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Regulation of social, anxiety, and sickness behaviors by forebrain vasopressin cells in mice

Jack Whylings

REGULATION OF SOCIAL, ANXIETY, AND SICKNESS BEHAVIORS BY FOREBRAIN VASOPRESSIN CELLS IN MICE

by

JACK WHYLINGS

Under the Direction of Geert J de Vries, Ph.D.

ABSTRACT

Sickness behavior is highly conserved across many species; inflammation causes a variety of physiological and behavioral changes, including lethargy, decreased social behavior, increased anxiety, and anhedonia. Many of these behavioral changes are also present in disorders such as depression and chronic fatigue, which likely have a substantial inflammatory component. While much is known about the physiology of inflammation, further understanding of neural

mechanisms driving sickness behavior is still needed. One potential modulator of sickness behavior is arginine vasopressin (AVP), a neuropeptide well known for its contributions to social and anxiety-like behaviors. AVP is expressed in multiple nuclei that regulate behaviors and respond to immune activation. We specifically target AVP cells in three of these nuclei: the paraventricular nucleus of the hypothalamus (PVN), the bed nucleus of the stria terminalis (BNST), and the suprachiasmatic nucleus (SCN) and test their role in regulating sickness behaviors. Using genetically modified mice expressing Cre-recombinase in AVP cells, we selectively ablated AVP cells in these nuclei, followed by tests of lipopolysaccharide (LPS) induced sickness behavior. Our results indicate that PVN AVP cells regulate changes in motivation during sickness and contribute to typical anxiety-like behavior; BNST AVP cells regulate male social behaviors and hedonic behavior in both sexes; and SCN AVP cells regulate anxiety-like behavior and hedonic behavior in both sexes.

INDEX WORDS: Vasopressin, LPS, Sickness behavior, BNST, PVN, SCN

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DEDICATION

To my loving husband, Jay, who patiently supported me in every way possible.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AVP: Arginine vasopressin
BNST: Bed nucleus of the stria terminalis
CeA: Central amygdala
CRF: Corticotropin releasing factor
DMH: Dorsomedial hypothalamus
EPM: Elevated plus maze
EZM: Elevated zero maze
OFT: Open field test
FISH: Fluorescent *in situ* hybridization
IL-1β: Interleukin-1 beta
IL-6: Interleukin-6
LPS: Lipopolysaccharide
LS: Lateral septum
MeA: Medial amygdala
NAc: Nucleus accumbens
NTS: Nucleus of the solitary tract
OVLT: Vascular organ of the lamina terminalis
PB: Parabrachial nucleus
PCR: Polymerase chain reaction
POA: Preoptic nucleus of the hypothalamus
PVH: Paraventricular hypothalamus
PVN: Paraventricular nucleus of the hypothalamus
SCN: Suprachiasmatic nucleus
shRNA: short hairpin ribonucleic acid
SON: Supraoptic nucleus
TLR4: Toll-like receptor 4
TNFα: Tumor necrosis factor alpha
TST: Tail suspension test
USV: Ultrasonic vocalization
V1aR: Vasopressin receptor 1a
VLM: Ventrolateral medulla
ZT: *Zeitgeber* time

1 INTRODUCTION

1.1 Inflammation and Sickness Behavior

Sickness is a universal experience among animals. Pathogens infect the body, which must then mount a response in order to maintain an animal's health. The immune system is broadly responsible for this, causing a cascade of physiological and behavioral changes in response to infection. These behavioral changes during inflammation, known as sickness behavior, are conserved across species, and serve a function of promoting recovery from infection[1,2]. Sickness behaviors include social withdrawal, reduction in activity, anhedonia, and reduced food and water intake[1,3,4]. These behavioral changes are similar to those seen in depressive disorders, and prolonged inflammation and sickness may be one contributing factor and treatment target for human depression[5–7]. Further understanding of the neural basis of sickness behavior may provide more specific targets for treating mental illnesses caused through neuroinflammatory mechanisms.

One common tool to study sickness behavior is bacterial lipopolysaccharide (LPS), a coat protein derived from gram-positive bacteria. Injections of LPS simulate bacterial infection and activate the innate immune system, leading to increased inflammation and sickness behaviors such as anhedonia, lethargy, reduced social behavior, and increased anxiety-like behavior[4,8–10]. LPS is detected by Toll-like receptor 4 (TLR4), a pathogen sensor on innate immune cells, and triggers production and release of pro-inflammatory cytokines[11], the main humoral signaling molecules of the immune system. The primary pro-inflammatory cytokines released after LPS challenge are interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα), which are critical for sickness behavior. Administration of these cytokines are effective at causing sickness behavior[12–15], while blocking their receptors inhibits sickness behavior[16–21].

These cytokines are then sensed by the vagus nerve, other immune cells, and cerebral epithelial cells to drive neuroinflammatory signaling[22,23].

A critical source of inflammatory signaling into the brain, and the primary pathway for LPS induced sickness behavior, is the vagus nerve[22]. The vagus nerve receives sensory information from visceral organs and transmits that information through the spinal cord and into the brain. In many models of LPS-induced sickness behavior, blocking vagus signaling to the brain reduces or eliminates behavioral effects of LPS[24–29] The vagus nerve detects pro-inflammatory cytokines[30,31], and activates the nucleus of the solitary tract (NTS) and ventral lateral medulla (VLM) through its hindbrain innervation[32,33]. The NTS, in concert with the VLM and parabrachial nucleus (PB), directly activate downstream nuclei via three parallel signaling pathways: one activating the paraventricular nucleus of the hypothalamus (PVN) and preoptic nucleus of the hypothalamus (POA), one activating the dorsolateral bed nucleus of the stria terminalis (BNST) and central amygdala (CeA), and one activating midline thalamus and cortical structures[22,34,35]. These nuclei are involved in physiological responses to inflammation; for example, the BNST, amygdala, and POA regulate fever response[36–41] , and the PVN regulates glucocorticoid release[42]. While these nuclei are involved in behavioral regulation, much of the evidence suggesting a role in sickness behavior is circumstantial, such as c-fos activation after immune challenge[32]. These regions are likely involved in sickness behavior, but there has not been enough specific targeting of these nuclei in order to better understand their role in generating sickness behaviors.

However, the vagal pathway is not the exclusive immune brain interface[23]. Serum cytokines can activate receptors on cerebral endothelial cells, as well as bypass the blood brain barrier in the circumventricular organs[43], and then in turn activate microglia and cause cytokine

release in the brain[44]. Activated peripheral immune cells may also signal through cerebral endothelial cells and microglia in order to increase microglia activation and cytokine release[45]. In addition to pathogen-mediated inflammatory and sickness response, non-pathogenic and commissural bacterial, such as those comprising the gut microbiota, signal through these immune pathways and through production and release of metabolic factors[23]. All of these pathways may also induce neuronal changes and contribute to sickness behaviors beyond the nuclei implicated in vagal-immune signaling.

1.2 Vasopressin Neuroanatomy and Function

Arginine vasopressin (AVP)-expressing cells are a promising target for regulating behavioral sickness, due to both established function of AVP in regulating behavior and neuroanatomical position of AVPergic cells. AVP regulates social[46,47] and anxiety-like behaviors[48–50], both of which are altered during sickness; additionally, AVP contributes to physiological aspects of sickness, such as fever[38,51] and HPA axis function[52]. AVP cells in PVN, BNST, and SCN all have potential to regulate vagal immune pathways and are thus well-positioned to change behavior in response to inflammation. In this thesis, I test the overarching hypothesis that **AVP in these forebrain nuclei (PVN, BNST, SCN) regulates behavioral sickness expression in mice by altering anhedonia, anxiety-like behavior, and social behavior in response to inflammation**. This is specifically tested by selectively ablating AVP cells in each of these nuclei followed by tests of LPS-induced sickness behavior.

1.2.1 Paraventricular Nucleus of the Hypothalamus

PVN AVP is highly likely to regulate sickness behaviors, as these cells receive direct activation from the NTS after LPS challenge, and increase AVP gene expression and release during sickness[32,53–55]. AVP, in conjunction with corticotropin releasing factor (CRF), controls

the hypothalamic-pituitary-adrenal (HPA) axis, including causing glucocorticoid release[42], which regulates immune responding as an anti-inflammatory signal[56]. PVN AVP contributes to social and anxiety behaviors, through HPA axis regulation and projections to the lateral septum (LS), BNST, CeA, and surrounding hypothalamic nuclei[48,57–60]. PVN AVP may regulate anxiety-like and social behaviors via actions in these nuclei. PVN AVP cells are activated during sickness, and regulate typical behavioral functions; therefore, **Chapter 2** tests the hypothesis that **PVN AVP cells contribute to expression of sickness behavior**. Specifically, if PVN AVP is involved in balancing inflammatory processes by increasing glucocorticoid release, or altering activity via central projections, removal of these cells will enhance sickness behavior.

1.2.2 Bed Nucleus of the Stria Terminalis

While the BNST AVP cells are located in the posteromedial BNST[61], separate from the dorsolateral BNST that receives NTS and PB projections[22], BNST AVP has been shown to play a role in regulating inflammation. In male rats, sex hormone-dependent AVP, primarily from the BNST, acts in the septal nuclei to reduce fever[38,51]. Additionally, intracerebroventricular manipulations of AVP, targeting BNST outputs such as the septum, suggest testosterone-dependent AVP reduces sickness behaviors[62]. BNST AVP is best known for its control of male social behaviors, which are also regulated by BNST-septal projections. BNST AVP expression is sexually dimorphic[63], and manipulations of BNST AVP changes male social behaviors, with minimal effects on females[64–67]. Due to its important role in male social behavior and evidence that hormone-dependent AVP regulates fever, **Chapter 3** tests the hypothesis that **BNST AVP cells contribute to expression of male sickness behavior**. If BNST AVP exerts similar effects on sickness behavior as it does fever, then removal of AVP cells will exacerbate behavioral sickness in males, with minimal effects in females.

1.2.3 Suprachiasmatic Nucleus

SCN AVP cells may regulate sickness behaviors via afferents onto vagal-activated nuclei. SCN AVP projects to midline thalamus and hypothalamus regions, including the PVN[57], which receive NTS and VLM innervation[22]. The SCN has a reciprocal relationship with neuroimmune signals[68–71], in which the SCN regulates inflammatory signaling while cytokines alter the activity of the SCN. The SCN is the central clock of the brain, and rhythms in clock genes organize physiological and behavioral rhythms, including circadian patterns in the immune system[72,73]. Neuroimmune mechanisms that increase cytokine signaling in the brain, such as humoral and immune-cell mediated signaling[23] are the likely source of inflammatory signals to the SCN. Cytokines such as TNF-a disrupt circadian rhythms by inhibition of the clock gene *Per*[74], and LPS-induced immune response can cause phase shifts if administered during the dark phase[75]. Conversely, the SCN regulates and keeps immune responding in check; light disruption and SCN lesions cause increased and over responsive reactions to LPS[68,76]. SCN AVP has been studied mainly in its contributions to circadian rhythm maintenance. Local AVP release in the SCN coordinates cellular clocks, and contributes to adjusting to light cycle changes, activity rhythms, and daily food and water intake[77–81]. However, AVP in the SCN has not been specifically studied as a mediator of this clock-immune interface, and it is unknown how disruptions to SCN AVP affect sickness. LPS has been shown to increase AVP release in SCN cell cultures[82], but the role this may play in sickness regulation is unknown. Additionally, SCN AVP contributions to baseline social and anxiety-like behaviors have not been characterized, although SCN AVP projects to nuclei that regulate these behaviors. **Chapter 4** tests the hypothesis that **SCN AVP cells contribute to expression of social, anxiety, and sickness behaviors**; if SCN AVP release

is one mechanism by which the SCN regulates immune responding, then removal of these cells will lead to exacerbated sickness behaviors.

1.3 Hypothesis and Specific Aims

To test the hypothesis that **AVP cells regulate expression of sickness behavior**, we directly target AVP-expressing cells. Mutant mice that express Cre-recombinase driven by AVP promoter, which causes Cre-expression only in AVP cells, were injected in target nuclei with viral mediated, Cre-dependent caspase. This causes programmed cell death, and selective ablation of AVP expressing cells with minimal damage to surrounding cells. To test this hypothesis, AVP cells in these three regions of interest: the **PVN (Specific Aim 1)**, the **BNST (Specific Aim 2)**, and the **SCN (Specific Aim 3)**, were selectively ablated, followed by tests of LPS-induced sickness behaviors. Characterization of these behaviors, along with observation of normative behaviors during healthy conditions, provide further insight into a role for AVP cells in regulating male social behavior, anxiety behavior in both sexes, and hedonic motivation in both sexes.

2 REMOVAL OF VASOPRESSIN CELLS FROM THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS ENHANCES LPS-INDUCED SICKNESS BEHAVIOR IN MICE

Jack Whylings, Nicole Rigney, Geert J de Vries, Aras Petrulis

JW designed and performed experiments, analyzed data, and wrote manuscript, NR contributed to performing experiments and data analysis, GJdV and AP contributed to experimental design, advisement, writing, and provided resources.[83]

2.1 Abstract

Vasopressin (AVP) cells in the paraventricular nucleus of the hypothalamus (PVN) are activated during sickness and project to multiple nuclei responsible for anxiety, social, and motivated behaviors affected during sickness, suggesting that these cells may play a role in sickness behaviors, typically expressed as reduced mobility, increased anxiety, anhedonia, and social withdrawal. In this study, we selectively ablated AVP neurons in the PVN of male and female mice (Mus musculus) and induced sickness behavior via injection of bacterial lipopolysaccharide (LPS). We found that PVN AVP ablation increased the effects of LPS, specifically by further decreasing sucrose preference in males and females and decreasing the social preference of males, monitored within 24 hours of LPS injection. These results suggest that PVN AVP contributes to the change in motivated behaviors during sickness and may help promote recovery from infection.

2.2 Introduction

The immune system is one of the main internal defenses of animals against infection. When the immune system is activated, cytokines are released systemically, body temperature increases, immune cells migrate to sites of infection and injury, and mobility and social

interactions are reduced[5]. While these alterations promote recovery from infection[1], inappropriate or overactive immune responses may contribute to mental health conditions such as depression, anxiety, PTSD, and chronic fatigue[84–87]. Indeed, prominent behavioral changes in sickness involve anhedonia and withdrawal from social stimuli and non-social anxiogenic stimuli, changes similar to those seen in depression, suggesting that immune activation may play a role in depression[5,88,89]. In fact, antidepressants can reduce depression-like components of sickness behavior triggered by administration of bacterial lipopolysaccharide (LPS), which supports an inflammatory component of depression[90,91]. Understanding the brain regions and circuits that give rise to sickness behavior may give insight what roles these structures may play in mood disorders.

One structure that may be important in sickness behavior is the paraventricular nucleus of the hypothalamus (PVN), a critical stress-response region[92,93]. The PVN receives information on systemic inflammation most prominently via vagus nerve signaling pathways[22,26,28], and is activated during an immune response, evidenced by immediate early gene expression in the PVN after LPS administration[22,32,54,55]. This activation causes increased release of corticotropin releasing factor (CRF) and vasopressin (AVP)[94]. These neuropeptides play an important role in glucocorticoid response to infections, but can also become over-responsive, leading to mental health deficits and prolonged neuroinflammation[95–98].

