an alteration of food preference or olfactory processing

resulting from DE71 treatment. Olfaction in DE71 treated mice merits further study. Following the completion of the battery of behavioral tests we measured plasma vasopressin. Prosocial effects of peripherally administered neuropeptides, vasopressin and oxytocin have been reported in rodents [257]. Although we suspect centrally released neuropeptides to have a more significant role in the social behaviors we assessed in this study, plasma vasopressin served a proxy variable to aid us in investigating systems that may underline the behavioral abnormalities in DE71 mice. We found no differences in plasma vasopressin in female mice. We did find significant differences in plasma vasopressin in our male mice. We found that vasopressin was elevated in our DE71 high dose males compared to both the low dose and oil controls. The plasma vasopressin of the low dose males was lower than the oil controls but did not meet statistical significance. While these findings do not completely match up and therefore can account for the social recognition deficit, they do suggest that neuropeptides like vasopressin may be affected during DE71 exposure. Previous work in our lab corroborate this conclusion. As for the lack of variability in female plasma vasopressin, it should be mentioned that while both vasopressin and oxytocin are involved in social behaviors in male and females, each neuropeptide plays different sex specific roles [264].

We used the results of the behavioral tests to guide our PCR analysis. The social recognition deficit was not sex dependent as both female and male low dose mice had an inability to recognize familiar mice. On the other hand, the aberrant plasma vasopressin was only found in the male mice. Therefore, we micropunched specific brains regions from frozen mouse brains in our male mice (oil controls, DE71 low dose and DE71 high dose). We found

expression of the PACAP receptor, PAC1 (*ADCYAP1r1*), to be significantly decreased in the cingulate cortex (**Figure 6.24**), lateral septum (**Figure 6.25**), paraventricular nucleus (**Figure 6.26**), supraoptic nucleus (**Figure 6.27**) and medial amygdala (**Figure 6.28**) of the high dose male mice. It has been shown that PACAP mRNA-expressing cell bodies are present are all of these regions in addition to expression of the PAC1 receptor [320], however their roles as it pertains to social recognition or social memory is unknown. The cingulate cortex is a center of high level contextual integration and behavior monitoring [184]. In humans, differences have been found in diffusion tensor imaging studies of the cingulate cortex of patients with attention-deficit hyperactivity-disorder (ADHD), but findings are inconsistent [307]. More relevant to this study, it has been demonstrated that lesions of the cingulate cortex diminish interest in conspecifics and elicited a reduction in memory for social stimuli [267]. Further strengthening the correlation between the reduction in PAC1 mRNA in the cingulate cortex and the social recognition deficit is a relatively recent study of *social synchrony*. Social synchrony is defined as the coordination of nonverbal behaviors that encode social information [86]. In humans, it is proposed that the recognition of social synchrony involves activation's in the dorsal anterior cingulate cortex (dACC) [10]. The role of PAC1 receptor in lateral septum has also been overlooked. It is known that the lateral septum is a key region for social recognition ability [22] in the rodent and that vasopressin signaling plays an important role. Early life stress can lead to impairments in vasopressin release within the lateral septum resulting in diminished social recognition ability [198]. Perhaps reduced PAC1 receptor activity in the lateral septum also impairs vasopressin release, as it does in the supraoptic nucleus [105]. However, we did not see

reduced PAC1 receptor mRNA levels in our male low dose, the group which had attenuated social recognition ability.

The medial amygdala was another region that showed significant gene expression changes. We found that in the high dose male mice, vasopressin (*Avp*) gene expression, was increased nearly 10-fold compared to oil dosed controls. While this was not a significant difference, this finding indicates a dysfunctional vasopressinergic system in the amygdala that may contribute to the social recognition deficit in the low dose males. Both vasopressin and oxytocin play vital roles in social information processing [42]. The lateral septum, which has been shown to be a vital region for social recognition processing, receives dense vasopressin projections from the medial amygdala [22]. So although we may not have detected any gene expression deficits in the lateral septum of the low dose males, other regions that project into the lateral septum could lead to abnormal social behaviors. It has been shown that a single polymorphism of the promoter region of the vasopressin gene has been associated with amygdala activation to face recognition [210]. Intraventricular vasopressin injections enhances social recognition, and this enhancement is blocked with application of V1a antagonist into the central nucleus of the amygdala [90]. Furthermore, amygdala responses to social stimulation have been associated with genetic variations of *Avpr1a* [233].

Also of interest is the decreased gene expression of oxytocin receptor in our male high dose group. It has been shown that mice lacking the oxytocin gene have social amnesia, while other forms of memory were unaffected [88]. More relevant was a finding in humans that decreased social recognition was positively associated with polymorphisms of

the oxytocin receptor gene [286]. What is perplexing with our findings is that our male high dose group still maintained the ability to recognize conspecifics in spite of the altered gene expression.

We hypothesize that these changes in gene expression are not imparted by direct action of PBDE in adult brains, but through epigenetic effects during early critical windows. It has been shown that PBDE can induce epigenetic changes via DNA methylation [344, 156, 181, 148]. One of the key components of the DNA methylation process is the binding proteins that bind CpG islands thus allowing for DNA methyltransferase proteins to lay down the methyl groups on cystines. Transient reductions in methyl-CpG-binding protein 2 (MeCP2) expression in the developing male amygdala of mice produces a lasting impact on vasopressin gene expression [92]. There is precedence for this type of epigenetic influence on social behaviors. Williams syndrome is characterized by abnormal social behavior and changes in oxytocin and vasopressin systems resulting from epigenetic modification of neuropeptide receptors [115].

Taken together, our data shows that PBDE exposure produces changes in vasopressin and PACAP systems in the mouse brain regions associated with social recognition. Unfortunately, while this study has revealed for the first time significant alterations of PACAP and neuropeptide receptor gene expression in the brain as a result of PBDE exposure, there is no clear correlation between the gene expression and the behavior deficits. What we seem to be left with is a mixed bag of conflicting findings. For this reason, its is important to consider our experimental design. First, we used an industrial mixture of penta-PBDEs, DE71. What few studies have worked on the consequences of *in utero*

PBDE exposure, have done so using single congeners. For those studies, the use of a single congener was both a strength and a weakness. The strength of that type of experimental design is that is reduces variability of results since only one type of PBDE is used. The weakness is that exposure to a single congener is unrealistic in the context of translating the results to human exposure. Humans are more likely to come into contact with mixtures of PBDEs like DE71. Since there are many PBDE congeners in a mixture like DE71, the list of possible interactions within a biological system is numerous. We may be looking at synergistic and/or opposing effects within this study. Given the seemingly endless possibilities of deleterious effects imparted by DE71 in this study, we dare only make general conclusions regarding the relationship between behavior and brain gene expression. First, we can say that exposure to DE71 has a negative impact on social interactions, specifically social recognition. Secondly, we can also conclude that exposure to DE71 significantly affects gene expression of PACAP, PAC1 receptor, and oxytocin receptor within brain regions involved in social interactions. Given these conclusions, we feel that more directed studies into the effects of PBDEs as they relate to disorders such as autism are merited.

Chapter 6

Conclusions

This dissertation presents a broad analysis of pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors and their involvement in stress and social behaviors. Focus on PACAP begin within the hypothalamus, as a master regulator of releasing factors targeted towards the pituitary gland. This is in fact where this dissertation work began. Our lab has previously established the requirement for PACAP in somatodentritic release of vasopressin within the supraoptic nucleus [105]. This early work was pivotal in the direction of the studies detailed here. When Gillard *et.al* 2006 established a novel role for PACAP in the release of vasopressin, it was done in an isolated tissue culture of hypothalamic punches. We now have provided for the first time, physiological data implicating a role for PACAP in acute osmotically stimulated systemic vasopressin release. When challenged with acute hyperosmotic stimulation, WT mice, both male and female, respond by increasing the release of vasopressin from the posterior pituitary (**Figure 6.4**). This vasopressin, targeting water resorption in the kidney, is attenuated in the PACAP KO mouse. The previous study

used bath application of osmotic stimulus to induce somatodentritic vasopressin release. In the study detailed here (**Chapter 3**), we used a physiological stimulus to trigger an activated state of the vasopressin producing supraoptic nucleus, prolonged salt water treatment. Tissue punches of supraoptic nuclei from WT animals maintained on salt water for a seven days, released more vasopressin compared to tissue punches from animals maintained on tap water. This effect was not present in tissue punches harvested from PACAP KO mice. These data further substantiate previous claims from our lab that PACAP is critical for vasopressin release. We now know that the role for PACAP in vasopressin release is physiologically relevant and not only observed *in vitro*.

Early work on the peptide vasopressin was focused on its role in water balance, hence its early name the *antidiuretic hormone*. However, there is another important role for vasopressin within the *social brain*. Both neuropeptides, vasopressin and oxytocin, have been demonstrated to play vital roles in social and anxiety behaviors via studies utilizing pharmacology and most recently transgenic models [264, 257, 91, 210]. Since the data presented in this dissertation has supported a permissive role for PACAP in the release of vasopressin for water balance, we sought to investigate whether or not this relationship was maintained in other aspects of the vasopressin system, namely behavior. The PACAP KO mice we utilized from the Waschek lab at UCLA, were generated on a pure C57Bl/6 background which is different from what others have used to characterize a behavioral profile for that particular transgenic model [137]. This is notable since others have reported behavioral difference amongst various strains of mice [53]. Therefore, we began by corroborating previous findings from other transgenic PACAP models. We found the same anxiety and

locomotion abnormalities that have been previously reported in our PACAP KO mouse. We then focused on social behaviors that have known associations with vasopressin signaling. We found that PACAP KO mice lack the ability to recognize novel from familiar mice. We then followed up this finding with another transgenic mouse with a gene deletion of a type II PACAP receptor, vasoactive intestinal peptide receptor (VPAC2r). We did not see the same social recognition deficit in the VPAC2r KO mouse. Given the role of PACAP in vasopressin release we hypothesize that the ablation of social recognition ability in the PACAP KO mouse is due, in part, by an attenuated vasopressin release resulting form the gene deletion. Additionally, we assayed plasma vasopressin content. We found that PACAP KO mice had significantly less circulating basal vasopressin than their WT counterparts. Since we did not see a change in social recognition ability in the VPAC2r KO mouse, and found them to have similar amounts of plasma vasopressin, we conclude that social recognition does not involve PACAP signaling through VPAC2 receptors. Since the PACAP KO mouse was not a site-specific knockout, future studies are needed to elaborate on the location and mechanism of PACAP signaling in recognizing social cues.