While the majority of work studying the role of inflammation in depression focuses on glucocorticoids, AVP is also likely involved in sickness behavior. In rats, LPS activates the centrally projecting parvocellular AVP cells of the PVN[55]. Similarly, the actions of central AVP in the basal forebrain temper fever[38]. Although the origin of antipyretic AVP action is thought to be the bed nucleus of the stria terminalis (BNST), the PVN also innervates areas of

the basal forebrain implicated in fever[57,61] and may therefore contribute to the febrile response as well[99]. In addition, AVP from the PVN is thought to play a role in modulation of social and anxiety behaviors in stressful contexts[47,58,100,101]. For example, hypothalamic AVP expression strongly correlates with anxiety phenotypes in mice and rats[48,49,102], suggesting that AVP from the PVN may alter social and anxiety-like behaviors during sickness as well. To test the hypothesis that PVN AVP cells contribute to social and emotional behavioral changes during sickness, we selectively ablated PVN AVP cells in male and female mice (Mus musculus) and measured their anxiety-like behavior, anhedonia, social preference and motor activity before and after challenge with illness-inducing LPS. We found that removing these cells affected behavioral responses to sickness in both sexes, but more so in males than in females.

2.3 Methods

2.3.1 Animals

All mice were maintained at 22°C on a 12:12 reverse light cycle with food and water available ad libitum, housed in individually ventilated cages (Animal Care Systems), and provided with corncob bedding, a nestlet square, and a housing tube. All animal procedures were approved by the Georgia State University Institutional Animal Care and Use Committee (IACUC) and were in accordance with the regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

AVP-IRES2-Cre-D (AVP-Cre) mice were obtained from The Jackson Laboratory (Stock No: 023530). AVP-Cre knock-in mice have Cre recombinase expression directed to AVP-expressing cells that are restricted to populations within the hypothalamus. Subjects were derived by crossing heterozygous Cre+ mutants to wildtype C57BL/6J mice and genotyped (via ear punch) by polymerase chain reaction (PCR) at 21-24 days of age (Transnetyx). Both Cre+ and

Cre- littermates were used in behavioral experiments. A total of 40 experimental animals were tested: 9 Cre- males, 12 Cre- females, 10 Cre+ males, and 9 Cre+ females. All subjects were used in a prior study35, and all surgical procedures described below were conducted as part of that study. In the prior study, behavioral tests were conducted 3-6 weeks after ablation surgery; in the present study, behavioral tests were conducted 8-10 weeks after the initial ablation surgery.. Stimulus animals for the three-chamber tests were adult C57BL/6J mice of both sexes, group-housed in the same room conditions as the experimental animals.

2.3.2 Viral Vectors

AVP promoter-driven-, Cre-expressing PVN neurons were ablated using an adeno-associated virus (AAV serotype 2/1 (3x1012 IU/mL) AAV-flex-taCasp3-TEVp; UNC Vector Core) that encodes, in a Cre-dependent fashion, a mutated pro-caspase-3 and its activator (TEVp). This system activates an apoptotic signaling cascade, cleaving multiple structural and regulatory proteins critical for cell survival and maintenance36,37 and killing cells with far less collateral inflammation than other lesion approaches35,38.

2.3.3 Stereotaxic surgery

All surgeries were carried out using 1.5-3% isoflurane gas anesthesia in 100% oxygen; 3 mg/kg of carprofen was given before surgery to reduce pain. Mice were positioned in a stereotaxic frame (David Kopf Instruments) with ear and incisor bars holding bregma and lambda at level. After a midline scalp incision, a hand-operated drill was used to make holes in the skull, exposing the dura. For both Cre+ and Cre- subjects, 500 nl of AAV-flex-taCasp3-TEVp was delivered bilaterally to the PVN (coordinates: AP -0.42 mm; ML ± 0.35 mm; DV 5.2 mm)39 at a rate of 100 nl/min using a 5 µl Hamilton syringe with a 30-gauge beveled needle

mounted on a stereotaxic injector. Following virus delivery, the syringe was left in place for 15 min and slowly withdrawn from the brain35.

2.3.4 Experimental Procedure

At least one week after tests for social and communicative behavior described in35, subjects were weighed and injected intraperitoneally with either 0.5 mg/kg LPS (from e. coli 0111:B4, Millipore-Sigma) or sterile saline one hour before dark phase (ZT11). While the LPS doses used across studies is highly variable, inflammation and sickness behavior after LPS administration occurs from 2 hours to over 24 hours post-injection 1,40. Therefore, the open field test (OFT) was conducted three hours following LPS injections, and the elevated zero maze (EZM) test was conducted immediately following the OFT. Sucrose preference was then assessed in the home cage during the following 24-hour period (5 hours after LPS). The day after LPS or saline injections, the three-chamber social interaction test was conducted during the dark phase. Animals were tested in all measures twice, first within ~24 hours after LPS/Saline, then again one week later, receiving both treatments as indicated in Figure 1. In all cases, animals were acclimated to the behavior testing suite for at least one hour before testing, and behavior tests were done in the dark phase under red lighting. Manual scoring of behaviors in the EZM and three-chamber tests was conducted by an experimenter blind to the subjects' genotype (Noldus Observer).

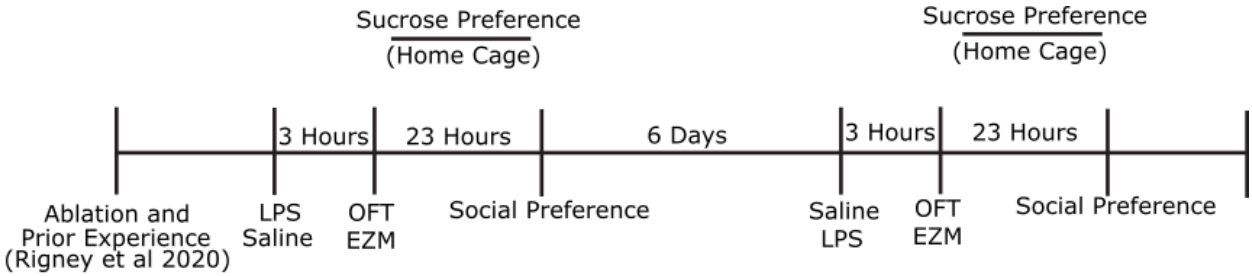

Figure 2-1
Timeline of behavioral experiments

2.3.5 Open-Field and Elevated Zero Maze

Three hours after LPS injections, animals were placed in a 43 cm x 43 cm x 30 cm open field chamber for 10 min and behavior was automatically tracked via infrared beam breaks (Med Associates). Distance traveled and time spent in the (anxiogenic) center area were analyzed as measures of locomotion and anxiety-like behavior, respectively.

Immediately after OFT, subjects were tested on an elevated zero maze (EZM). This apparatus consists of a 5.5 cm wide circular platform (internal diameter 35 cm) raised 50 cm off the ground, with two equally spaced enclosed compartments covering half of the platform. Video was manually scored for time spent in both open and closed arms as a measure of anxiety-like behavior, and for zone crosses (subject crossing from open to closed arm and vice versa) as a measure of activity.

2.3.6 Sucrose Preference

For at least 2 days before LPS/saline injections, subjects were acclimated to having two water bottles placed in their home cage. After OFT/EZM assessment, subjects were returned to their home cage, and bottles were replaced with pre-weighed bottles, one containing sucrose solution (2.5% in tap water) and the other tap water. Animals had access to both sucrose solution and water for the next 20 hours, until the start of the next day's testing. The bottles were then removed and weighed to measure consumption. A control water bottle in a nearby empty cage showed a <1 g loss over the same period and room conditions. Sucrose consumption, water consumption and preference, calculated as the percentage of sucrose consumed of total consumption (Sucrose / (Sucrose + Water) *100%), were analyzed.

2.3.7 Social Preference

To measure social preference and social novelty-preference, animals were tested in a large plexiglass chamber (20.3 x 42 x 22 cm) divided into three equal compartments with openings between sections, 26 hours after LPS injection. Subjects were placed in the apparatus for 5 min before testing in order to habituate the subjects to the environment and were then temporarily removed while stimulus animals/objects, contained within smaller cages (8 cm diameter, 18 cm height, 3-mm diameter bars, 7.4 mm spacing) were placed in the center of each of the two outer chambers. First, to test for social preference, a novel toy object (either a mouse, robot, or small car figurine) and a novel same-sex stimulus animal were placed in opposite cages. Subjects and stimulus animals had limited ability to directly contact each other; they were able to pass extremities (e.g. paws, tail) through the smaller cage bars during investigation. The subjects were then returned to the apparatus and allowed 10 min to explore the apparatus. At the end of this test, the subjects were removed again, and the toy object replaced with a novel stimulus animal (from a different cage from the first stimulus) to test for recognition of social novelty. The subject was then placed into the center chamber again and given 10 min to explore the apparatus. The position of object and original animal was counterbalanced across trials but did not change between social preference and social novelty preference tests. Videos were manually scored for outer chamber entries as a measure of activity, time spent in each chamber, and active investigation, defined by the subject's snout within 2 cm of the stimulus cage.

2.3.8 Histology

After completion of all behavioral testing, animals were sacrificed via CO2 asphyxiation. Brains were rapidly removed and flash-frozen in 2-methylbutane before storage at -80° C. Frozen tissue was cut coronally in 20 μm sections, and processed for AVP mRNA fluorescent in

situ hybridization, as described in detail in Rigney et al.35. Bilateral PVN images were taken across four sections covering the entire anterior to posterior extent of the PVN; averaged cell counts per hemisphere were used to estimate percentage of AVP cells ablated.

2.3.9 Data Analysis

Statistical analysis was conducted using SPSS (IBM). OFT, EZM, sucrose preference test, and social preference data were analyzed using a mixed-model, three-way ANOVA with treatment (LPS, Saline) as within-subjects variable, and sex (M, F) and genotype (Cre+, Cre-) as between-subject variables. Results with p-values equal to or less than 0.05 were considered significant, and results with p-values between 0.05 to 0.09 were considered trends. All data sets maintained sphericity as determined by Mauchly's test of sphericity, and thus degrees of freedom were not adjusted. Post-hoc t-tests were used to analyze genotype and sex differences following significant or trending ANOVA main effects and interactions. In addition, to analyze the relationship between PVN AVP cell loss and behavior, Pearson's correlation coefficients were calculated using the number of remaining PVN AVP cells/mm2 in Cre+ subjects.

2.4 Results

2.4.1 Removal of AVP Cells in PVN

As described in Rigney et al., Cre+ subjects were considered to have a successful ablation of PVN AVP cells when more than 60% of cells were removed. Of subjects that met this criteria, the average ablation percentages for male and female subjects were 73.14 ± 2.67% and 83.11 ± 3.19%, respectively. Figure 2 shows an example of AVP expression in a Cre- subject, which shows normal distribution of AVP cells, and in a Cre+ subject, which shows significant AVP cell loss. No correlation between extent of AVP cell ablation and behavior was observed, except where noted below.

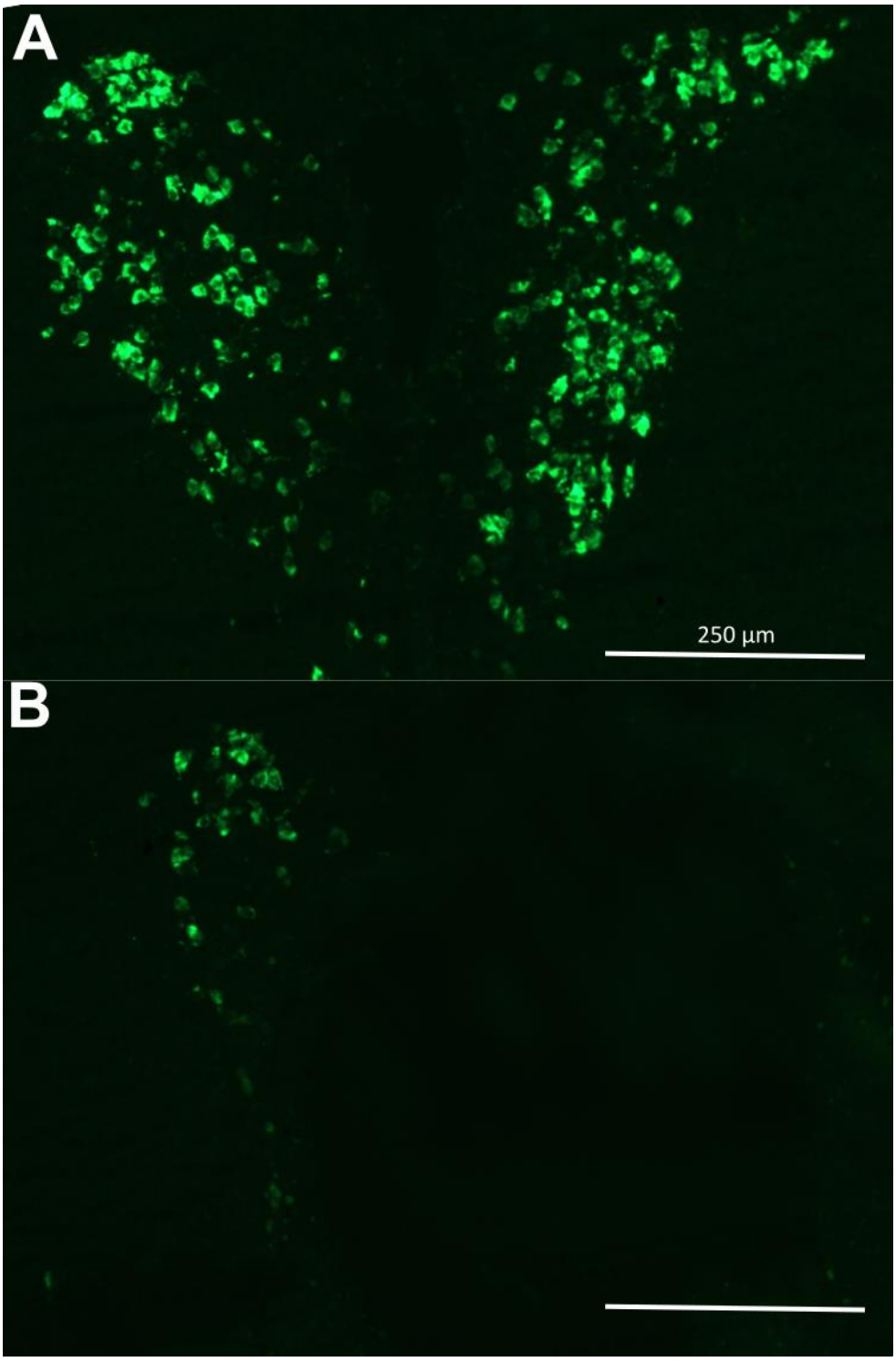

Figure 2-2

AVP in situ hybridization. Example photomicrographs of PVN AVP cells in (A) Cre- and (B) Cre+ animals.

2.4.2 Anxiety-Like Behaviors in Open Field and Elevated Zero Maze

In both the open field test (OFT) and elevated zero maze (EZM), there were strong effects of LPS, but no main effects or interactions of sex and genotype on either mobility or anxiety-like behaviors. In the open field test (OFT), LPS treatment significantly reduced total distance traveled ($F_{LPS} = 481.68$, $p < 0.01$, $\eta p2 = 0.94$), indicating reduced locomotion, with a significant interaction of treatment and sex ($F_{LPS \times Sex} = 4.39$, $p = 0.044$, $\eta p2 = 0.12$); there were no main effects or interactions of genotype. To explore the treatment by sex interaction effect, post-hoc t-tests revealed that males traveled significantly less after saline treatment than females ($t = -2.12$, $p = 0.042$), but the sexes did not differ in locomotion after LPS treatment ($t = 0.52$, $p = 0.61$). LPS also reduced time spent in the center area of the open field ($F_{LPS} = 115.51$, $p < 0.001$, $\eta p2 = 0.78$), indicating increased anxiety-like behavior, while no other main effects or interactions of sex and genotype were observed (Table 1). However, there was a positive correlation between the number of AVP cells remaining/mm2 in Cre+ males and female subjects and time spent in the center of the open field after saline treatment ($r = 0.55$, $p = 0.017$), but not after LPS ($r = 0.81$, $p = 0.749$), suggesting that increased PVN AVP cell deletion correlates with increased anxiety (Figure 3).

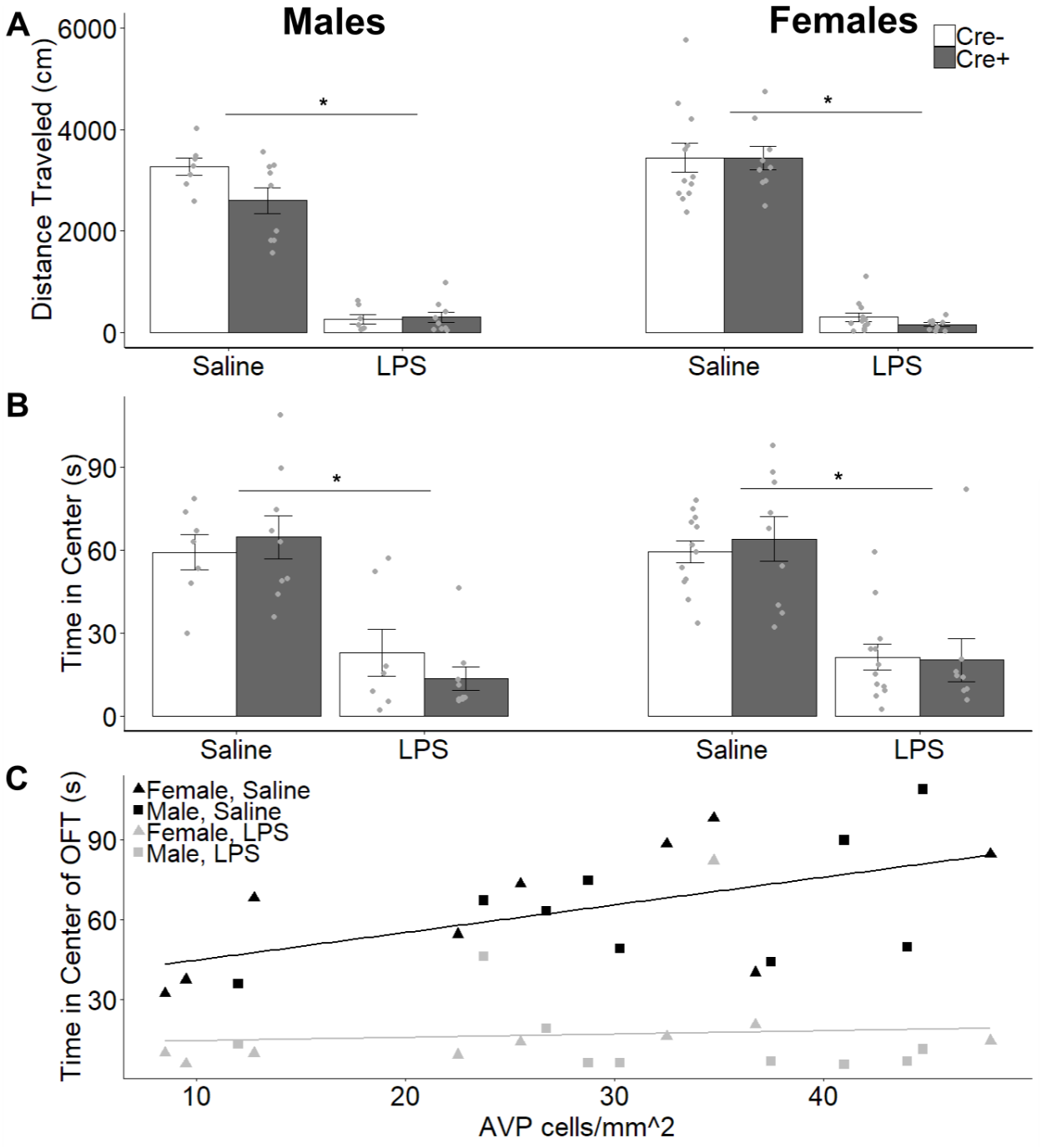

Figure 2-3

Open Field Test. Mean ± SEM of (a) the total distance traveled in the OFT, and (B) time spent in the center zone of the OFT for Cre- (white bars) and Cre+ (gray bars) male and female mice after LPS and saline treatments. All data were analyzed in a 3 way mixed-model ANOVA (Treatment x Genotype x Sex). Points indicate individual data; asterisks indicate significant effects of LPS treatment. (C) Scatterplot showing the relationship of time spent in the center of the OFT and AVP cells remaining/mm2 in Cre+ (ablated) animals after both saline (black points) and LPS (gray points) treatment in males (squares) and females (triangles).

In the elevated zero maze (EZM) test, LPS treatment significantly reduced both total zone crosses (F LPS = 54.71, $p < 0.001$, $\eta p2 = 0.6$), indicating reduced locomotion, with no main effect or interactions of sex and genotype. LPS also reduced time spent in the open arms of the EZM (F LPS 40.11, $p < 0.001$, $\eta p2 = 0.97$), indicating increased anxiety-like behavior, with no main effects or interactions of sex and genotype (Table 1; Figure 4).

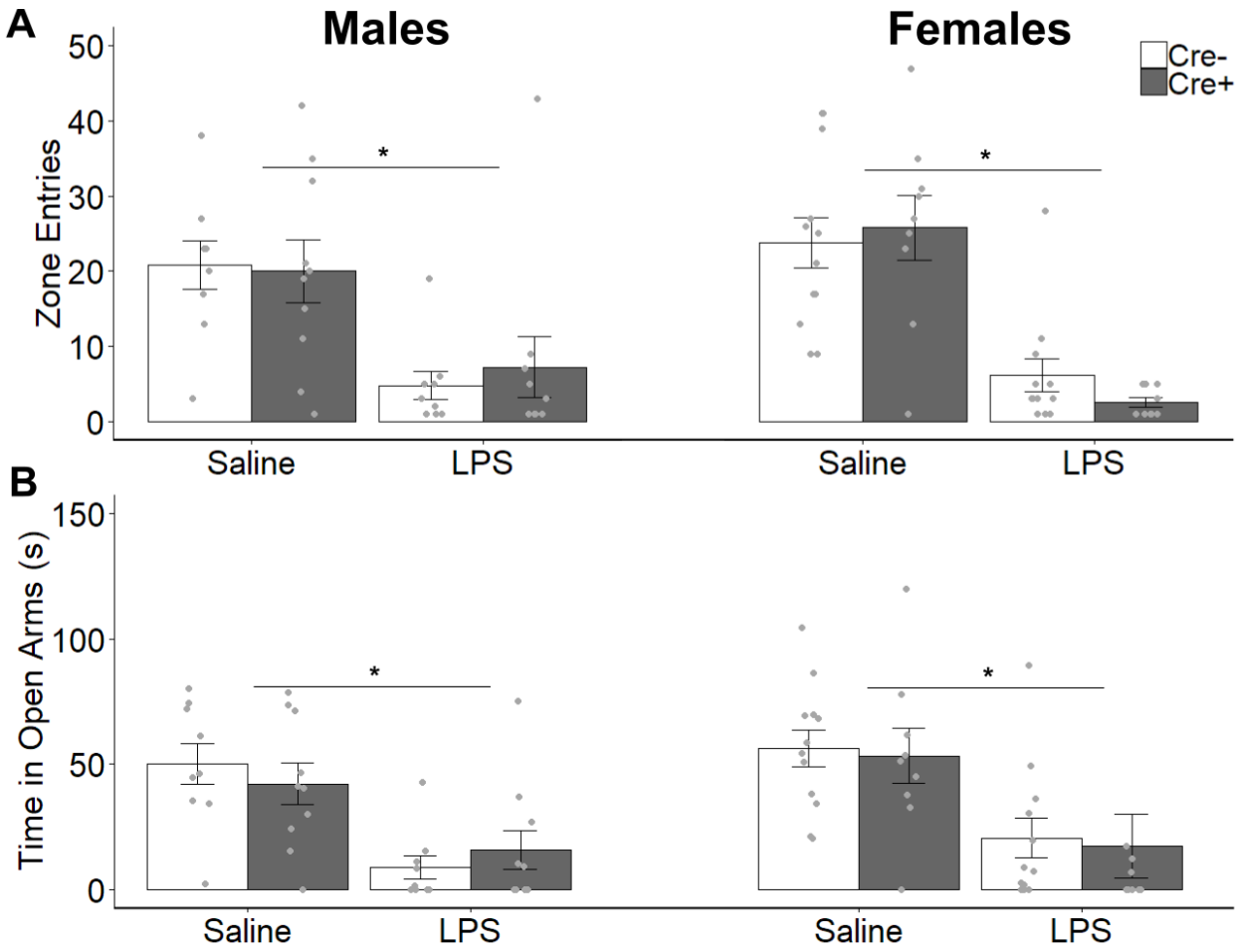

Figure 2-4

Elevated Zero Maze. Mean ± SEM of (a) the number of zone entries in the Elevated Zero Maze, and (B) time spent in the open arms of the Elevated Zero maze for Cre- (white bars) and Cre+ (gray bars) mice after LPS and saline treatments. All data were analyzed in a 3 way mixed-model ANOVA (Treatment x Genotype x Sex). Points indicate individual data; asterisks indicate significant effects of LPS treatment.

2.4.3 Sucrose Preference Test

Treatment with LPS as well as genotype altered sucrose preference (F LPS = 41.686, $p < 0.01$, $\eta p2 = 0.54$; F Genotype = 5.8, $p = 0.021$, $\eta p2 = 0.14$) with a trending interaction between treatment and sex (F Treatment x sex = 3.190, $p = 0.083$) and no other main effects or interactions (Table 1). A post-hoc t-test for LPS treatment confirmed that LPS reduced preference for sucrose ($t = -6.27$, $p < 0.001$). Post-hoc tests for genotype indicated no difference between genotypes when treated with saline but did show that LPS reduced preference for sucrose more in Cre+ than in Cre- animals ($t = 2.082$, $p = 0.044$). Post-hoc t-tests for sex indicated no sex differences in sucrose preference after either saline ($t = -1.40$, $p = 0.17$) or LPS ($t = 1.11$, $p = 0.27$) treatment (Figure 5).

Changes in sucrose preference was not due to changes in water consumption, as there were no differences between treatment conditions or genotype in amount of water consumed (Table 1), although there was a trending interaction of LPS treatment and sex (F Treatment x Sex = 3.49, $p = 0.07$). Specifically, while there was no sex difference in water consumption after saline treatment ($t = 0.41$, $p = 0.69$), males consumed less water than females following LPS treatment ($t = -2.15$, $p = 0.038$).

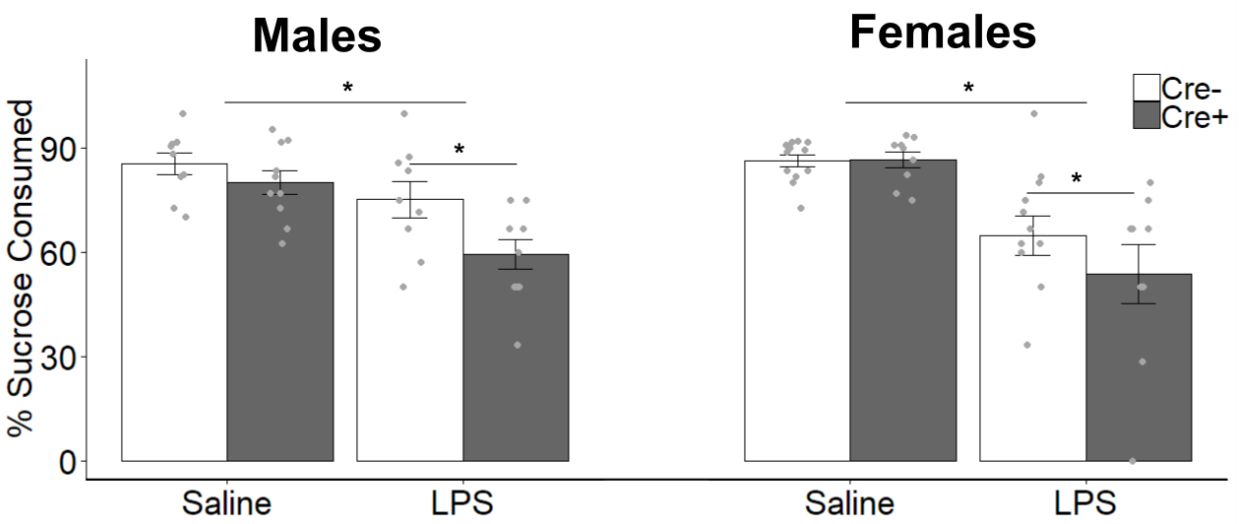

Figure 2-5

Sucrose preference. Mean ± SEM of the percentage of sucrose solution consumed out of total fluid consumption (sucrose + water) for Cre- (white bars) and Cre+ (gray bars) mice after LPS and saline treatments. All data were analyzed in a 3 way mixed-model ANOVA (Treatment x Genotype x Sex). Points indicate individual data. Asterisks represent significant effects of treatment and genotype.

2.4.4 Social preference test

In the social preference test, when animals were given a choice between a conspecific and an object, there were no main effects of treatment, genotype, or sex on preference for the animal stimulus (Table 1), but there was a significant treatment by genotype interaction in the percentage of time investigating the stimulus animal compared to the object (F LPS x Genotype = 6.8, $p = .013$, $\eta p2 = 0.16$). Post-hoc analysis for genotype revealed that Cre+ animals have less preference for an animal after LPS treatment ($t = 2.07$, $p = 0.047$), but not after saline treatment ($t = -0.61$, $p = 0.54$). To further examine potential influence of sex, exploratory post-hoc t-tests were performed, which showed a trend of Cre+ males reducing preference for an animal over an object after LPS treatment ($t = 2.12$, $p = 0.058$), whereas females maintained their preference ($t = 0.72$, $p = 0.48$; Figure 6). This suggests that the effect of genotype is primarily driven by males, but not strongly enough to generate a significant interaction of sex by genotype.

In the social novelty preference test, in which the object was replaced with a novel conspecific, there were no main effects of treatment, genotype, or sex on preference for the novel animal (Table 1; Figure 6). Using zone entries as an indicator of activity, LPS reduced activity in both social preference and social novelty preference tests ($F = 124.27$, $p < 0.001$, $\eta p2 = 0.78$), with a trend towards a treatment x sex interaction ($F = 3.314$, $p = 0.077$). Post-hoc t-tests did not reveal a sex difference in zone crosses in either LPS-treated ($t = 1.52$, $p = 0.14$) or saline-treated ($t = -0.45$, $p = 0.65$) animals (Figure 7).