We wanted to analyze the *real world* implications of PACAP's role in social recognition. Unpublished work from our lab has shown that polybrominated diphenyl ethers (PBDEs) can reduce expression of PACAP and vasopressin. In this unpublished work, we analyzed expression of vasopressin and PACAP in the osmoregulation centers of the hypothalamus, the supraoptic nucleus and the paraventricular nucleus, and found profound decreases. Other work from our lab has shown that PBDE exposure debilitates osmoregulation in the rat [274]. Therefore, we hypothesized that PBDE exposure would produce the

behavioral deficits we found in the PACAP KO model by globally impacting PACAP and vasopressin systems in the mouse brain. We used an industrial mixture of penta-PBDEs, DE71, to expose mouse pups *in utero*. We found that a low dose of DE71 impaired social recognition ability while oil controls and DE71 high dose did not produce this effect (**Figure 6.19**). This impairment of social recognition ability had no effect on general sociability. To understand how these changes in behavior arose, we used quantitative PCR to measure gene expression of neuropeptides, PACAP and their receptors along the social recognition pathway outlined in the literature [22]. We found abnormal gene expression of the PACAP receptor, PAC1r in the corpus collosum of low dose mice. We additionally found a profound increase in gene expression of vasopressin in the medial amygdala of the low dose male mice. Both regions have been implicated in social and anxiety behaviors. Plasma vasopressin was also affected in the low and high dose males. Although the results are inconsistent with findings from PACAP KO mice, they still allude to an altered vasopressin system caused by PBDE exposure. Taken together, this work provides the first evidence of a social recognition deficit concomitant with changes in vasopressin signaling as a result of an environmental toxin. The work presented in this dissertation supports the notion that PBDEs may be an environmental trigger for social disorders such as autism spectrum disorder. We hope that this study will be able to focus future inquires into the ramifications of PBDE exposure.

Finally, we utilized the PACAP receptor, VPAC2r, knockout mouse to investigate its role in stress responses. PACAP has been implicated in stress responses and even post-traumatic stress disorder [260, 227]. Given the comorbidity of stress and anxiety with disorders such as autism spectrum disorder, we wanted to understand the role, if any, of

PACAP. By the time of this study, PACAP was had an established role in stress responses [228, 239, 121, 265, 313, 227, 260]. A recent study found that these stress responses were not mediated via signaling though the type I PACAP receptor, PAC1r [227]. Having ruled out a role for the type I PACAP receptors in stress responses, the role for type II receptors such as VPAC2r seemed plausible. **Chapter 2** outlines a critical role for VPAC2Rs in immediate epinephrine secretory responses to psychogenic stress. We found that acute restraint stress significantly increases plasma epinephrine content and that this response is absent in VPAC2r KO mice. Furthermore, VPAC2r gene expression is decreased in WT mouse adrenal glands concurrent with the plasma epinephrine increase. We hypothesize that psychogenic stress increases plasma catecholamine levels by PACAP signaling in the adrenal gland via VPAC2 receptors. Whether or not toxins like PBDEs can affect stress responses is to be determined. However, this dissertation provides evidence that by affecting PACAP systems, PBDEs may also be involved in aberrant responses to stress.

Taken together, the studies summarized in this dissertation provide new insights into the role of PACAP and its receptors in social behaviors and stress responses. More importantly, these studies highlight the role of polybrominated flame retardants in abnormal PACAP signaling and their potential influence in producing behavioral phenotypes resembling autism. Hopefully, future investigations deriving from these studies will provide novel targets for treating or preventing social disorders in humans.

Tables & Figures

Table 6.1: **List of Abbreviations**

Abbreviation	
ADCYAP1	pituitary adenylate cyclase-activating polypeptide (*gene*)
ASD	Autism Spectrum Disorder
Avp	arginine vasopressin (*gene*)
Avpr1a	arginine vasopressin receptor 1A (*gene*)
β-actin	beta actin (*gene*)
EPM	elevated plus maze
KO	knockout (gene deletion)
Oxtr	oxytocin receptor (*gene*)
Oxy	oxytocin
PACAP	Pituitary adenylate cyclase activating polypeptide
PBDE	polybrominated diphenyl ethers
PCR	polymerase chain reaction
PVN	paraventricular nucleus
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
SON	supraoptic nucleus
SRT	Social Recognition Task
VP	vasopressin (peptide)
VPAC2r	vasoactive intestinal peptide receptor 2 (*gene*)
WT	wild type

Table 6.2: **Quantitative PCR Primers**

Gene	Accession #	Forward primer 5'-3'	Reverse primer 5'-3'	$T_m{}^oC$ (F/R)	Length (bp)	Efficency
β-Actin	NM 007393	TAGGCACCAGGGTGTGATGGTGG	GCAGCACAGGGTGCTCCTCAG	63.2/62.9	200	102.1 %
Avp	$NM_0$09732.2	CTCAACACTACGCTCTCCGC	CAGCAGATGCTTGGTCCGA	60.8/57.89	173	110.3 %
Avp1r	$NM_0$16847.2	GCTGGACACCTTTCTTCATCGTC	CTGTTCAAGGAAGCCAGTAACG	61.71/59.52	115	91.0 %
PAC1r	$NM_0$07407	TTCACTACTGCGTGGTGTCCAACT	ATATCCCAGCATCCCGCATCATCA	60.3/60.3	199	110.4 %
PACAP	$NM_0$09625	AGGTGCTGGTGTTGGAATGAATGC	AATGCATGAGGGCAAGGGTAGGAA	60.2/60.7	176	96.0 %
Oxtr	$NM_0$01081147.1	GTGCTGGACGCCTTTCTTCTTC	CTTGCTAATGCTCGTCTCTCCAG	62.01/61.04	235	103.0 %
TH	NM 009377	AGTCAGTCAGTCAGTCAGTC	AGTCAGTCAGTCAGTCAGTC	60.3/60.4	177	104.15 %
PNMT	NM 008890	TGCCCACTTTGAGGACATCACCAT	TGAGGCAGGCATGCTGACTATACA	60.7/60.0	125	105.0 %
VPAC2	NM 009511.2	GCGGTGTCTGGGACAACATC	CTGTGACATTTTCCCCAACGT	58.6/55.7	60	102.1 %

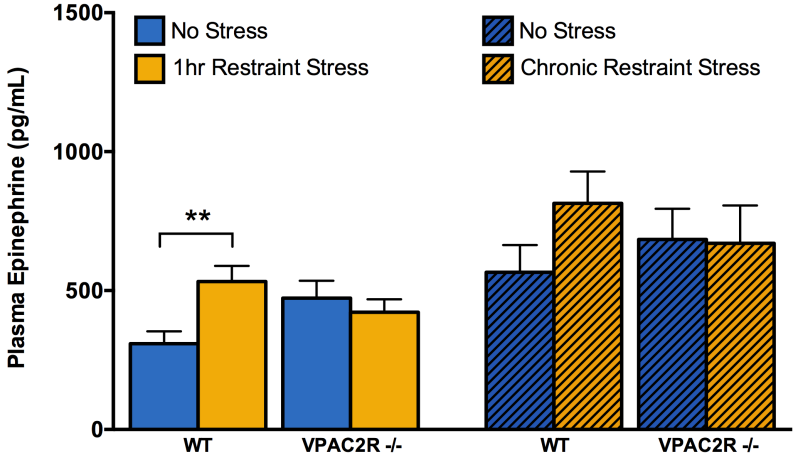

Figure 6.1: **Effect of VPAC2 gene deletion on plasma epinephrine content following acute and chronic restraint stress.**

Epinephrine was assayed from plasma of male VPAC2 KO and WT C57Bl6 mice after a single restraint stress or sham. Plasma assay data was used to construct a *Between Subjects General Linear Model* with *Genotype* and *Stress* representing main factors. The *Analysis of Variance* revealed a significant *Interaction* (p=0.02204). Asterisks indicate statistical significance of multiple comparison test between *WT No Stress* and *WT 1hr. Restraint Stress* groups at p=0.00594 (∗∗).

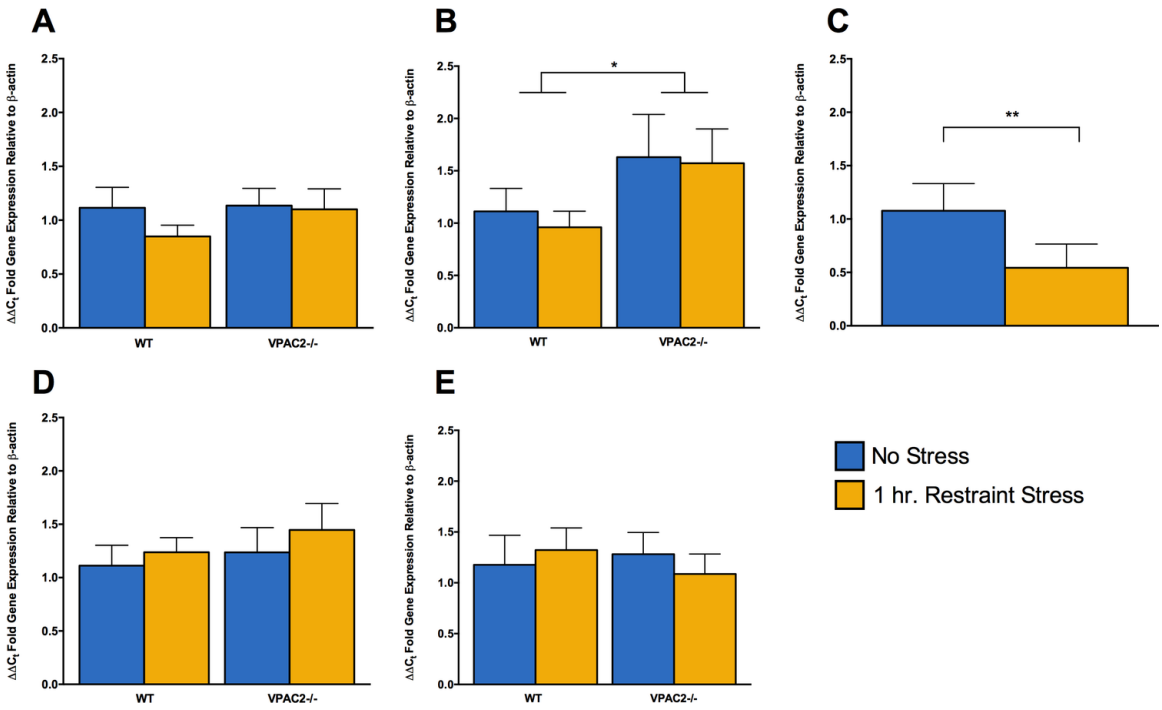

Figure 6.2: **Effect of acute 1 hr. restraint stress on gene expression of WT and VPAC2 KO adrenal glands.**

qPCR analysis was performed on the adrenal glands of male WT C57Bl6 mice after a single restraint stress or sham. Transcript expression levels were normalized to the reference gene β-actin. **A** Adrenal expression of pituitary adenylate cyclase-activating peptide (PACAP) **B** Adrenal expression of pituitary adenylate cyclase-activating peptide receptor type 1 (PAC1r) was significantly elevated in VPAC2R KO mice compared to WT (p=0.0203). **C** Adrenal expression of vasoactive intestinal peptide receptor 2 (VPAC2) **D** Adrenal expression of phenylethanolamine N-methyltransferase (PNMT) **E** Adrenal expression of tyrosine hydroxylase (TH) Asterisks indicates statistical significance from a Two Sample t-Test in comparison to unstressed controls at $p \leq 0.05$ $(*)$