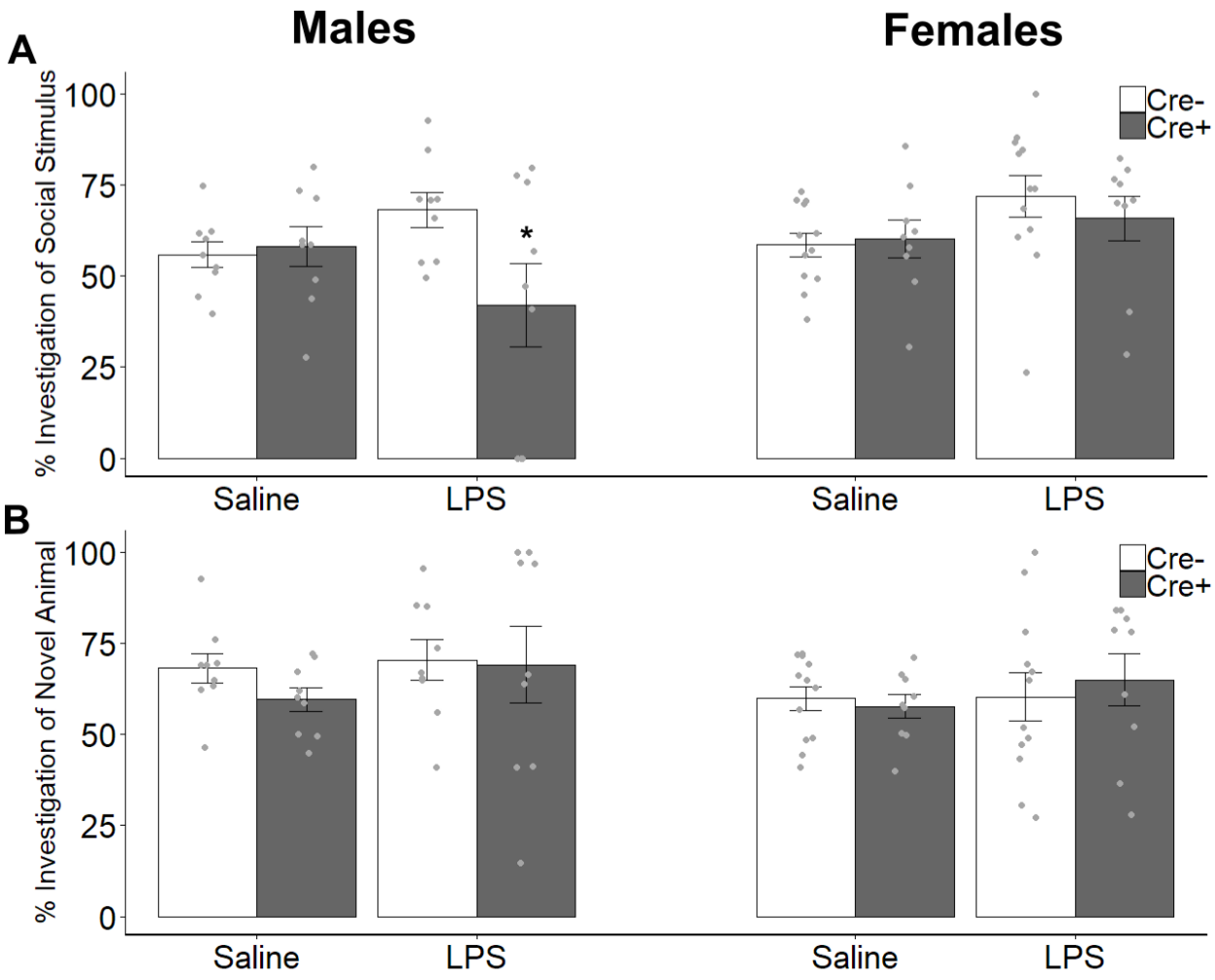

Figure 2-6

Social preference. (A) Mean ± SEM of the percentage of time spent investigating an animal to total time investigating animal and object stimuli in the three-chamber apparatus of Cre- (white bars) and Cre+ (gray bars) male and female mice after LPS and saline treatments.

(B) Mean ± SEM of the percentage of time spent investigating a novel animal to total time investigating the novel and original animal of Cre- (white bars) and Cre+ (gray bars) mice after LPS and saline treatments. All data were analyzed in a 3 way mixed-model ANOVA (Treatment x Genotype x Sex). Points indicate individual data. Asterisk represents a significant effect of treatment and genotype.

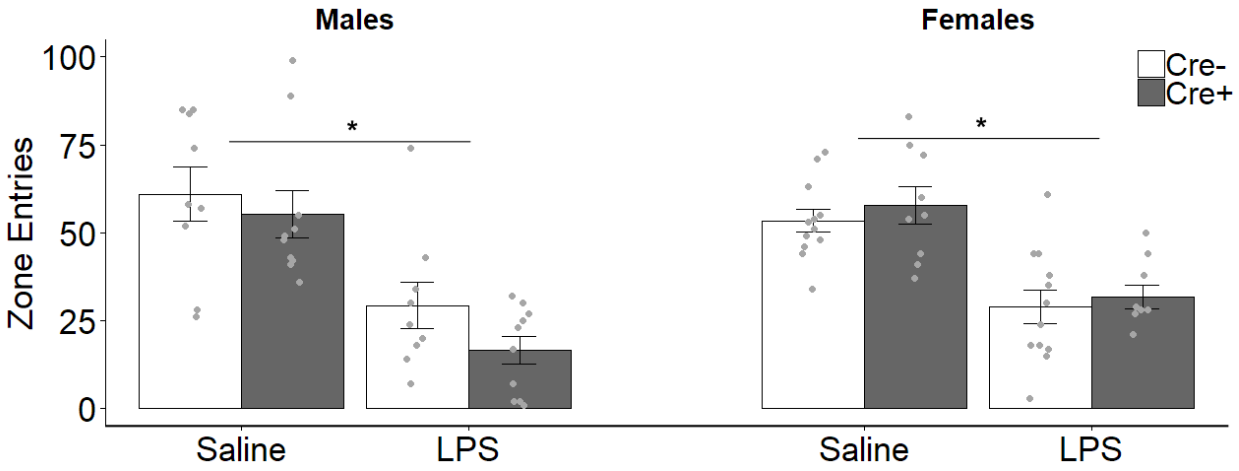

Figure 2-7

Zone crosses in 3 chamber preference test. Mean ± SEM of zone crosses in both the social preference and social novelty preference phases of the three-chamber test combined of Cre- (white bars) and Cre+ (gray bars) mice after LPS and saline treatments. All data were analyzed in a 3 way mixed-model ANOVA (Treatment x Genotype x Sex). Points indicate individual data. Asterisks represent significant effects of LPS treatment.

Table 2-1

OFT			
	Distance Traveled		
		LPS	F (1,33) = 481.68	p < 0.001
		Genotype	F (1,33) = 1.84	p = 0.185
		Sex	F (1,33) = 2.46	p = 0.13
		LPS x Genotype	F (1,33) = 1.12	p = 0.3
		LPS x Sex	F (1,33) = 4.39	p = 0.044
		Genotype x Sex	F (1,33) = 0.684	p = 0.41
		LPS x Genotype x Sex	F (1,33) = 2.48	p = 0.125
	Time in Center		
		LPS	F (1,33) = 115.51	p < 0.001
		Genotype	F (1,33) = 0	p = 0.99
		Sex	F (1,33) = 0.054	p = 0.82

		LPS x Genotype	F (1,33) = 1.71	p = 0.2
		LPS x Sex	F (1,33) = 0.13	p = 0.72
		Genotype x Sex	F (1,33) = 0.13	p = 0.72
		LPS x Genotype x Sex	F (1,33) = 0.33	p = 0.57
EZM				
	Zone Crosses			
		LPS	F (1, 36) = 54.71	p < 0.001
		Genotype	F (1, 36) = 0	p = 0.99
		Sex	F (1, 36) = 0.355	p = 0.55
		LPS x Genotype	F (1, 36) = 0.63	p = 0.8
		LPS x Sex	F (1, 36) = 1.65	p = 0.21
		Genotype x Sex	F (1, 36) = 0.12	p = 0.73
		LPS x Genotype x Sex	F (1, 36) = 0.86	p = 0.36
	Time in Open Arms			
		LPS	F (1, 36) = 40.11	p < 0.001
		Genotype	F (1, 36) = 0.071	p = 0.79
		Sex	F (1, 36) = 1.27	p = 0.27
		LPS x Genotype	F (1, 36) = 0.46	p = 0.5
		LPS x Sex	F (1, 36) = 0.38	p = 0.85
		Genotype x Sex	F (1, 36) = 0.4	p = 0.84
		LPS x Genotype x Sex	F (1, 36) = 0.48	p = 0.49
Sucrose Preference				
	Preference (%)			
		LPS	F (1, 36) = 41.686	p < 0.001
		Genotype	F (1, 36) = 5.8	p = 0.021
		Sex	F (1, 36) = 0.42	p = 0.52
		LPS x Genotype	F (1, 36) = 2.7	p = 0.11
		LPS x Sex	F (1, 36) = 3.19	p = 0.083
		Genotype x Sex	F (1, 36) = 0.63	p = 0.43
		LPS x Genotype x Sex	F (1, 36) = 0.004	p = 0.95
	Water Consumed			
		LPS	F (1, 36) = 2.51	p = 0.12
		Genotype	F (1, 36) = 0.98	p = 0.76
		Sex	F (1, 36) = 1.57	p = 0.22
		LPS x Genotype	F (1, 36) = 0.029	p = 0.87
		LPS x Sex	F (1, 36) = 3.49	p = 0.07
		Genotype x Sex	F (1, 36) = 0.221	p = 0.641
		LPS x Genotype x Sex	F (1, 36) = 0.21	p = 0.64
3 chamber test				

Social Preference			
	LPS	F (1, 36) = 1.24	p = 0.27
	Genotype	F (1, 36) = 2.06	p = 0.16
	Sex	F (1, 36) = 2.65	p = 0.11
	LPS x Genotype	F (1, 36) = 6.8	p = 0.013
	LPS x Sex	F (1, 36) = 2.67	p = 0.11
	Genotype x Sex	F (1, 36) = 0.97	p = 0.33
	LPS x Genotype x Sex	F (1, 36) = 2.25	p = 0.14
Social Novelty Preference			
	LPS	F (1, 36) = 1.62	p = 0.21
	Genotype	F (1, 36) = 0.17	p = 0.68
	Sex	F (1, 36) = 1.79	p = 0.19
	LPS x Genotype	F (1, 36) = 0.85	p = 0.36
	LPS x Sex	F (1, 36) = 0.071	p = 0.79
	Genotype x Sex	F (1, 36) = 0.46	p = 0.5
	LPS x Genotype x Sex	F (1, 36) = 0	p = 0.99
Zone Crosses			
	LPS	F (1, 36) = 124.27	p < 0.001
	Genotype	F (1, 36) = 0.37	p = 0.55
	Sex	F (1, 36) = 0.29	p = 0.6
	LPS x Genotype	F (1, 36) = 0.64	p = 0.43
	LPS x Sex	F (1, 36) = 3.314	p = 0.077
	Genotype x Sex	F (1, 36) = 2	p = 0.17
	LPS x Genotype x Sex	F (1, 36) = 0.25	p = 0.62

2.5 Discussion

In this study, we tested the role of PVN AVP cells in modulating sickness behaviors and found that removal of these cells exacerbated LPS effects on anhedonia in both sexes, as measured by reduced sucrose consumption, and on social preference, which was eliminated in males but not in females. Removal of PVN AVP cells also modestly increased anxiety-related behavior in the open field test (OFT), in line with an increase of anxiety previously demonstrated in these animals[103]. However, we did not detect a role of PVN AVP cells in modulating anxiety-

like behaviors in other tests of anxiety either at baseline or during sickness. These data suggest that PVN AVP cells may normally reduce sickness effects on positively valenced behaviors (sucrose consumption, social interaction), but have less impact on negatively valenced behavior (anxiety-like behaviors).

We cannot conclude that the effects of PVN AVP cell ablation on behavior are solely due to the elimination of AVP transmission from those cells. This study eliminated entire cells, which do contain and release more than AVP. For example, these cells may also release glutamate, CRF, galanin, and other neurotransmitters[58,104–108]. CRF, which is increased after LPS administration[109–111], contributes to a rise in glucocorticoid release in sickness along with AVP. However, these peptides are produced in mostly separate cell populations in unstressed mice[112]. Moreover, PVN CRF cell deletion reduces anxiety behaviors[113], whereas our male subjects showed increased anxiety in the current study and in previous tests in our laboratory[103], suggesting that our results cannot be explained by a loss of CRF cells. Nevertheless, the loss of other non-AVP signals from PVN could contribute to our results; this could be tested in future experiments with more specific targeting of AVP production.

Additionally, PVN AVP populations are heterogenous and consist of median eminence and pituitary-projecting cells as well as centrally projecting cells[63,112] As cell ablation did not discriminate for any subtype of PVN AVP cells, we cannot limit interpretation of the results to ablation of one set of these cells. Remaining PVN AVP cells or other populations may also compensate for the loss of these cell types and reduce potential effects of ablation. For example, given our results that show no effect of PVN AVP cell ablation on water intake under baseline or LPS conditions, and previous data in these animals that showed no difference in urine output[103], we do not believe that cell ablation noticeably affected overall fluid balance, potentially because

other sources of peripheral AVP such as the supraoptic nucleus and, possibly, remaining PVN AVP cells may have compensated for the loss of neurosecretory AVP cells in the PVN. We cannot exclude that ablation of PVN AVP cells did not affect physiological indicators of sickness ,as measuring body temperature or collecting blood would have introduced additional stressors, which would confound behavioral analysis.

We did not observe a strong effect of PVN AVP cell ablation on anxiety-like behavior in saline-treated mice, even though we did see an increase in anxiety-like behavior in an elevated plus maze test in males in an earlier study with these mice[103]. This discrepancy may be explained by differences in anxiogenesis of different tests[114] and/or the timing of testing. In our previous study, we tested anxiety-like behaviors prior to all other behavioral tests[103] in order to minimize effects of repeated handling on anxiety measures[115,116]. Consequently, the testing and handling before LPS injections in the current study may have reduced the anxiogenicity of the OFT and elevated zero maze (EZM) in later tests, which may explain why we observed less strong effects of ablation on anxiety. Nevertheless, we did observe a positive correlation between the degree of PVN AVP cell ablation and avoidance of the anxiogenic center of the OFT, which supports a role for these cells in anxiety-like behavior.

Even though we did not find that deletion of PVN AVP cells significantly enhanced the effects of LPS treatment on anxiety-like behaviors in the OFT or EZM, we cannot exclude a role for these cells in reducing anxiety-like behavior during sickness. If, in this study, LPS-induced increases in anxiety-like behavior reached a ceiling level in our subjects, ablation may not have been able to further enhance the increase in anxiety caused by LPS. A milder immune stimulus might reveal effects of AVP cell ablation on these tasks. Alternatively, PVN AVP cells may have a time-dependent effect on sickness expression. In the EZM and OFT, which were performed 3

hours after LPS injection, only the effects of LPS and not cell ablation were observed. In contrast, cell ablation enhanced LPS effects during the overnight sucrose preference test and the social behavior tests performed 24 hours after LPS administration. It is, therefore, possible that sickness behavior may have been less severe during these later measures, allowing for detection of sickness behavior-enhancing ablation effects. Conversely, PVN AVP cells may act later in sickness to promote recovery. Future experiments would be needed to explore PVN AVP effects on sickness progression and recovery.

The effects of PVN AVP cell deletion were most pronounced on positively valenced behaviors: the non-social motivation to consume sucrose and the social motivation to investigate a conspecific. These cells are well-positioned to effect reward circuitry and social behavior, leading to behavioral changes during sickness. PVN AVP cells project to reward and salience-sensitive regions such as the nucleus accumbens (NAc)[57,117], in which AVP increases during drug taking and cocaine-place preference[118–120]. The PVN also interacts with the social behavior neural network[121–123], and is one source of AVP afferents to the lateral septum[57] and therefore may contribute to septal AVP effects on social interaction and aggression[65,100,124,125] as well as fever regulation in males[126–128]. However, behavioral effects of cell ablation in this study were seen only after LPS injection, not under control saline conditions, suggesting that PVN AVP input may not significantly modulate positively valenced behavior under normal circumstances, but is instead involved in adjusting these behaviors in contexts such as sickness. As the expression of sickness behavior can change depending on social context[129] and motivational states[130], PVN AVP and its outputs may integrate social and motivational context to adjust sickness behaviors.

Removal of PVN AVP cells lowered social motivation after LPS, but only in males. Given that AVP exerts sex-dependent effects on social behavior, typically stronger in males than in females[50], our results are consistent with reported sex differences in AVP function. AVP effects on fever are also male-biased: AVP, presumed to be derived from the male-biased AVP cell populations in the BNST and MeA, acts in the septal area to reduce fever in males only[51,62]. BNST AVP cells contribute to baseline sex differences in social behavior, but do not affect LPS-induced changes[131,132]. Conversely, we found no baseline changes in social preference after PVN AVP cell ablation, but did find that these ablations changed social preference, but only in LPS-treated male mice. Even though PVN AVP expression as a whole is neither strongly sexually dimorphic[103] nor the main source of AVP to areas like the septal region involved in social behavior[57], our results confirm sexually different impacts of PVN AVP cells on behavior. For example, PVN AVP cell ablations only enhanced male, but not female, anxiety-like behavior[103]. The results of the current study suggest that PVN AVP projections may interact with AVP projections from other sources, such as the BNST, to contribute to the sex difference in social behavior we observed.

Sickness enhances anxiety-related behaviors as well as reduces positively motivated behaviors. Our results identify PVN AVP cells as modulators of appetitive behaviors during sickness, such as the anhedonic reduction in sucrose intake[133,134]. In rodents, hypothalamic AVP expression increases in response to inflammation[22,32], and our results indicate that a loss of PVN AVP enhances anhedonia. This suggests a modulatory role for PVN AVP during sickness in opposing the loss of positive motivation, similarly to how AVP reduces febrile response during sickness[38,126,135]. Anhedonia during sickness resembles anhedonia observed in melancholic depression in humans, which can have an inflammatory component[136–138]. An increase of PVN

AVP during both sickness and depression may counter a loss of motivated behaviors during illness, and suggest a potential role of PVN AVP cells in modulating anhedonia in models of depression.

3 SEXUALLY DIMPORPHIC ROLE OF BNST VASOPESSIN CELLS IN SICKNESS AND SOCIAL BEHAVIOR IN MALE AND FEMALE MICE

Jack Whylings, Nicole Rigney, Nicole V Peters, Geert J de Vries, Aras Petrulis

JW designed and performed experiments, analyzed data and wrote manuscript, NR and NVP contributed to performing experiments, NR contributed to data analysis, GJdV and AP contributed to experimental design, advisement, writing, and provided resources.[132]

3.1 Abstract

Circumstantial evidence supports the hypothesis that the sexually dimorphic vasopressin (AVP) innervation of the brain tempers sickness behavior in males. Here we test this hypothesis directly, by comparing sickness behavior in animals with or without ablations of BNST AVP cells, a major source of sexually dimorphic AVP in the brain. We treated male and female AVP-iCre+ and AVP-iCre- mice that had been injected with viral Cre-dependent caspase-3 executioner construct into the BNST with lipopolysaccharide (LPS) or sterile saline, followed by behavioral analysis. In all groups, LPS treatment reliably reduced motor behavior, increased anxiety-related behavior, and reduced sucrose preference and consumption. Male mice, whose BNST AVP cells had been ablated (AVP-iCre+), displayed only minor reductions in LPS-induced sickness behavior, whereas their female counterparts displayed, if anything, an increase in sickness behaviors. All saline-treated mice with BNST AVP cell ablations consumed more sucrose than did control mice, and males, but not females, with BNST AVP cell ablations showed reduced preference for novel conspecifics compared to control mice. These data confirm that BNST AVP cells control social behavior in a sexually dimorphic way, but do not play a critical role in altering sickness behavior.

3.2 Introduction

Most animals experience pathogen-induced sickness during their lifetime. While each pathogen brings its own set of inflammatory responses and other symptoms, sickness often causes general behavioral changes such as lethargy, reduced ingestive behavior, and social withdrawal [3]. These behavioral changes are generally thought to complement physiological responses, such as fever, in speeding up recovery [1]. While such behavioral changes may be beneficial for survival, long-term or inappropriate inflammation may contribute to mental health conditions such as depression [139]. Consequently, understanding the ways in which inflammation alters behavior may help treat such conditions.

The physiological basis of sickness behavior involves multiple pathways that relay information about peripheral inflammation, such as vagus nerve activity and humoral immune signaling [23]. Ultimately, peripheral inflammation activates brain regions such as the paraventricular hypothalamus (PVH), medial amygdala (MeA), bed nucleus of the stria terminalis (BNST), and preoptic area [22,32], all of which have been associated with behavioral profiles altered during sickness [8,54,140].

Some of these areas, such as the posterior BNST and MeA, contain arginine vasopressin (AVP) cells that have been indirectly implicated in regulating fever and sickness behavior [10,38,51]. For example, in rats, fever increases BNST neuronal activity [141], and electrical stimulation of the BNST reduces fever [142]. This may be due to effects of AVP in the septum, a target of BNST/MeA AVP projections, as septal AVP administration also reduces fever. This effect is testosterone-dependent, and is found in males but not in females [51,143], mirroring the sex differences in BNST AVP expression, which is more pronounced in males than in females [63], which suggest that BNST AVP cells modulate the fever response.

The same cells may also regulate sickness behavior. For example, in male rats intracerebroventricular injections of AVP reduce sickness behavior, whereas AVP antagonism exacerbates sickness behavior [62]. These effects are also testosterone-dependent; effects of inflammation and AVP administration are more pronounced in castrated animals, which cease to produce AVP in the BNST [62,63]. However, whether BNST AVP cells modulate sickness behavior has not been directly tested. We do so here by selectively ablating AVP cells in the BNST of male and female AVP-iCre mice via injections of viral vector containing a Cre-dependent cell death construct (caspase-3/Tev) and testing effects of ablation on sickness behavior. We induced sickness behavior via intraperitoneal injections of lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, commonly used as a proxy for bacterial infections [144], followed by tests for behaviors altered in sickness. We predicted that BNST AVP cell ablation would intensify sickness behavior, more so in males than in females.

3.3 Methods

3.3.1 Animals

All mice were maintained at 22°C on a 12:12 reverse light cycle with food and water available *ad libitum*, housed in individually ventilated cages (Animal Care Systems), and provided with corncob bedding, a nestlet square, and a housing tube. All animal procedures were approved by the Georgia State University Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Founding AVP-iCre mice were obtained from Dr. Michihiro Mieda (Kanazawa University, Japan). These mice were generated using a bacterial artificial chromosome that expressed codon-improved Cre recombinase [145] under the transcriptional control of the AVP

promoter (AVP-iCre mice). In these animals, iCre expression is found in the bed nucleus of the stria terminalis and the medial amygdala, as well as in hypothalamic areas [77]. Subjects were derived by crossing heterozygous iCre+ mutants to wildtype C57Bl/6J mice and genotyped (ear punch) by polymerase chain reaction (PCR) at 21-24 days of age (Transnetyx). Both iCre+ and iCre- littermates were used in behavioral experiments. All subjects were used in a prior experiment [131] and all surgical procedures described below were conducted as part of that study. Stimulus animals for the three-chamber test were adult C57B6/J mice of both sexes, group housed in the same room conditions as the experimental animals.

In total, 45 animals with confirmed BNST AVP cell ablation [131] were used for the behavioral testing described below: 11 Cre- males, 13 Cre – females, 13 Cre+ males, and 8 Cre+ females. Two female subjects (1 Cre+ and 1 Cre-) did not recover from initial LPS treatment and were euthanized and removed from all analyses. The remaining 43 animals were tested on all behavioral measures described below. Video recording error forced removal of 1 Cre+ female from the tail suspension test, and removal of 1 Cre+ and 1 Cre- female from the three-chamber social test analyses. Bottle failure and fluid leakage forced the removal of 6 females (5 Cre-, 1 Cre+) from the sucrose preference analysis.

3.3.2 Viral Vectors

BNST neurons with AVP promoter-driven Cre-expression were ablated using an adeno-associated virus (AAV-flex-taCasp3-TEVp) (University of North Carolina at Chapel Hill Vector Core) that encodes Cre-dependent pro-caspase-3. This enzyme activates an apoptotic signaling cascade, cleaving multiple structural and regulatory proteins critical for cell survival and maintenance [146,147] and thereby inducing far less inflammation than other lesion approaches [148].

High titer AAV of serotype 2/1 (3×10^{12} IU/mL) was purchased from the University of North Carolina at Chapel Hill Vector Core [131].

3.3.3 Stereotaxic surgery

All surgeries were carried out using 1.5-3% isoflurane gas anesthesia in 100% oxygen; 3 mg/kg of carprofen was given before surgery to reduce pain. Mice were positioned in a stereotaxic frame (David Kopf Instruments) with ear and incisor bars holding bregma and lambda level. After a midline scalp incision, a hand operated drill was used to make holes in the skull, exposing the dura. For all subjects, 500 nl of AAV-flex-taCasp3-TEVp was delivered bilaterally to the BNST (coordinates: AP -0.01 mm; ML ±0.75 mm; DV 4.8 mm [149] at a rate of 100 nl/min using a 5 μl Hamilton syringe with a 30-gauge beveled needle mounted on a stereotaxic injector. Following virus delivery, the syringe was left in place for 15 minutes and slowly withdrawn from the brain [131].

3.3.4 Experimental Procedure

All behavior tests were done in the dark phase under red lighting, and animals were acclimated to the behavior testing suite for at least one hour before testing. At least one week after tests for social and communicative behavior described in [131] and seven weeks after viral ablation, subjects were weighed and injected intraperitoneally with either 1 mg/kg LPS (from *e. coli* 0111:B4, Millipore-Sigma) or sterile saline one hour before dark phase (ZT11). While the LPS dose used is highly variable across previous studies, inflammation and sickness behavior after LPS administration have generally been reported to occur from 2 hours to over 24 hours post-injection [5,9], and the dose of LPS used in this study reliably induces sickness behavior [8]. Therefore, the open field test (OFT) was conducted three hours following LPS or saline injections, and the elevated zero maze (EZM) test was conducted immediately following the

OFT. Sucrose preference was then assessed in the home cage over a 20-hour period, starting 5 hours after LPS or saline injections). Immediately following this (25 hours post-injection), animals were tested in the three-chamber social interaction test and tail suspension test (TST). This entire sequence was repeated one week later, with animals that first received LPS now receiving saline and vice versa as indicated in Fig. 1. An interval of one week between treatments was chosen, because LPS causes sickness for only up to four days post-injection [150]. To make sure there were no residual effects of the initial LPS treatment results were compared across treatment order.

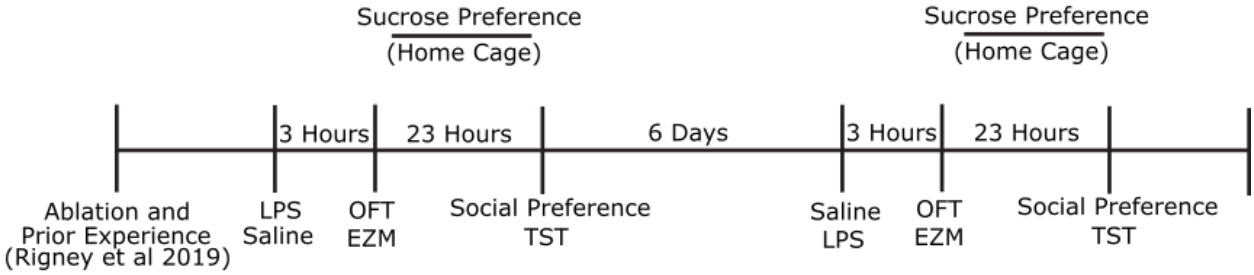

Figure 3-1
Experimental Timeline

3.3.5 Open-Field and Elevated Zero Maze

Three hours after injections, animals were placed in an 43cm x 43cm x 30cm open field chamber for 10 min and behavior was automatically tracked (Med Associates). Data was analyzed in two 5-min blocks, to separate behavior during the initial exploration period (first 5-min block, early exploration phase) from behavior after habituation to the chamber (second 5-min block, late exploration phase) [151,152]. Distance traveled and time spent in the center area were analyzed as measures of locomotion and anxiety-like behavior, respectively.

Immediately after OFT, subjects were tested on an elevated zero maze (EZM). This apparatus consists of a 5.5 cm wide circular platform of internal diameter 35 cm raised 50 cm off

the ground, with two equally spaced enclosed compartments covering half of the platform. Subject activity was tracked using automated software (AnyMaze) with the time and speed in each zone (closed, open), total distance traveled, and immobility time recorded.

3.3.6 Sucrose Preference

For at least 2 days before LPS/saline injections, subjects were acclimated to having two water bottles placed in their home cage. After OFT/EZM assessment, approximately 5 hours after LPS injections, subjects were returned to their home cage, and bottles were replaced with pre-weighed bottles, one containing sucrose solution (2.5% in tap water) and the other tap water. Animals had access to both sucrose solution and water for the next 20 hours, until the start of the next day's testing. The bottles were then removed and reduction in liquid was measured. A control water bottle in a nearby empty cage showed a <1 mL loss over the same period and room conditions. Preference was calculated as the percentage of sucrose consumed compared to total consumption (Sucrose / (Sucrose + Water) *100%).

3.3.7 Social Preference

To measure social preference and social novelty-preference, animals were tested in a large plexiglass chamber (20.3 x 42 x 22 cm) divided into three equal compartments with openings between sections, 26 hours after LPS injections. Subjects were habituated to the apparatus for 5 min before testing. Subjects were temporarily removed while stimuli, contained within smaller cages (8cm diameter, 18cm height, 3-mm diameter bars, 7.4mm spacing) were placed in the center of each of the two outer chambers. First, to test for social preference, a novel toy object and a novel, same-sex stimulus animal were placed in opposite cages. Subjects and stimuli animals had limited ability to directly contact each other; they were able to pass extremities (e.g. paws, tail) through the smaller cage bars during investigation. The subjects were

then returned to the apparatus and allowed 10 minutes to explore the apparatus. At the end of this test, the subjects were removed again, and the toy object replaced with a novel same-sex stimulus animal (from a different cage from the first stimulus) to test for recognition of social novelty. The subject was then placed into the center chamber again and given 10 minutes to explore the apparatus. The position of object and original animal was counterbalanced across trials but did not change between social preference and social novelty preference tests. Each trial was video-recorded, and the time spent in each chamber and in active investigation, defined by the subject's snout within 2 cm of the stimulus cage were manually scored from video files (Noldus Observer) by an experimenter blind to the subjects' genotype.