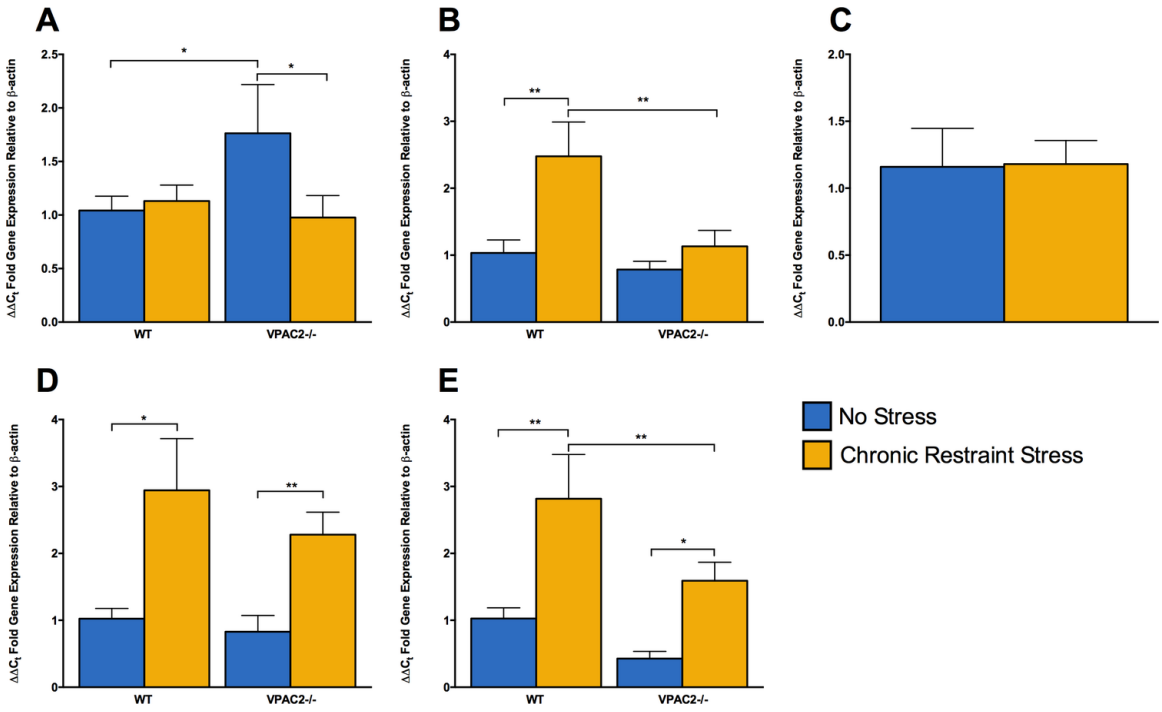

Figure 6.3: **Effect of chronic restraint stress on gene expression of WT and VPAC2 KO adrenal glands.**

A Pituitary adenylate cyclase-activating peptide (PACAP) was increased in VPAC2R KO compared to WT (p=0.0256). PACAP expression was decreased in VPAC2R KO chronic stress compared to VPAC2R KO no stress (p=0.0153). **B** Pituitary adenylate cyclase-activating peptide receptor type 1 (PAC1r) was significantly elevated in WT chronic stress compared to WT controls (p=0.00693). Additionally, VPAC2R KO chronic stress expression was significantly lower than WT chronic stress (p=0.00295). **C** Vasoactive intestinal peptide receptor 2 (VPAC2). **D** Phenylethanolamine N-methyltransferase (PNMT) was significantly increased by chronic stress in both WT (p=0.01217) and VPAC2R KO (p=0.00872). **E** Tyrosine hydroxylase (TH) was significantly elevated by chronic stress in both WT (p=0.00366) and VPAC2R KO (p=0.02229). VPAC2R KO chronic stress was significantly lower compared to WT chronic stress (p=0.03191).

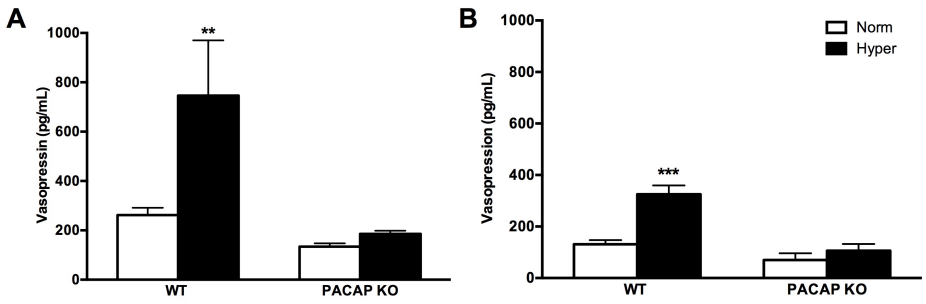

Figure 6.4: **PACAP KO mice have attenuated plasma vasopressin release.**

A. Male Mice were stimulated via i.p. injection of normosmotic and hyperosmotic saline then anesthetized with isoflurane before cardiac blood was taken for analysis of VP release. Hyperosmotic saline stimulated a significant increase in VP release in only WT but not PACAP KO. Treatment has a very significant effect on vasopressin release, $F(1,26)=6.16$, $p=0.02$, but only for WT. **B.** Female Mice were stimulated via i.p. injection of normosmotic and hyperosmotic saline then anesthetized with isoflurane before cardiac blood was taken for analysis of VP release. Hyperosmotic saline stimulated a significant increase in VP release in only WT but not PACAP KO. Treatment has a very significant effect on vasopressin release, $F(1,28)=16.53$, $p=0.0004$, but only for WT.

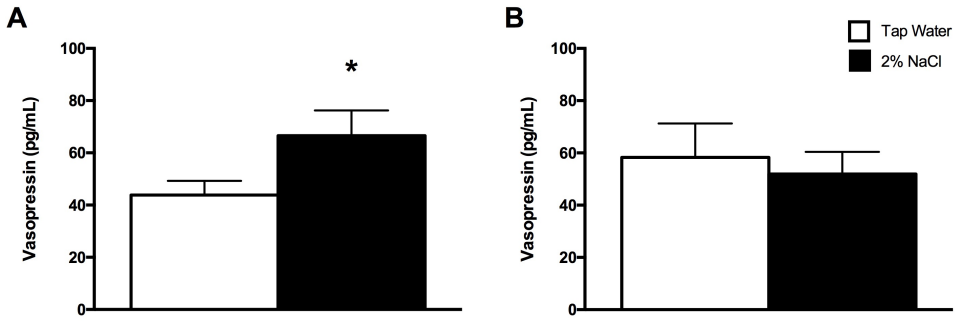

Figure 6.5: **PACAP KO mice have blunted somatodendritic vasopressin release after chronic in vivo stimulation.**

A. Acutely dissected SON punches from dehydrated WT mice show increased somatodendritic vasopressin release. $p<0.05$. Tap water n=16; Salt water n=19. **B.** SON punches from PACAP KO mice do not show similar increased somatodendritic vasopressin release. Tap water n=10; Salt water n=13.

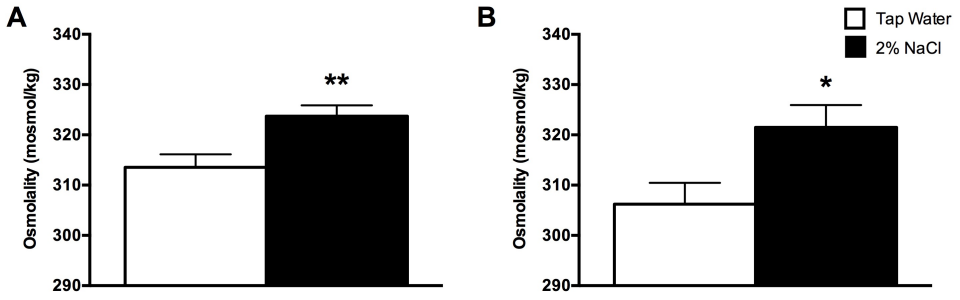

Figure 6.6: **Plasma osmolality during salt loading.**

WT and PACAP KO mice have increased osmolality during a week of salt loading on 2% salt water. **A.** WT mice responded to salt water treatment with elevated osmolalities. **B.** PACAP KO mice also displayed increased plasma osmolality when treated with salt water. $*$ Indicates statistically different from WT tap water, $p<0.05$. $**$ Indicates statistically different from KO tap water, $p<0.01$

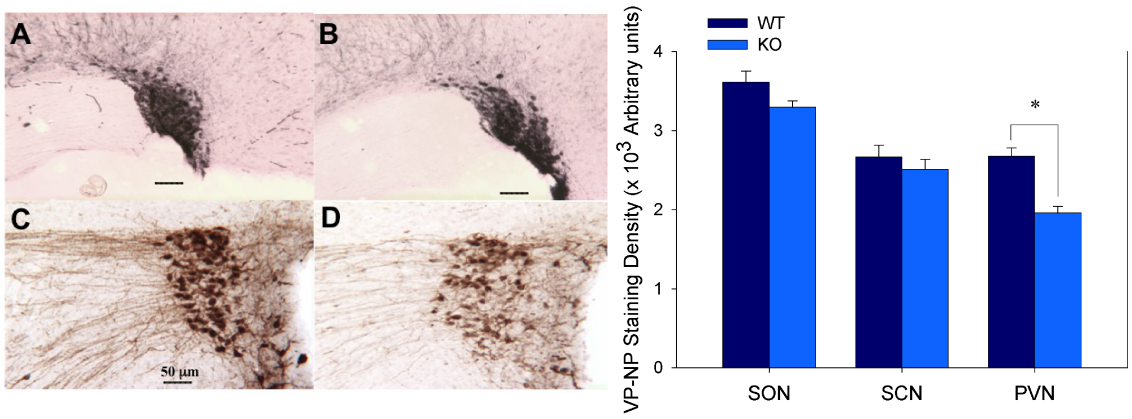

Figure 6.7: **Effect of PACAP gene deletion on vasopressin content in the mouse hypothalamic nuclei.**

Brain sections from perfused mouse brains were subjected to VP-neurophysin (VP-NP) immunohistochemistry and images were taken from regions of interest and analyzed using computer-assisted densitometry. **A-D**: Micrographs of VP-NP images from WT **(A,C)** and PACAP KO **(B,D)**. SON images are shown on top and PVN on bottom. Reduced VP-NP immunoreactivity in the magnocellular PVN is seen in both the cell bodies and in axons projecting to the posterior pituitary. **E**: Pooled VP-NP staining density for WT and PACAP KO cohorts. Basal VP-NP staining in SON was robust in both KO and WT. In contrast, the magnocellular PVN of PACAP KO mice displayed significantly reduced VP-NP immunostaining. $p < 0.05$. Calibration bar= 50 μm.