3.3.8 Tail Suspension

After the social preference/novelty tests, subjects underwent a 5-minute tail suspension test (TST) as a measure of stress-coping behavior [153]. Time spent hanging immobile (not struggling) was scored as was latency to first immobile period. Animals were suspended by their tails by a strip of tape (~15cm) attached to an overhang, during which they were recorded and later scored (Noldus Observer) by an experimenter blind to the genotype of the subjects.

3.3.9 Tissue Collection and FISH

After completion of all testing, animals were sacrificed via CO_2 asphyxiation. Brains were rapidly removed and flash frozen in 2-methylbutane before storage at -80° C. Frozen tissue was sectioned coronally in 20μm sections, and processed for AVP mRNA fluorescent *in situ* hybridization (FISH) to confirm AVP cell deletion, as described in detail in Rigney et al. (2019). Ablation was specific to AVP cells in the BNST with no indication of non-targeted cell loss in the BNST. Nearby AVP hypothalamic cell populations were intact. Only data from subjects with confirmed BNST ablation (defined as over 90% AVP cell loss) were analyzed in this report.

3.3.10 Data Analysis

Statistical analysis was conducted using SPSS (IBM). OFT, EZM, Sucrose preference test, and TST, data was analyzed using a mixed-model three-way ANOVA. Treatment (LPS, Saline) was the within-subjects variable; both sex (M, F) and genotype (Cre+, Cre-) were the between-subject variables. Post-hoc t-tests were used to analyze genotype effects following significant ANOVA interactions. The social preference data was analyzed using a mixed-model three-way ANOVA with chamber (stimulus 1, stimulus 2) as a within-subjects variable, sex (M, F) as a between-subjects variable, and genotype (Cre+, Cre-) as a between-subjects variable. This analysis was performed for each treatment (LPS, Saline) separately, in order to better analyze the impact of genotype and sex on social investigation.

3.4 Results

3.4.1 Open Field Test and Elevated Zero Maze

LPS caused acute sickness behavior in all animals, with some specific measures affected by sex and genotype. As expected [8,154], LPS injections decreased the overall distance traveled in the OFT by all subjects, in both early exploration ($F_{Treatment\ 1,39} = 396.29$, $p < 0.001$, $\eta_p^2 = 0.91$) and late exploration phases ($F_{Treatment\ 1,39} = 309.54$, $p < 0.001$, $\eta_p^2 = 0.89$); no other differences were apparent (Fig 2A, B).

LPS treatment did not affect time spent in the center zone during the early exploration phase of the OFT ($F_{Treatment\ 1,39} = 0.45$, $p= 0.51$) (Fig 2C) but did decrease time spent in the anxiogenic center zone during the late exploration phase in the OFT test ($F_{Treatment\ 1,39} = 74.847$, $p < 0.001$, $\eta_p^2 = 0.66$) (Fig 2D). There were no genotype or sex effects on any other measures.

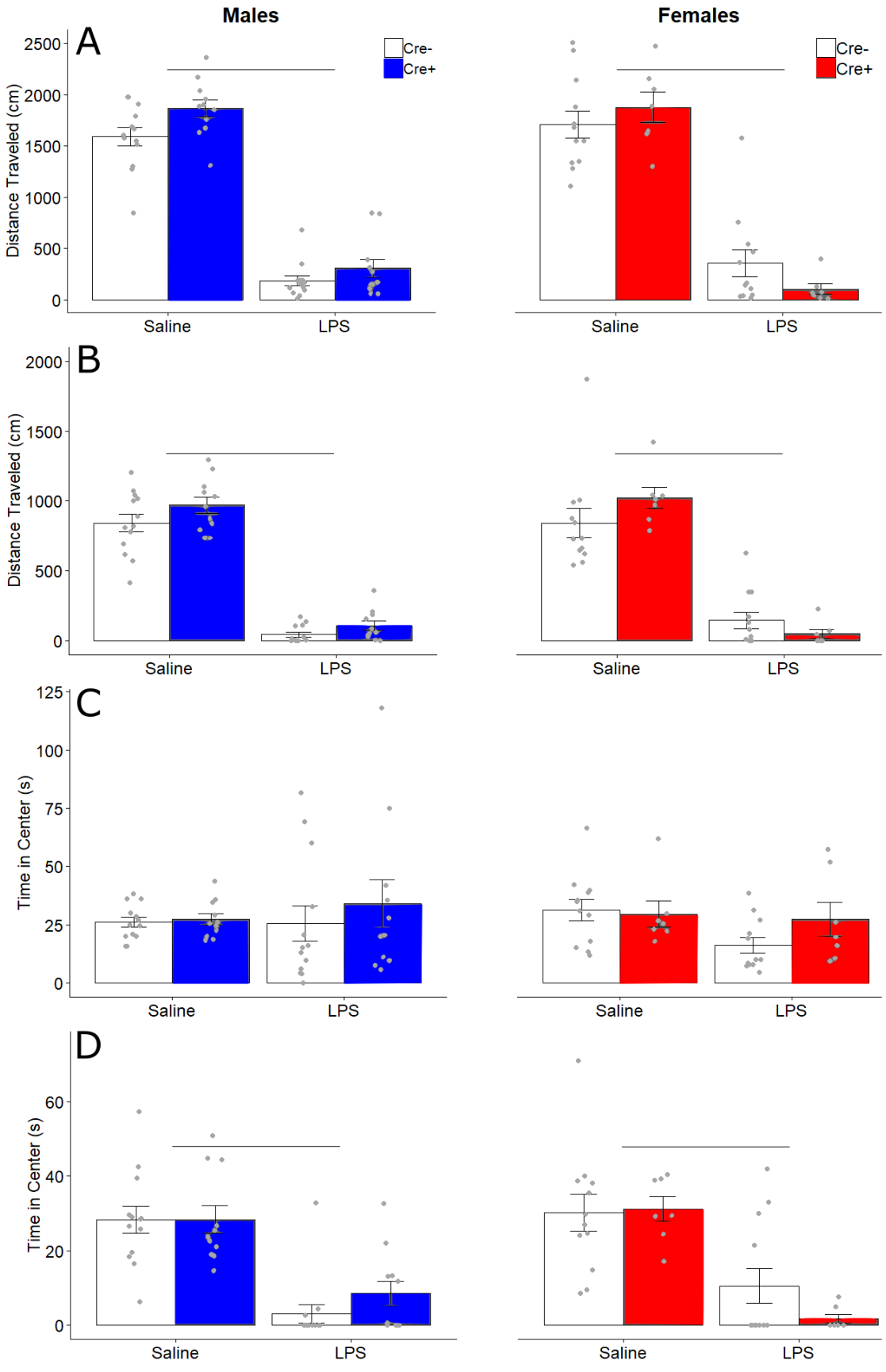
Males
Females
A
B
C
D
Cre-
Cre+
Distance Traveled (cm)
Time in Center (s)
Saline
LPS
0
500
1000
1500
2000
2500
25
50
75
100
125
20
40
60

Figure 3-2

Open field behavior. Mean ± SEM of total distance (cm) traveled in the early (A) and late (B) exploration phases, and of time (seconds) spent in the center of the open field in early (C) and late (D) exploration phases for male and female Cre- (white bars) and Cre+ (filled bars) mice. Points indicate individual data. Horizontal bars indicate significant differences between treatments.

In the elevated zero maze, LPS treatment decreased distance traveled ($F_{Treatment\ 1,39}$ = 285.55, $p < 0.001$, $\eta_p^2 = 0.88$) with an interaction of Treatment by Sex by Genotype ($F_{1,\ 39}$ = 4.723, $p = 0.036$, $\eta_p^2 = 0.108$). Post hoc comparisons for effects of genotype trended towards decreased distance in Cre+ females as compared to Cre- females after LPS treatment ($t = 2.17$, $p = 0.052$), but no obvious differences between Cre+ and Cre- males after LPS treatment ($t = -1.11$, $p = 0.28$). There were no apparent differences between genotypes for saline treated males ($t = 0.27$, $p= 0.98$) or females ($t = -0.99$, $p = 0.35$) (Fig 3A).

LPS treatment decreased time spent in the anxiogenic open arms ($F_{1,39} = 30.37$, $p < 0.001$, $\eta_p^2 = 0.44$), with an interaction of Treatment by Sex ($F_{Treatment\ x\ Sex\ 1,39} = 0.47$, $p= 0.037$, $\eta_p^2 = 0.107$). No other effects or interactions were detected (Fig 3B). LPS injections decreased subjects' speed in the open arms ($F_{1,39} = 31.50$, $p < 0.001$, $\eta_p^2 = 0.48$) in a manner dependent on sex and genotype ($F_{Treatment\ x\ Genotype\ x\ Sex\ 1,39} = 11.668$, $p = 0.001$, $\eta_p^2 = 0.23$; $F_{Genotype\ x\ Sex\ 1,39}$ = 5.343, $p = 0.026$, $\eta_p^2 = 0.12$). Post-hoc comparisons for genotype revealed that in LPS-treated subjects, Cre+ males move faster than Cre- males ($t=-2.8$, $p=.017$), while Cre+ females move slower than Cre- females ($t= 2.52$, $p = 0.022$). No differences in genotype were detected in saline treated males ($t = 0.69$, $p= 0.50$) or females ($t = -0.47$, $p = 0.64$) (Fig 3C), suggesting that ablating AVP cells does not affect overall activity levels.

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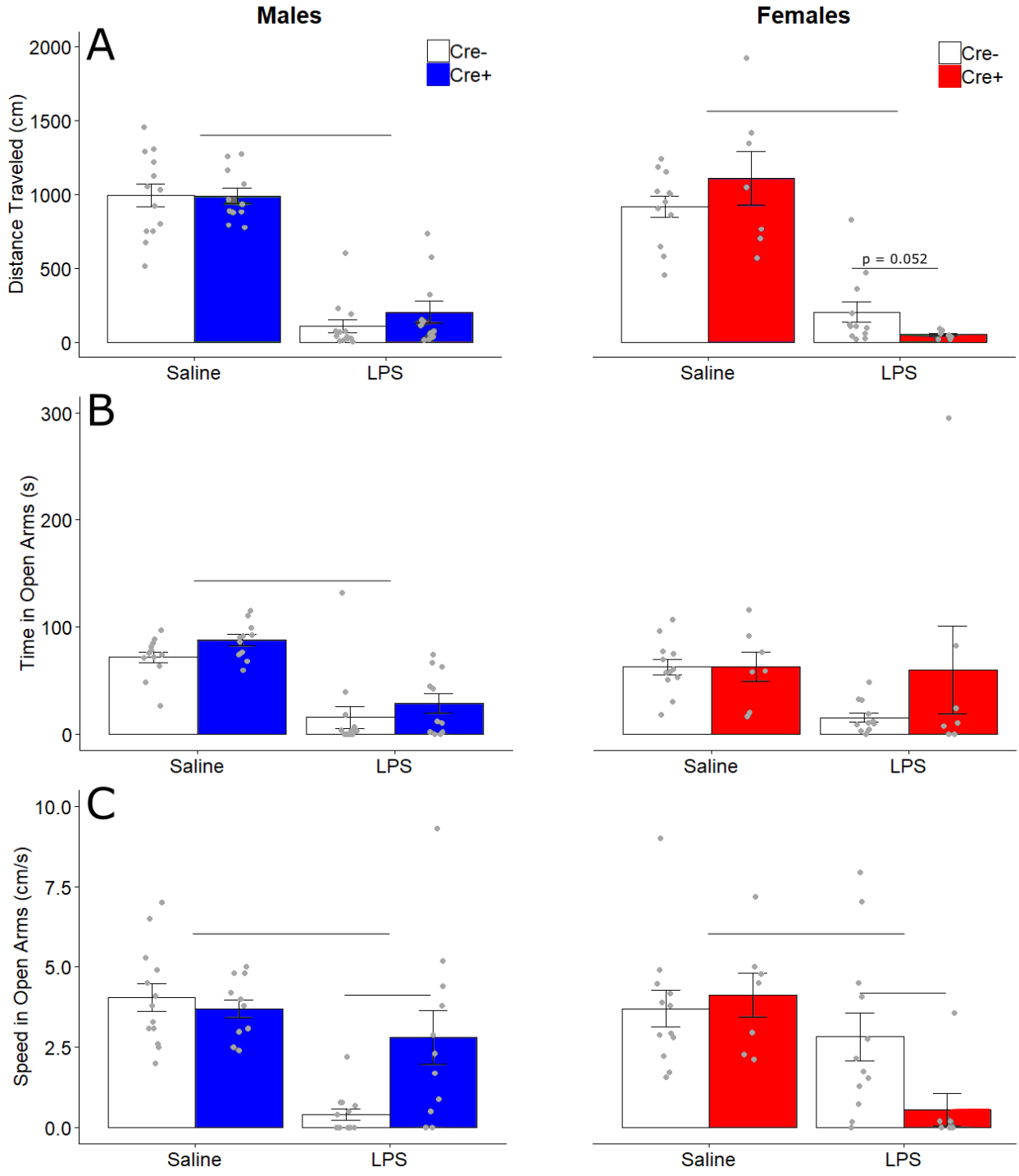

Figure 3-3

Elevated zero maze behavior. Mean ± SEM of (A) total distance traveled (cm), (B) time spent (seconds) in open arms of the maze, and (C) average speed (cm/seconds) in the open arms

of the maze for male and female Cre- (white bars) and Cre+ (filled bars) mice. Points indicate individual data. Horizontal bars indicate treatment differences and genotype differences.

3.4.2 Sucrose Preference and Tail Suspension Tests

Technical problems with several water bottles caused leakage, thus data from six female subjects (5 Cre-, 1 Cre+) were removed from this analysis. LPS treatment decreased preference (percentage of sucrose consumed) for sucrose in all animals ($F_{1,33} = 32.88$, $p < 0.001$, $\eta_p^2 = 0.499$). Overall, males had a higher preference for sucrose than females ($F_{1,33} = 4.761$, $p = 0.036$, $\eta_p^2 = 0.126$). No other significant effects or interactions were detected for preference (Fig 4A). Total consumption was lowered by LPS ($F_{1,33} = 38.959$, $p< 0.001$). This difference was most likely driven by changes in sucrose consumption rather than water consumption as LPS decreased sucrose ($F_{1,33} = 50.111$, $p < 0.001$, $\eta_p^2 = 0.603$) but not water consumption (Fig 4 B, C). There was also a Treatment x Genotype interaction ($F_{1,33} = 4.64$, $p = 0.039$, $\eta_p^2 = 0.123$), and a trending effect of genotype ($F_{1,33} = 3.796$, $p = 0.06$, $\eta_p^2 = 0.1$) for sucrose consumption. Follow up post-hoc tests for genotype effects show Cre+ animals (combined sexes) consumed more sucrose than Cre- animals after saline treatment ($t = -2.27$, $p = 0.031$), but not after LPS treatment ($t = 0.20$, $p = 0.84$). There were no other significant effects or interactions for sucrose consumption (Fig 4B). Body weights for all animals, taken immediately before injection, did not differ by treatment or genotypes, but, unsurprisingly, males were heavier than females ($F_{Sex\ 1,33} = 41.29$, $p < 0.001$, $\eta_p^2 = 0.51$) (Table 1).

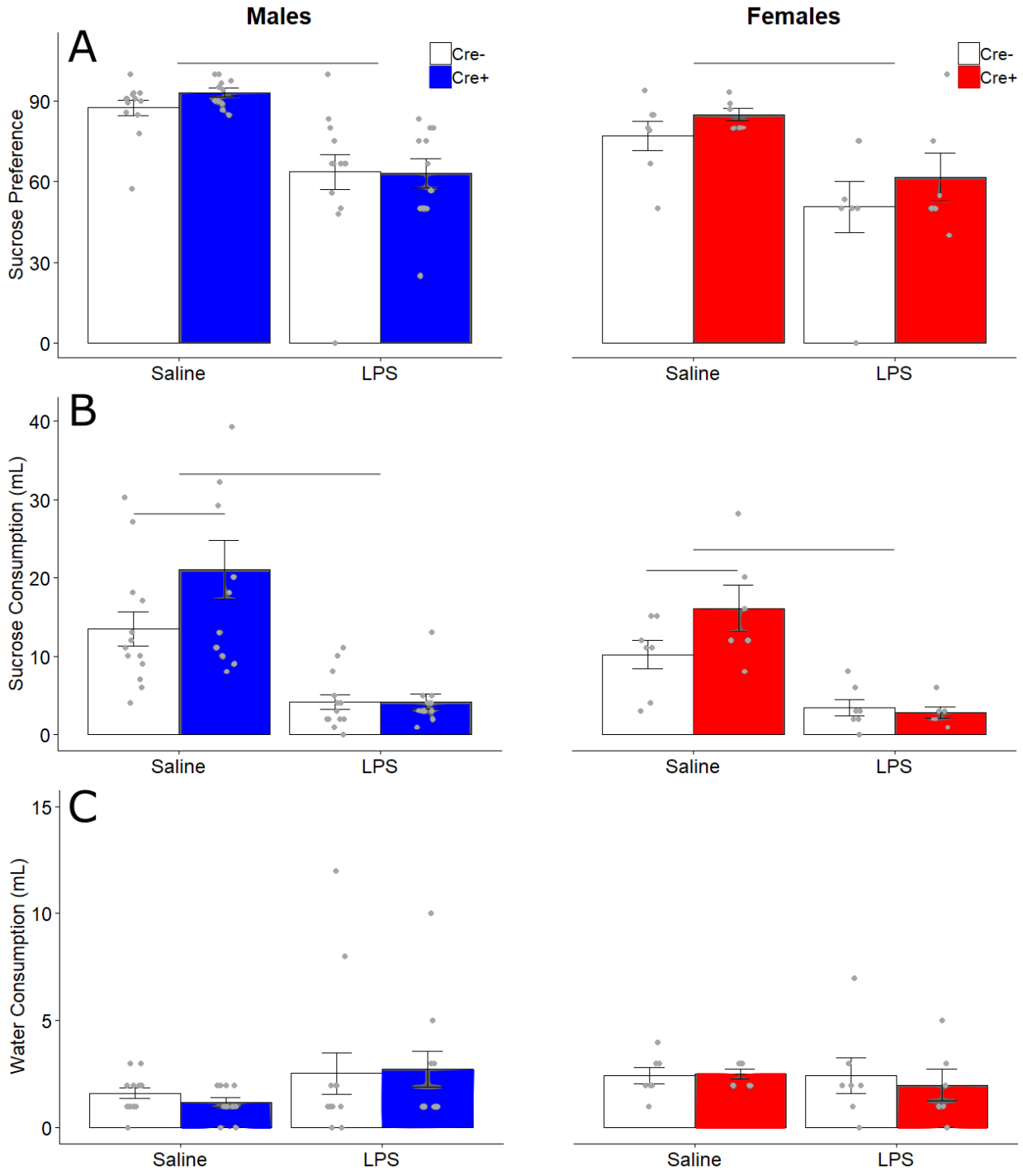

Figure 3-4

Sucrose and water consumption and preference. Mean ± SEM of (A) preference score (percentage of sucrose consumption compared to total consumption), (B) consumption (mL) of 2.5% sucrose solution, and (C) consumption (mL) of water for male and female Cre- (white bars) and Cre+ (filled bars) groups. Points indicate individual data. Horizontal bars indicate treatment differences and genotype differences

Time spent immobile, latency to first immobile period, and the number of immobile periods were measured in the tail suspension test (TST). Males spent more time immobile than females ($F_{Sex\ 1,38} = 4.893$, $p = 0.033$, $\eta_p^2 = 0.114$); there were no other effects detected. There were no effects on latency to first immobile period. LPS treatment caused an increase in the number of immobile periods ($F_{Treatment\ 1,38} = 4.534$, $p=.040$, $\eta_p^2=.107$); there were no other effects on immobile periods. (Table 1).

Table 3-1

Body weight and tail suspension test data. Mean ± SEM for body weight at time of injection and TST measures.

Measure	Male Cre- Sal	Male Cre+ Sal	Male Cre- LPS	Male Cre+ LPS	Female Cre- Saline	Female Cre+ Saline	Female Cre- LPS	Female Cre+ LPS
Body weight	27.08 ± 1.706	26.82 ± 1.537	27.46 ± 2.367	27.00 ± 2.431	23.67 ± 2.103	23.14 ± 1.215	24.17 ± 1.850	23.29 ± 1.604
Time immobile	168.54 ± 31.16	155.72 ± 31.57	155.93 ± 42.72	178 ± 29.1	151.2 ± 42.18	156 ± 39.82	130.76 ± 44.9	134.55 ± 29.25
Latency to first immobile period	31.93 ± 18.55	37.75 ± 19.56	29.89 ± 20.95	26.06 ± 18.96	51.48 ± 37.96	51.67 ± 50.44	32.13 ± 24.18	41.62 ± 25.92

Number of immobile periods	12.62 ± 2.84	13.36 ± 3.35	13.31 ±2.84	14.64 ± 4.589	10.08 ± 2.54	12.33 ± 3.386	13 ± 5.170	13.33 ± 4.179

3.4.3 Sociability tests

. All subjects preferred investigating an animal over an object following both LPS and saline treatment (Saline $F_{1,37} = 38.538$, $p < 0.001$, $\eta_p^2 = 0.510$; LPS $F = 21.895$, $p < 0.001$, $\eta_p^2 = 0.372$). In saline-treated animals, there was a trend towards an interaction of Stimulus by Genotype by Sex ($F_{1,37} = 3.59$, $p = 0.066$). There were no other significant effects or interaction in saline conditions (Fig 5). LPS effects lasted at least one day, as LPS-treated animals showed significantly fewer zone crosses the day following injection than did saline-treated animals (12.34±7.97 vs. 24.32±7.29, mean ± SEM.; $F_{1,37} = 64.47$, $p < 0.001$, $\eta_p^2 = 0.64$).

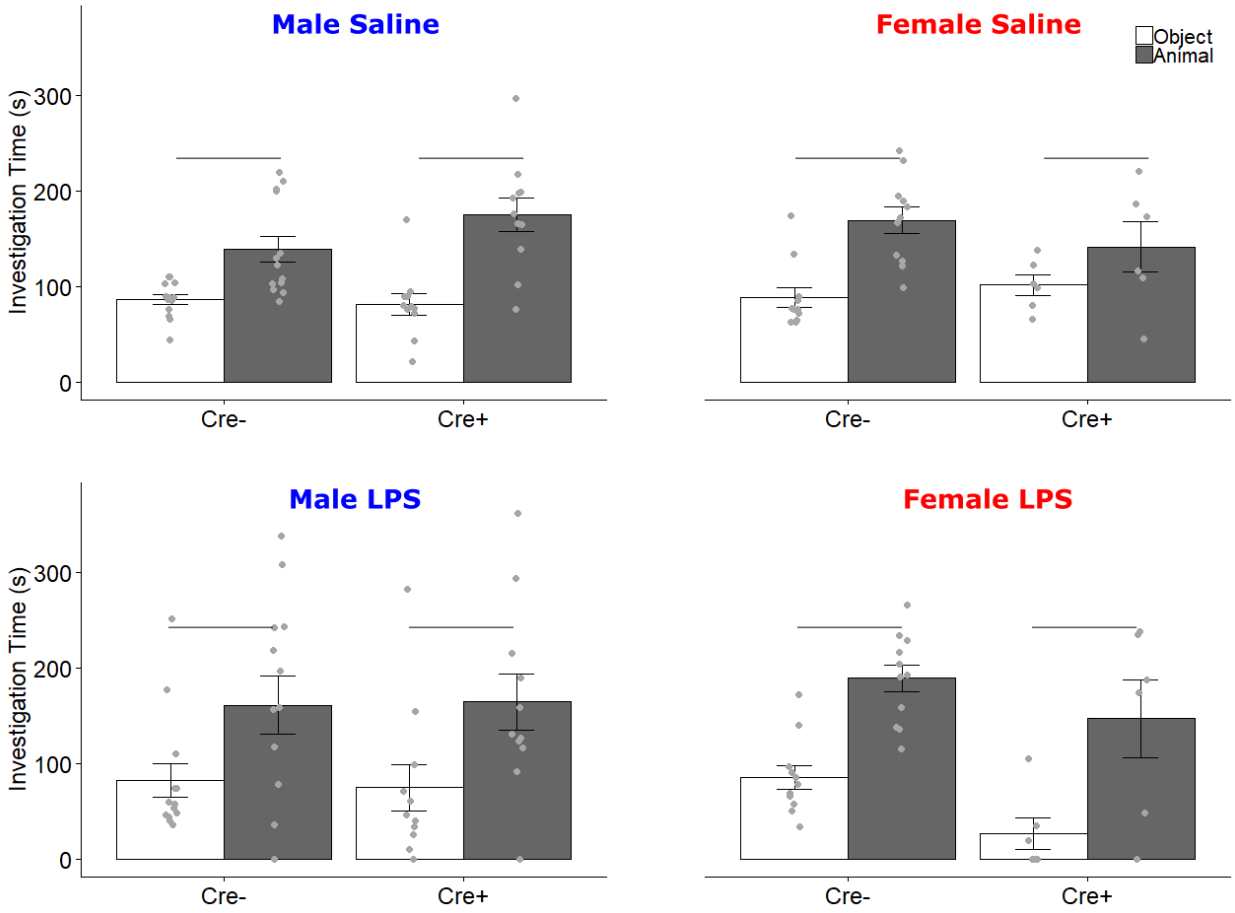

Figure 3-5

Social Preference Test. Data for Saline and LPS treatments are presented separately. Mean ± SEM of active investigation (s) of both object (white bars) and same-sex stimulus animal (filled bars) during the social preference test. Points indicate individual data. Horizontal bars indicate investigation differences.

To test subjects' preference for social novelty, the object was replaced with a novel stimulus animal. For saline-treated subjects, there was a main effect of stimulus ($F_{1,37} = 20.56$, $p < 0.001$, $\eta_p^2 = 0.36$), and an interaction of Stimulus by Genotype by Sex ($F_{1,37} = 4.916$, $p = 0.033$, $\eta_p^2 = 0.117$). In males, there was a main effect of stimulus ($F_{1,22} = 10.79$, $p = 0.003$) and an interaction of Stimulus by Genotype ($F_{1,22} = 5.56$, $p = 0.028$). To further elucidate this interaction, post-hoc t-tests were conducted for each genotype; Cre- males showed a preference

for the novel animal (t = -4.9, p = 0.0004), while Cre+ males did not (t = -.55, p = 0.59). In females, there was a main effect of stimulus ($F_{1,16}$ = 9.11, p = 0.008) but no interaction of Stimulus x Genotype. There were no other main effects or interactions in saline-treated animals. After LPS treatment, there was no preference for either the novel or original stimulus across subjects (Fig 6). Once again, LPS effects lasted at least one day, as LPS-treated animals showed significantly fewer zone crosses the day following injection than did saline-treated animals (9.12 ± 6.90 vs., 18.63 ± 5.14, mean ± SEM.; $F_{1,37}$ = 56.41, p < 0.001, η_p^2 = 0.60)

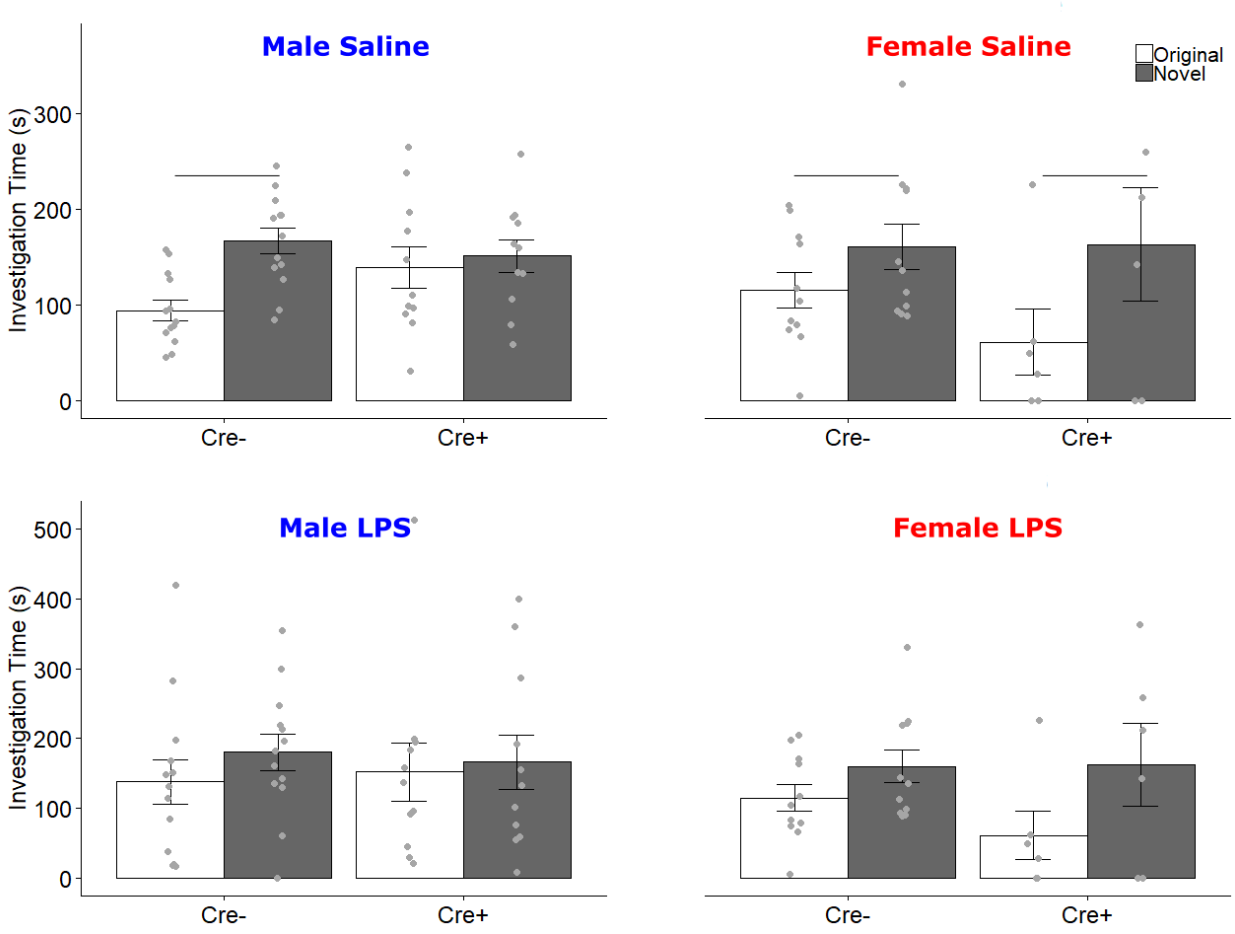

Figure 3-6

Social Novelty Preference Test. Data for Saline and LPS treatments are presented separately. Mean ± SEM of active investigation (s) of both original (white bars) and novel (filled

bars) same-sex stimulus animals during the social novelty preference test. Points indicate individual data. Horizontal bars indicate investigation differences.