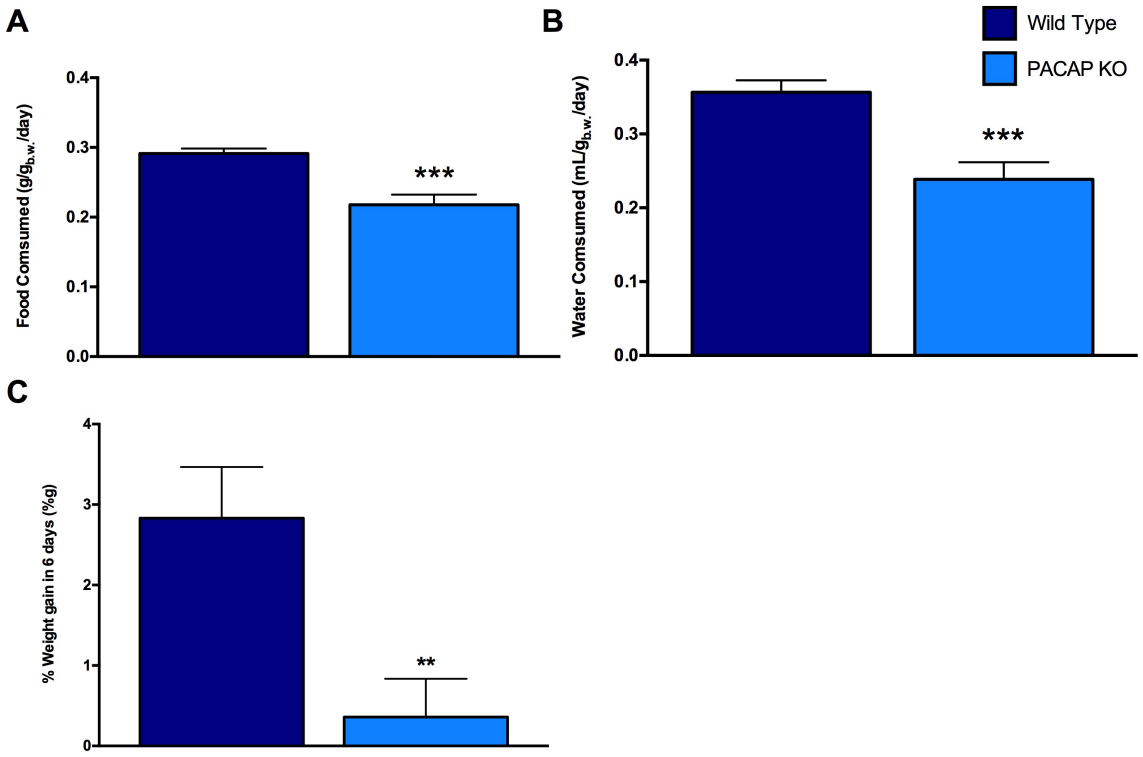

Figure 6.8: **PACAP gene deletion affects water and food intake.**

A. Animal body weights and food intake were recorded. PACAP gene deletion resulted in a significantly lower daily food intake. WT n=23 & KO n=17. **B.** PACAP KO mice drink less water. Animal body weights and water intake were recorded. PACAP gene deletion resulted in a significantly lower daily water intake. WT n=23 & KO n=16. **C.** PACAP KO animals have significantly decrease weight gain over 6 days; WT n=15 & KO n=17. $**$ p=0.0035; $***$ p<0.0001.

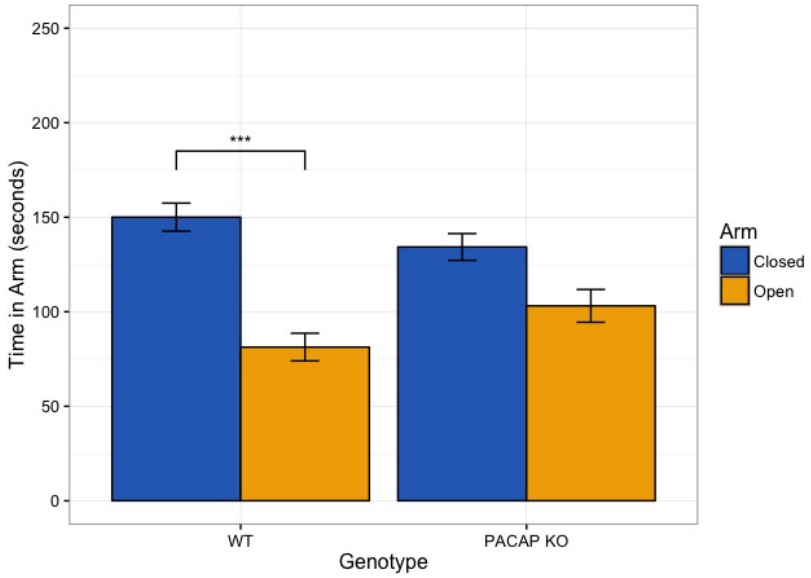

Figure 6.9: **PACAP KO Elevated Plus Maze**

The elevated plus maze measures anxiety via monitoring an animals preference for thigmotaxis. Our WT mice spent significantly more time in the open arm of the maze compared to the closed (p-value $=5.709e^{-06}$). PACAP KO mice also spent less time in the open arm of the maze, however, the difference in time spent in each arm of the maze was not significant.

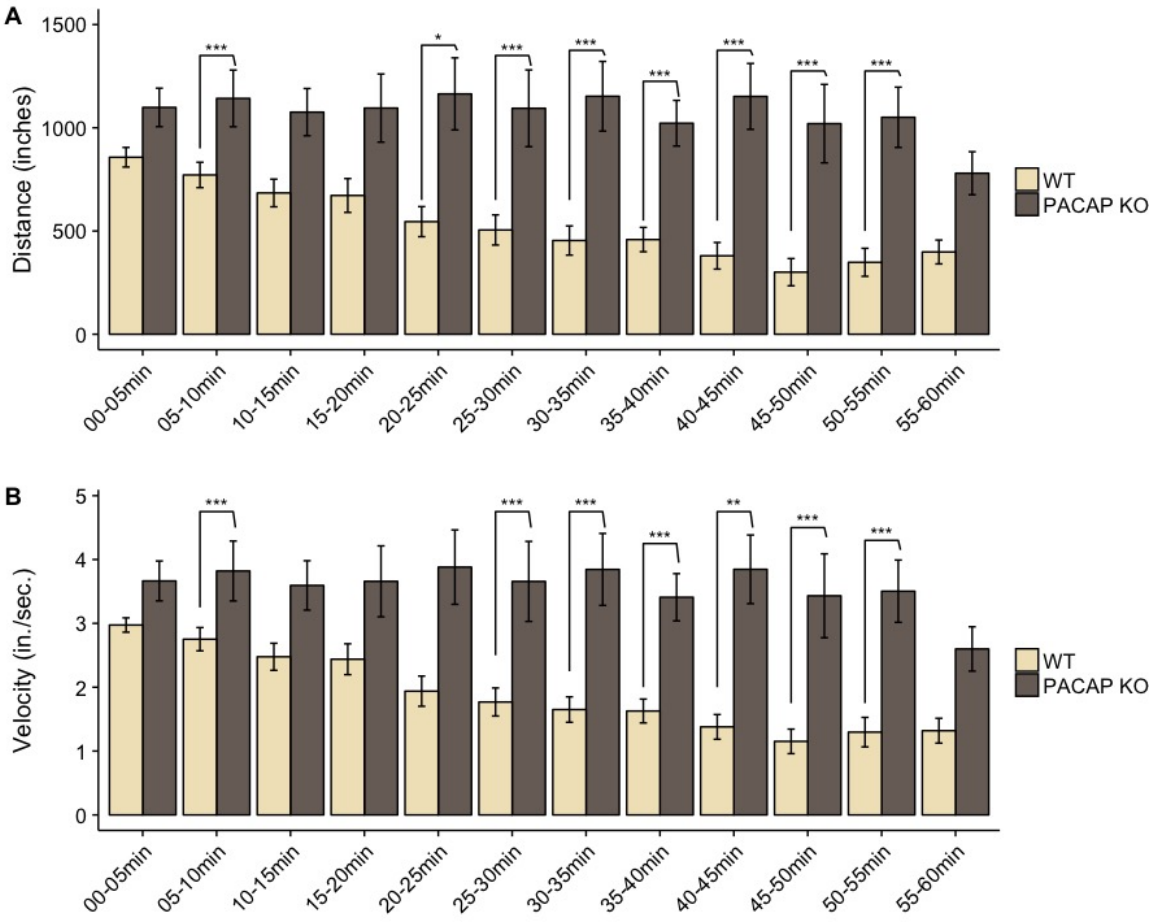

Figure 6.10: **PACAP KO Open Field Test**

A. *Distance:* PACAP KO mice travel significantly more inches per 5 minute time bin compared to WT for the following time bins: 05-10min: p< $1e^{-04}$, 20-25min: p=0.032790, 25-30min: p=0.000203, 30-35min: p=0.000477, 35-40min: p<$1e^{-04}$, 40-45min: p=0.000988, 45-50min: p<$1e^{-04}$, 50-55min: p-value=<$1e^{-04}$. **B.** *Velocity:* PACAP KO mice travel significantly faster (inches/second) per 5 minute time bin compared to WT for the following time bins: 05-10min: p=$1.35e^{-05}$, 25-30min: p=0.000334, 30-35min: p=0.000543, 35-40min: p=$3.15e^{-05}$, 40-45min; p=0.001361, 45-50min: p< $1e^{-05}$, 50-55min: p=$2.78e^{-05}$.

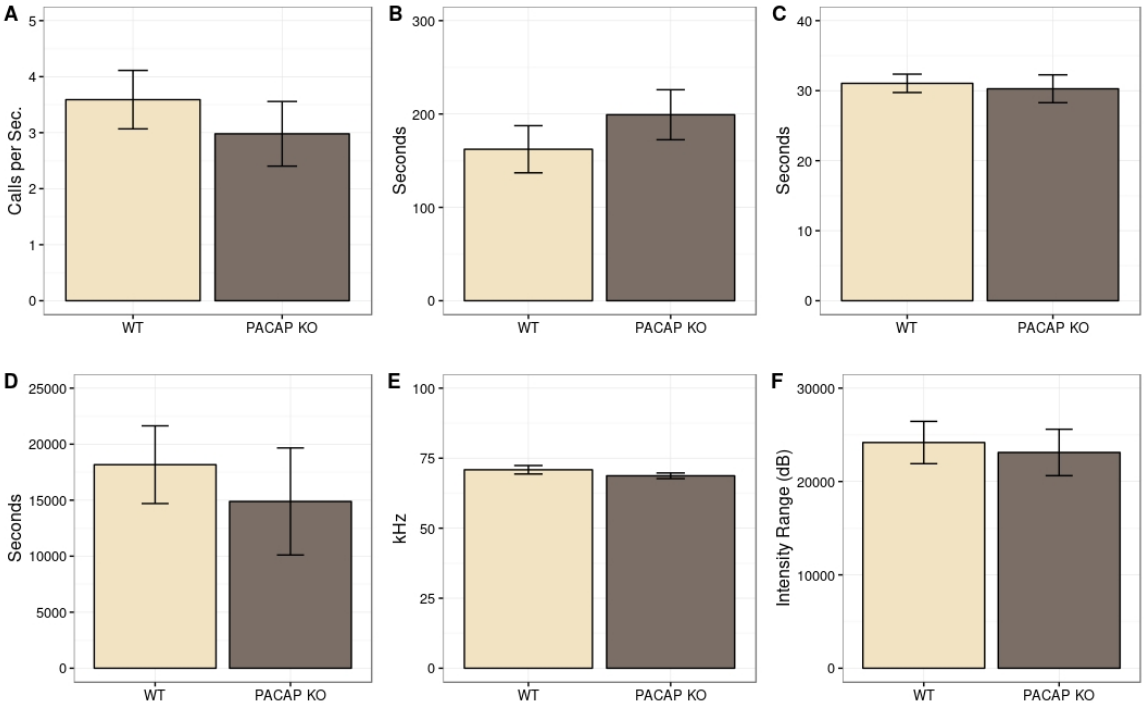

Figure 6.11: **PACAP gene deletion has no apparent effect on ultrasonic vocalizations**

A. *Calls per second:* PACAP KO had a superficial decrease in C.P.S. but did not significantly deviate from WT recordings. **B.** *Seconds without calls:* The average time in which no calls were emitted by PACAP KO mice was similar WT. **C.** *Average call duration:* The duration of each call was recorded and averages from both PACAP KO and WT were nearly identical. **D.** *Total call duration:* The total duration of calls emitted during the 5 min. trial also displayed no difference between PACAP KO and WT mice. **E.** *Average call frequency (kHz):* The frequency of the emitted call was compared across genotype. PACAP KO mice emitted near identical kHz calls. **F.** *Dynamic Range:* The range of intensity in which calls were emitted also showed no difference between PACAP KO and WT mice. WT n= 8, PACAP KO n=9.

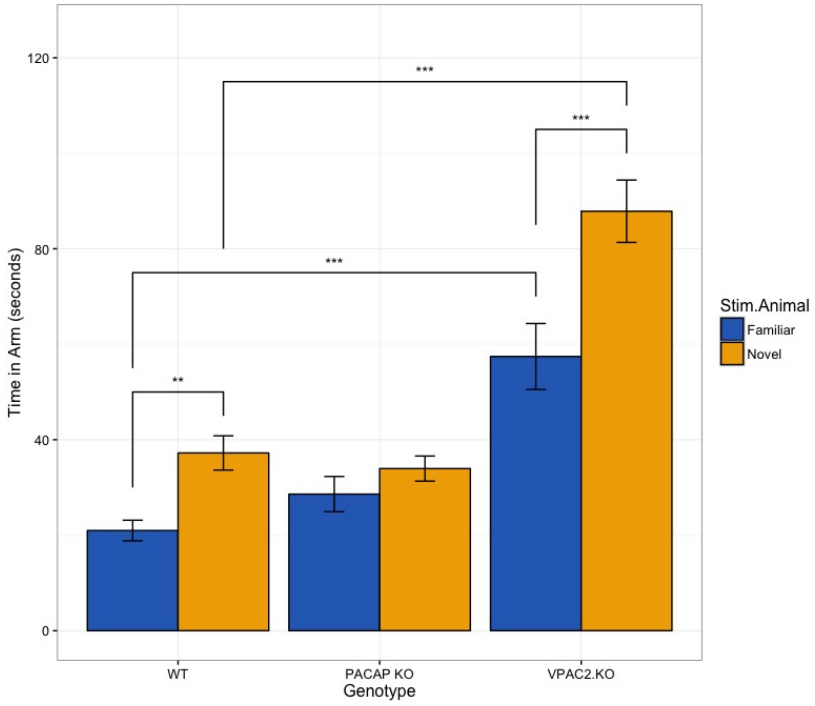

Figure 6.12: **PACAP KO but not VPAC2r KO Mice Have Ablated Social Recognition.**

In this social recognition task, greater time spent investigating a novel over a familiar mouse indicated social recognition. Here we show that WT mice spend significantly more time investigating novel stimulus mice (p-value= 0.00172). PACAP KO do not have the ability to recognize a familiar from a novel mouse and spend equivalent amounts of time investigating each (p-value= 0.51264). VPAC2r KO mice also spend significantly more time investigating novel stimulus mice (p-value= < 0.001. However, VPAC2r KO mice overall spend more investigating familiar (p-value= < 0.001) and novel (p-value= < 0.001) mice compared to WT.

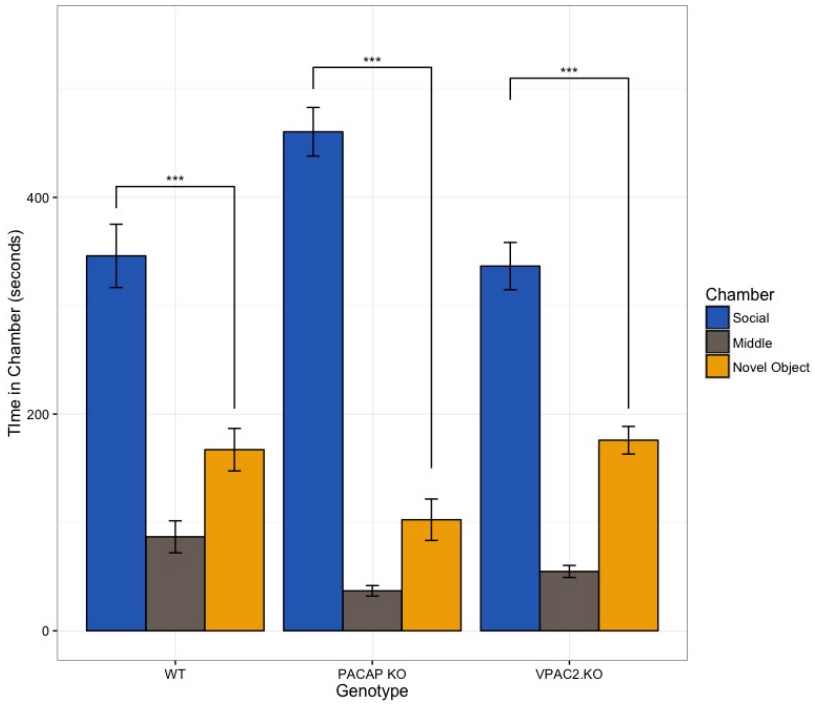

Figure 6.13: **PACAP KO and VPAC2r KO Mice Display Normal Sociability.** Sociability was measured in a three-chamber apparatus, where one chamber contained a corral housing a mouse (social chamber), one chamber was empty (middle chamber), and one chamber contained only a corral without the animal inside (novel object chamber). All three genotypes (PACAP KO mice, VPAC2r KO mice and WT) have a preference for social interaction. WT mice spent significantly more time in the social chamber vs. the novel object chamber (p-value=$2.23e^{-09}$). VPAC2r KO mice spent significantly more time in the social chamber vs. the novel object chamber (p-value=$2.20e^{-07}$). PACAP KO spent significantly more time in the social chamber vs. the novel object chamber (p-value<$2e^{-16}$).

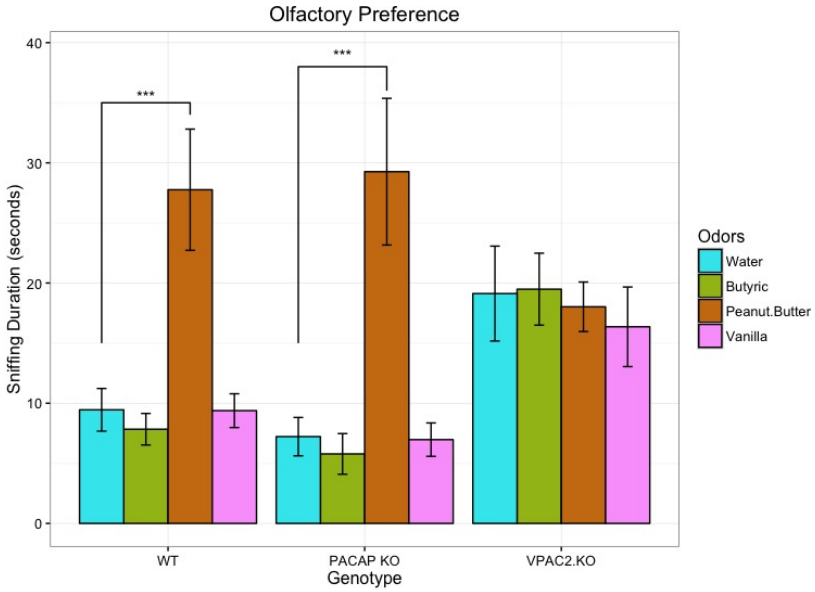

Figure 6.14: **VPAC2r KO Mice Have Attenuated Olfaction.**

We performed a simple olfactory preference test to assess any deficits in olfactory ability in both KO models. WT mice show a significant preference towards the scent of peanut butter compared to water (p-value= 0.000323). PACAP KO mice show a similar preference for peanut butter (p-value= 0.000174). However, VPAC2r KO mice displayed equal preference for all odors.

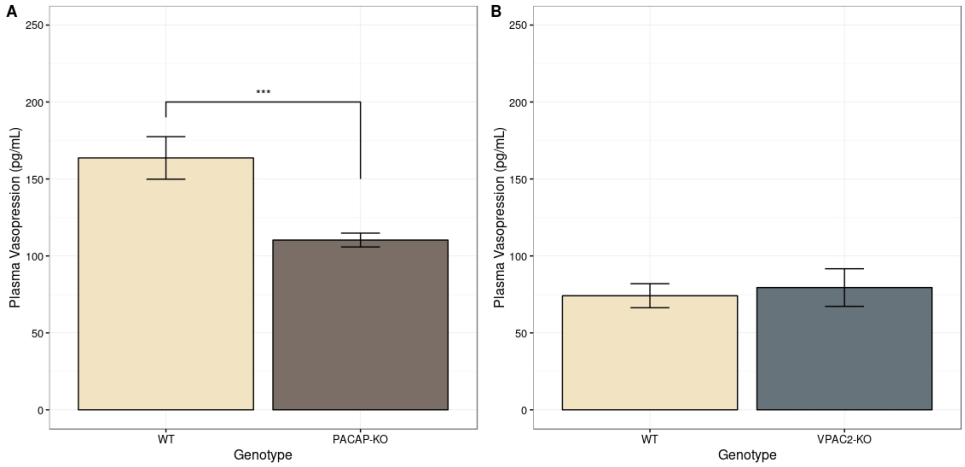

Figure 6.15: **PACAP KO but not VPAC2r KO Mice Have Reduced Basal Plasma Vasopressin.**

Plasma vasopressin was assayed from cardiac blood via ELISA. **A.** Our PACAP KO cohort had significantly less basal plasma vasopressin (Approximative Two-Sample Fisher-Pitman Permutation Test: Z =-2.8679, p-value =0.0019). **B.** The VPAC2r KO had a similar amount of basal plasma vasopressin compared to WT.

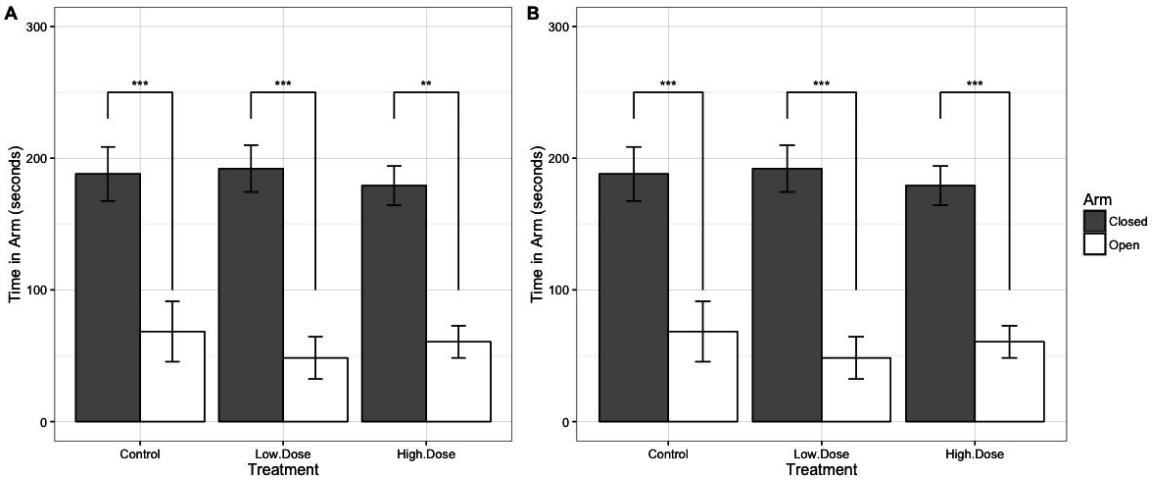

Figure 6.16: **DE71 Exposure Does Not Affect Anxiety in the Elevated Plus Maze** The elevated plus maze was used to assess anxiety in male and female mice exposed to DE71. **A.** *Male mice*: all three treatment groups spent significantly more time in the closed arm of the EPM (male oil control open-closed arm p-value=0.000177; male high dose open-closed p-value= 0.003820; male low dose open-closed p-value=0.000563). **B.** *Female mice*: all three groups spent significantly more time in the closed arm of the EPM (female oil control open-closed p-value<$1e^{-04}$; female high dose open-closed p-value<$1e^{-04}$; female low dose open-closed arm p-value=0.000388)

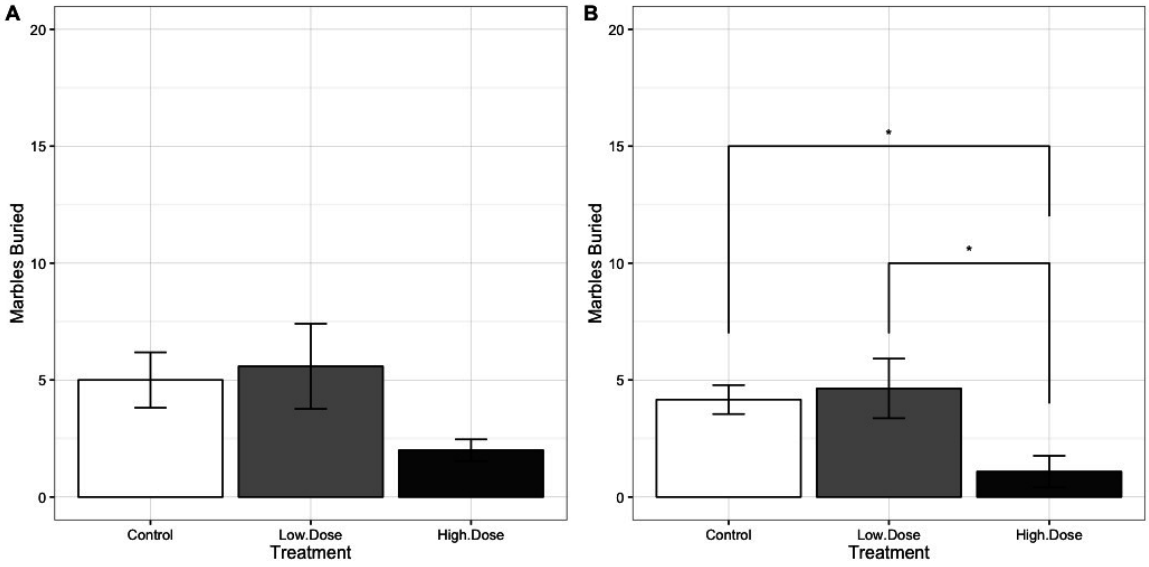

Figure 6.17: **DE71 High Dose Exposed Mice Spontaneously Dig Less Frequently** **A.** *Male* across all treatment levels there were no significant differences in marbles buried, although there was an apparent decrease in marbles buried for the high dose group. **B.** *Female* High dose females buried fewer marbles than both controls (p-value=0.0170) and low dose (p-value=0.0323).

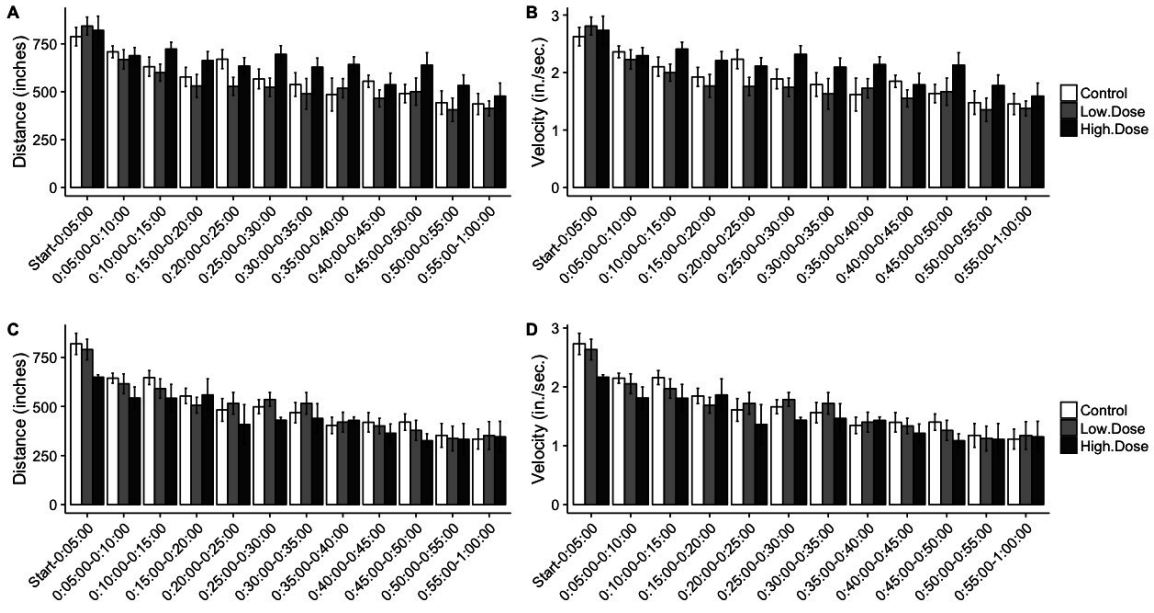

Figure 6.18: **DE71 Open Field Test**

DE71 exposure has no apparent effect on locomotion in male and female mice. **A** *Male Distance*: All three groups treatment groups show no difference in distance covered per five minute time bin. **B** *Male Velocity*: All three treatment groups exhibit similar velocity in each five minute time bin. **C** *Female Distance*: All three groups treatment groups show no difference in distance covered per five minute time bin. **D** *Female Velocity*: All three treatment groups exhibit similar velocity in each five minute time bin.

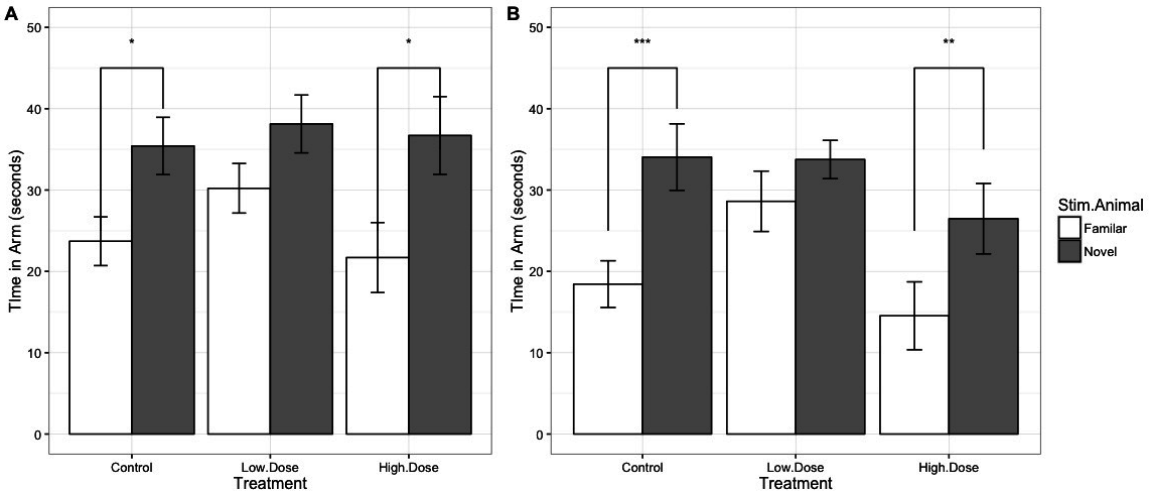

Figure 6.19: **Low Dose DE71 Exposure Ablates Social Recognition Ability Independent of Sex**

A. Male oil controls spent significantly more time investigating novel over familiar mice (p-value=0.0465). High dose male mice also spent more time investigating familiar over novel mice (p-value=0.0153). Male low dose mice failed to recognize familiar from novel mice (p-value=0.1611). **B.** Female oil control mice spend significantly more time (p-value=0.000835). Female high dose also spend more time investigating novel compared to familiar mice (p-value=0.001203). Comparable to males, the female low dose group did not spend more time investigating novel over familiar (p-value=0.325931).

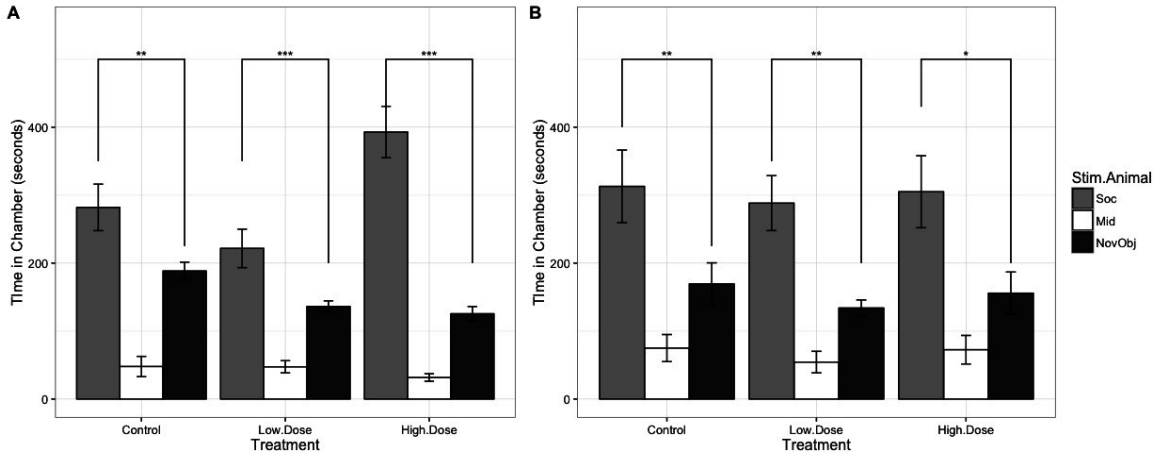

Figure 6.20: **DE71 Exposure Has No Effect on General Sociability**

We used the three-chambered sociability apparatus to measure social approach as an indication of general sociability. **A.** All three male treatment groups spent significantly more time in the social chamber compared to the novel object chamber (oil control: p-value=0.006722; low dose: p-value=0.000369; high dose: p-value$<2e^{-16}$. **B.** Al three female groups spent significantly more time in the social chamber compared to the novel object chamber (oil control: p-value=0.00871; low dose: p-value=0.00127; high dose: p-value=0.03886)

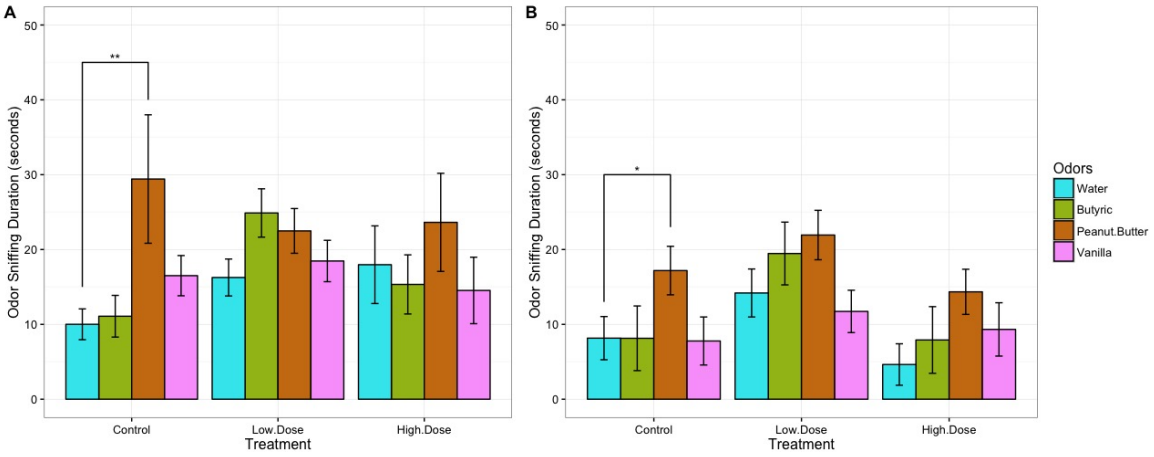

Figure 6.21: **DE71 Exposure Impairs Olfactory Preference**

We saturated 2x2 in. squares of absorbent paper with one of 4 odors and measured the time spent investigating each odor. Increased time spent investigating the odor was interpreted as increased preference towards that odor. **A.** *Male olfactory preference test.* Only the male oil control showed a significant preference for peanut butter compared to water (p-value=0.00679). **B.** *Female olfactory preference test.* As with the males, only the female oil control showed a significant preference for peanut butter compared to water (p-value=0.0184).

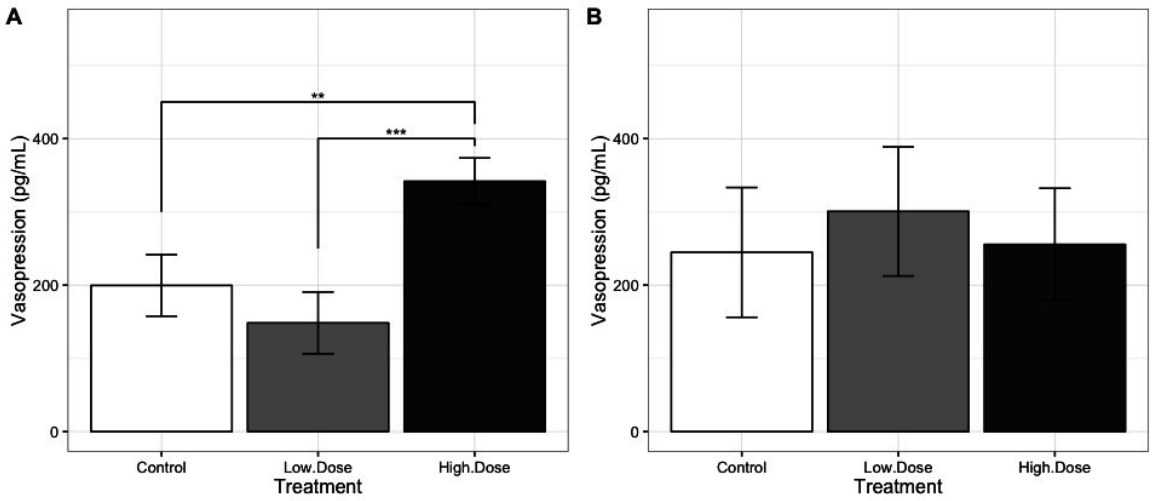

Figure 6.22: **Plasma Vasopressin in DE71 Exposed Mice.**

A. Male high dose mice had significantly higher basal plasma vasopressin in comparison to oil controls (p-value=0.00932). There was also a significant difference in basal plasma vasopressin between male low dose and male high dose (p-value < 0.001). **B.** There was no difference in female basal plasma vasopressin.

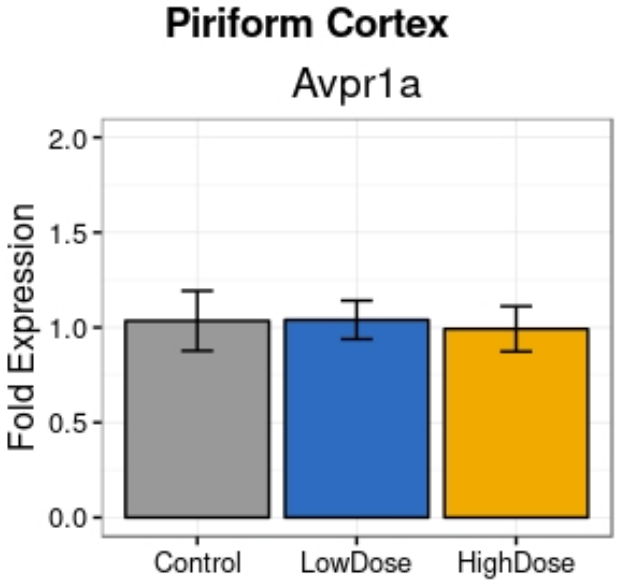

Figure 6.23: **DE71 Exposure Did Not Change Expression of Avpr1a in the Piriform Cortex**

Quantitative PCR was used to analyze gene expression differences of arginine vasopressin receptor 1A (*Avpr1a*) in the piriform cortex of male oil controls, DE71 low dose and DE71 high dose mice. Transcript expression levels were normalized to the reference gene β-actin. No apparent difference in gene expression was found. Grey= Oil Controls; Blue= Low Dose; Yellow= High Dose.

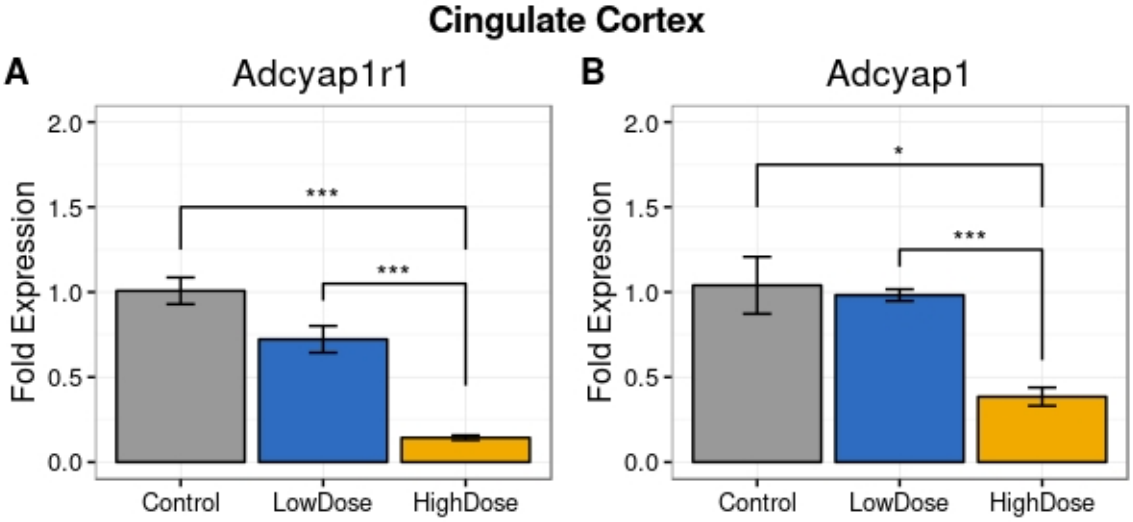

Figure 6.24: **High Dose DE71 Exposure Decreases Gene Expression of Adcyap1r1 and Adcyap1 in the Cingulate Cortex**

A. Gene expression of pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) within the cingulate cortex was significantly lower in male high dose mice compared to male oil controls (p-value <0.001) and low dose (p-value <0.001). **B.** Gene expression of pituitary adenylate cyclase-activating polypeptide (*ADCYAP1*) in the cingulate cortex was significantly lower in male high dose mice compared to oil controls (p-value=0.0147) and male low dose (p-value <0.001). Data represents fold expression relative to β-actin. Grey= Oil Controls; Blue= Low Dose; Yellow= High Dose.

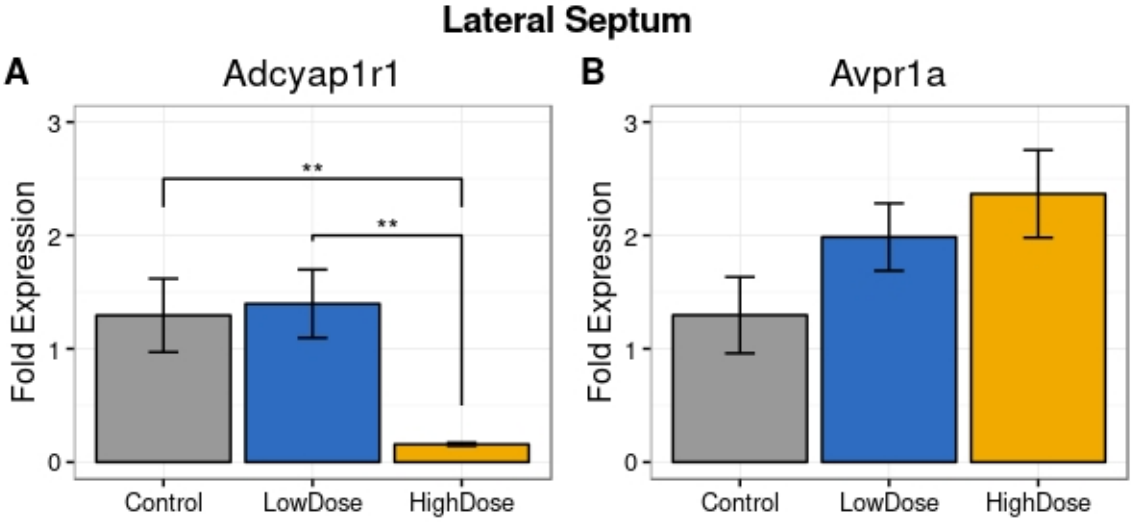

Figure 6.25: **High Dose DE71 Exposure Decreases Gene Expression of Adcyap1r1 in the Lateral Septum**

A. Gene expression of pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) within the lateral septum was significantly lower in male high dose compared to male oil controls (p-value=0.00781) and male low dose mice (p-value=0.00173). **B.** Gene expression of arginine vasopressin receptor 1A (*Avpr1a*) within the lateral septum was also similar between male oil controls, low dose and high dose mice. Data represents fold expression relative to β-actin. Grey= Oil Controls; Blue= Low Dose; Yellow= High Dose.

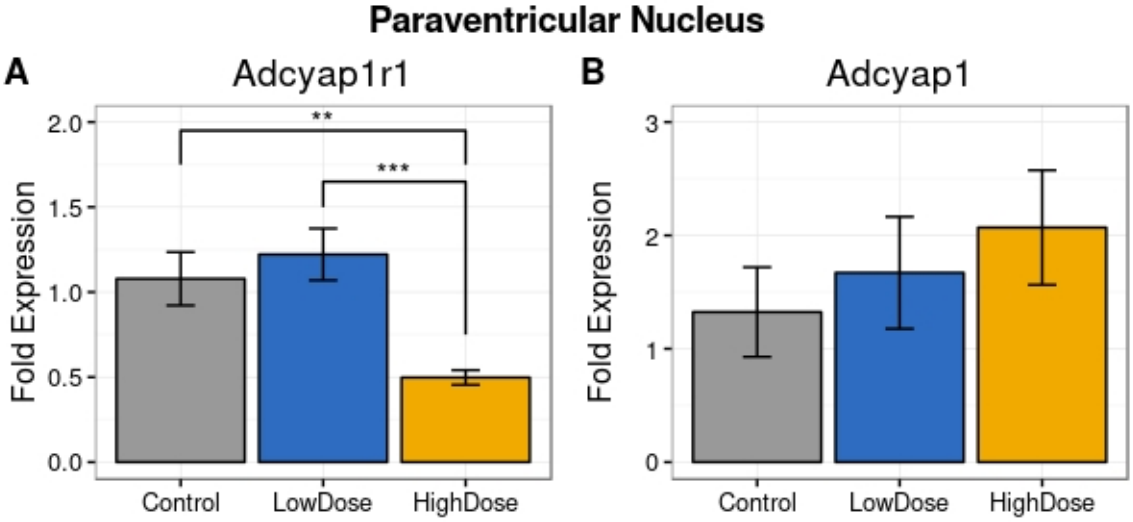

Figure 6.26: **High Dose DE71 Exposures Decreases Gene Expression of Adcyap1r1 in the Paraventricular Nucleus**

A. Gene expression of pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) was significantly decreased in male high dose mice compared to low dose (p-value=0.00173) and oil control mice (p-value=0.00781). **B.** Gene expression of pituitary adenylate cyclase-activating polypeptide (*ADCYAP1*) in the paraventricular nucleus was not different across treatment groups. Data represents fold expression relative to β-actin. Grey= Oil Controls; Blue= Low Dose; Yellow= High Dose.

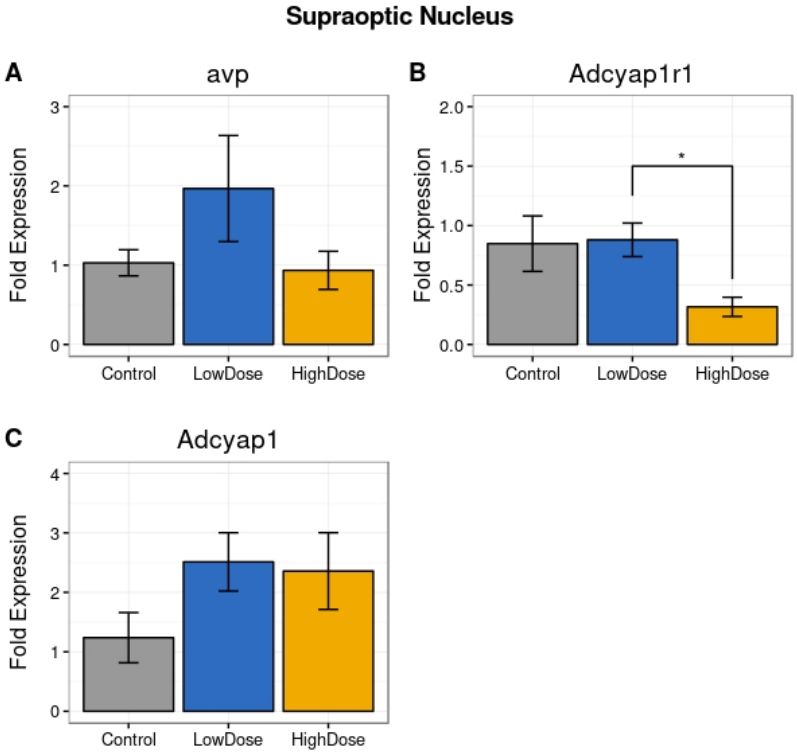

Figure 6.27: **High Dose DE71 Exposure Decreases Gene Expression of Adcyap1r1 in the Supraoptic Nucleus**

A. Gene expression of arginine vasopressin (*Avp*) in the supraoptic nucleus was elevated in low but the difference were not significant. **B.** Gene expression of pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) was significantly lower in male high dose compared to low dose (p-value=0.0221). **C.** Gene expression of pituitary adenylate cyclase-activating polypeptide (*ADCYAP1* in the supraoptic nucleus. Data represents fold expression relative to β-actin. Grey= Oil Controls; Blue= Low Dose; Yellow= High Dose.

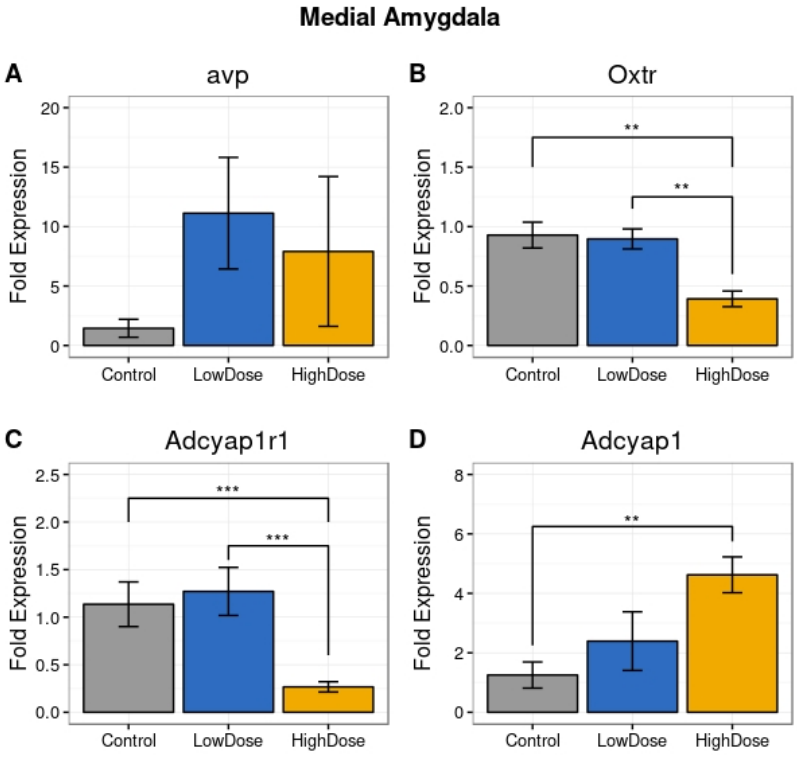

Figure 6.28: **High Dose DE71 Increases Adcyap1 Gene Expression and Decreases Oxtr and Adcyap1r1 in the Medial Amygdala**

A. Gene expression of arginine vasopressin (*Avp*) was higher in male high and low dose compared to oil controls in the medial amygdala but the differences were not significant. **B.** Gene expression of oxytocin receptor (*Oxtr*) was significantly decreased in the medial amygdala in high dose mice compared to low dose (p-value=0.00406) and oil controls (p-value=0.00565). **C.** Gene expression of pituitary adenylate cyclase-activating polypeptide type 1 receptor (*ADCYAP1r1*) was significantly decreased in male high dose compared to low dose (p-value=0.000228) and oil control (p-value=0.000866). **D.** Gene expression of pituitary adenylate cyclase-activating polypeptide (*ADCYAP1*) was significantly elevated in the male high dose compared to oil controls (p-value=0.00371). Data represents fold expression relative to β-actin. Grey= Oil Controls; Blue= Low Dose; Yellow= High Dose.

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