3.4.4 Testing order

We compared behavioral results between animals that received LPS first with those that received saline first. We did not find an effect in the majority of measures, except that in the first block of the OFT, saline-treated animals in the second trial (which received LPS in the first trial) traveled slightly less distance ($F_{1,39} = 4.955$, p=.032) than saline-treated animals in the first trial, irrespective of genotype. Additionally, all tail suspension measures, which were not affected by treatment or genotype, were affected by testing order (Time Immobile: $F_{1,38} = 18.35$, $p < 0.001$; Latency: $F_{1,38} = 29.65$, $p < 0.001$; Immobile Periods: $F_{1,38} = 9.63$, $p = 0.004$). This suggests that only the TST was significantly affected by repeated testing.

3.5 Discussion

Although previous studies have suggested that BNST AVP cells in rats may play a role in reducing sickness behavior (e.g., [62]), our results in mice do not align with this; if anything, males were affected opposite to our prediction. For example, ablating BNST AVP cells reduced sickness behavior in response to LPS in males. The same treatment significantly altered other behaviors in control conditions. Specifically, it reduced preference for social novelty in males and increased sucrose intake in both sexes. Therefore, these data support the idea that BNST AVP cells play a more prominent role in male than in female behavior, but they do not suggest a critical role in LPS-induced sickness behavior.

LPS reliably induced sickness behavior, seen especially as reductions in mobility and increases in anhedonia, but did so in all animals, irrespective of BNST AVP cell ablation. If BNST AVP cells reduce sickness behavior, one would expect that removal of these cells enhance

behavioral effects of LPS. On the contrary, ablating BNST AVP cells mitigated the effects of LPS treatment on speed of locomotion in the elevated zero maze in males. However, ablating these cells in females produced effects in the predicted direction, enhancing the reduction in speed after LPS treatment. While this sex difference is still in line with the sex- and steroid-dependent function of these cells, if AVP significantly modulates sickness behavior in mice as it does in rats, it suggests that other AVP-expressing systems, such as the paraventricular nucleus of the hypothalamus (PVN) and suprachiasmatic nucleus (SCN), may be more significant players. PVN cells, including a large number of its AVP-expressing cells, respond to LPS with increased fos activation [55]. A central role of the PVN in sickness behavior is also suggested by studies showing correlated changes in behavior and neurochemical parameters in the PVN following LPS treatment [8,155]. Likewise, the SCN is an important regulator of immune responding, as lesions of this structure exacerbate reactions to LPS [68]. Additionally, the SCN sends AVP projections to nuclei that regulate behaviors changed by LPS injection, such as the PVN, BNST, and medial preoptic area [57], and is therefore well positioned to play a role in sickness behavior.

Our results do not eliminate a possible role of sex- and steroid-dependent AVP in sickness, as there are sexually dimorphic AVP populations in the MeA as well as the BNST [61,63], both of which have been implicated in fever suppression in rats [36–38]. Therefore, one may have to ablate both populations to see a significant effect on sickness behavior as the remaining AVP cells in the MeA may compensate for the loss of BNST AVP and be sufficient to reduce sickness behavior in response to LPS. However, ablating BNST AVP cells strongly affected male social behavior, indicating that these lesions were extensive enough to impair AVP-dependent behavior.

Our data add strong evidence for a sexually dimorphic role of BNST AVP cells in the control of social behavior. As mentioned above, indirect evidence had already implicated these cells in social behavior. For example, injections of AVP antagonists [46,66,67,100,156] or V1aR gene manipulations [64,65] affect social behavior, often in a sex-dependent manner. As we previously reported, BNST AVP cell deletion generates deficits in social communication in males only [131]. Here we find BNST cell ablation in males eliminates social recognition memory as measured by a bias for investigating novel individuals (social novelty preference), without changing social interest.

The effects of BNST AVP cell ablation on behavior found in the present study cannot be unequivocally linked to sexually-dimorphic AVP expression as we removed whole cells, which not only express AVP but also other neuroactive substances, such as galanin [157,158], which also contributes to social behavior [159]. Consequently, behavioral effects may be caused by removal of AVP, co-transmitters, or both. However, since this manipulation eliminated male social recognition, a behavior that depends on AVP within BNST projection sites [46,64–67,160], it is likely that our results are due primarily to removal of BNST AVP production.

Unexpectedly, BNST AVP cell ablation caused an increase in sucrose consumption in both males and females. While it is possible that this increase is due to increased fluid intake, this is not likely because water consumption was not altered by cell deletion. It is also possible that this increase was driven by increased hunger or overactivity. However, subjects whose BNST AVP cells were ablated did not differ from controls in body weight or activity levels during behavioral tests, suggesting no difference in caloric intake or energy expenditure. Perhaps these cells can modulate hedonic drive, such that their removal increased the desire for sucrose. Subnuclei of the BNST respond to sucrose with changes in dopamine and norepinephrine release

[161], so it is possible that AVP cells may contribute to sucrose reward. At any rate, the effects of BNST AVP cells on sucrose consumption suggest a wider role for these cells than just modulation of social behaviors.

3.5.1 Conclusions

Given the sex- and steroid-dependent nature of BNST AVP cells, we expected to find sex differences in the effects of their ablation on behavior, with males exhibiting greater impacts than females. AVP had been previously shown to reduce sickness behaviors, specifically in males, with the BNST indirectly implicated as a source for this effect. Our data, however, suggest that these cells are not critical for reducing LPS-induced sickness behavior, but they are critical for recognition of social novelty in males. This highlights the BNST, and specifically its AVP cells, as critical nodes for male social behavior.

4 REDUCTION OF VASOPRESSIN CELLS IN THE SUPRACHIASMATIC NUCLEUS IN MICE INCREASES ANXIETY AND ALTERS FLUID BALANCE

Jack Whylings, Nicole Rigney, Geert J de Vries, Aras Petrulis

JW designed and performed experiments, analyzed data, and wrote manuscript, NR contributed to experimental design and provided assistance in conducting and analyzing experiments. GJdV and AP contributed to experimental design, advisement, writing, and provided resources.

4.1 Introduction

Animals do not live in isolation; they must respond to both internal and external contexts in order to thrive and reproduce. These needs are met by a combination of homeostatic physiological mechanisms and behavioral interactions. For many organisms, including humans, social behavior is critical for survival, and must be tuned to multiple contexts. One neuromodulatory system heavily involved in regulating both physiology and behavioral outputs is arginine vasopressin (AVP), a nonapeptide neuromodulator and hormone. AVP from various sources[57], such as the paraventricular nucleus of the hypothalamus (PVN), the bed nucleus of the stria terminalis (BNST), the supraoptic nucleus (SON), and the suprachiasmatic nucleus (SCN), is important for regulating stress responses, anxiety, social behaviors, and water balance[47,100,162–164].

Additionally, AVP contributes to sickness: the body's response to infection and inflammation. Activation of the immune system causes multiple physiological and behavioral changes, such as fever, altered metabolic processes, reduced activity, social withdrawal, and anhedonia[1,3,5]. AVP mpdulates some of these changes, for example, AVP from the BNST and medial amygdala regulates fever in male rats[36–38,51]. Previous work in our laboratory has shown

that PVN AVP contributes to changes in motivated behaviors, such as anhedonia, during sickness caused by lipopolysaccharide (LPS), a gram-negative bacterial coat protein[83]. AVP is, therefore, well-positioned to act in concert with other inflammatory-responding systems to generate physiological and behavioral sickness responses.

The source of AVP that regulates social and anxiety-like behaviors, in both healthy and sick contexts, is primarily AVP-expressing cells in the PVN and BNST, and most studies have centered on these two regions. However, another major source of centrally-projecting AVP, the SCN[165,166], may also contribute to these behaviors. SCN AVP cells project to multiple midline nuclei, such as the PVN, paraventricular thalamus, dorsomedial hypothalamus, and preoptic nucleus[57,167], as well as within the SCN[168]. SCN AVP cells are critical for coordinating biological clock function[169,170] and behavioral circadian rhythms[77,79,171], and have been implicated in regulating food intake[80] and circadian thirst[81,172,173]. However, the role of SCN AVP in regulating emotional and social behavior has not been as extensively studied.

In addition to circadian rhythms, the SCN as a whole regulates inflammatory responding. Circadian rhythms alter responses to immune stimuli[174,175], and immune challenges can disrupt circadian rhythms[70,75,176]. Disruptions of the SCN, ranging from dim light exposure during the dark phase[76] to ablation of the entire SCN[68], increase the inflammatory response to endotoxin challenges. Although LPS has been shown to increase AVP release from SCN cells[82], it is unknown whether SCN AVP is involved in regulating sickness behaviors.

Due to the role of AVP in anxiety and social behaviors[48,67,121,156], the function of the SCN in regulating inflammatory responding, and the SCN AVP projections to behaviorally-important hypothalamic nuclei, we assessed the role of SCN AVP cells in social and emotional behavior across two experiments. In the first experiment, we measured the effects of selectively ablating

SCN AVP cells on anxiety and social behaviors. In the second experiment, we examined whether deletion of SCN AVP cells would alter sickness behavior and so we injected subjects with either LPS or saline in a counterbalanced design, and then tested them on anxiety-like, depressive-like, and social behaviors.

4.2 Methods

4.2.1 Animals

All mice were maintained at 22°C on a 12:12 reverse light cycle with food and water available ad libitum, housed in individually ventilated cages (Animal Care Systems, Centennial, CO, USA), and provided with corncob bedding, a nestlet square, and a housing tube. All animal procedures were approved by the Georgia State University Institutional Animal Care and Use Committee (IACUC) and were in accordance with the regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

4.2.1.1 Subjects

AVP-IRES2-Cre-D (AVP-Cre) mice were obtained from The Jackson Laboratory (Stock No: 023530; Bar Harbor, Maine, USA). AVP-Cre knockin mice have Cre recombinase expression directed to vasopressin-expressing cells that are restricted to populations within the hypothalamus. Subjects were derived by crossing heterozygous Cre+ mutants to wildtype C57Bl/6J mice and genotyped (ear punch) by polymerase chain reaction (PCR) at 21-24 days of age (Transnetyx, Cordova, TN, USA). Both Cre+ and Cre- littermates were used in behavioral experiments. A total of 54 experimental animals were tested: 14 Cre- males, 11 Cre- females, 16 Cre+ males, and 13 Cre+ females.

4.2.1.2 Stimulus Animals

Stimulus animals,C57B6/J and CD-1 mice of both sexes, were housed in same-sex groups. CD-1 female stimulus mice were ovariectomized and implanted subcutaneously with a Silastic implant of estradiol (0.7 cm active length; 1.02 mm inner diameter, 2.16 mm outer diameter, Dow Corning, Midland, MI, USA; 1:1 estradiol benzoate:cholesterol). Before any testing for sex behavior (social experience and copulatory behavior, as described below), female stimulus mice were injected with .1mL progesterone (500 μg dissolved in sesame oil, Sigma, St. Louis, USA) to induce behavioral estrus. Male CD-1 stimulus mice were gonadectomized, and group housed. A subset of male CD-1 stimulus mice, receiving subcutaneous implants of testosterone (0.7 cm active length; 1.02 mm inner diameter, 2.16 mm outer diameter, Dow Corning, Midland, MI, USA; crystalline testosterone, Sigma, St. Louis, USA) and singly-housed, were used for providing social experience and copulatory behavior. Surgery and implant procedures were performed as described previously in detail in prior publications[103,131].

4.2.2 Social Experience

All subjects received social experience (described in detail in prior publications[103,131]) prior to viral vector injections as sexual and aggressive experiences promote communication behaviors in mice[177,178] Subjects received sexual and aggressive experience in four separate instances; each sex experience was followed by an aggressive experience; there was at least one day between the first and second sets of sex-aggression experiences.

Sexual experience was provided by placing a sexually-experienced, hormone-implanted, opposite-sex CD-1 stimulus mouse overnight in the subjects' home cage (first experience) or for ninety minutes (second experience). For aggressive experience, subjects were exposed to a same-sex, non-territorial (gonadectomized and group-housed), CD-1 mouse as an intruder in the

subject's home cage. The stimulus animal was removed after the subject's first offensive attack (biting) or after ten minutes if no fighting occurred. Female subjects were exposed to female intruders; however, this did not elicit attacks from either animal.

4.2.3 Viral Vectors

AVP driven-, Cre-expressing SCN neurons were ablated using an adeno-associated virus (AAV; serotype 2/1 (3x1012 IIU/mL) AAV-flex-taCasp3-TEVp; UNC Vector Core) that encodes, in a Cre-dependent fashion, a mutated pro-caspase-3 and its activator (TEVp). This system activates an apoptotic signaling cascade, cleaving multiple structural and regulatory proteins critical for cell survival and maintenance[146,147] and killing cells with far less collateral inflammation than other lesion approaches[103,148].

4.2.4 Stereotaxic surgery

All surgeries were carried out using 1.5-3% isoflurane gas anesthesia in 100% oxygen; 3 mg/kg of carprofen was given before surgery to reduce pain. Mice were positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with ear and incisor bars holding bregma and lambda level. After a midline scalp incision, a hand operated drill was used to make holes in the skull, exposing the dura. For all subjects, 250 nl of AAV-flex-taCasp3-TEVp was delivered bilaterally to the SCN (coordinates: -.2 mm AP; +- 1.73 mm ML; -5.74 mm DV, 15 degree angle) (Paxinos and Franklin, 2012) at a rate of 100 nl/min using a 5 µl Hamilton syringe with a 30-gauge beveled needle mounted on a stereotaxic injector. Following virus delivery, the syringe was left in place for 15 minutes and then slowly withdrawn from the brain.

4.2.5 Experiment 1: Effects of ablation of SCN AVP cells on social and emotional behaviors

All testing occurred within the first eight hours of the dark cycle under red light illumination. All tests were scored by an experimenter blind to the genotype of the subject. On experimental days, subjects were adapted to the experimental room for fifteen minutes prior to testing. First, we tested mice on an elevated plus maze to test for anxiety-related behavior. Mice were then tested in the three-chamber apparatus over five days. Lastly, copulatory and aggressive behavior were measured sequentially in the subject's home cage. Female subjects were tested irrespective of estrous cycle day, except during copulation testing, when they were in behavioral estrus, confirmed by analysis of vaginal cell morphology.

4.2.5.1 Elevated-Plus Maze

The elevated plus maze (EPM) consisted of two open arms (30 x 5 x 0 cm) and two closed arms (30 x 5 x 25 cm) crossed perpendicularly and raised 60 cm above the floor. Subjects were placed in the intersecting center zone and were habituated to the apparatus for one minute; subjects' behavior was scored over the following five minutes. Due to software failure, results from a subset of animals could not be reliably quantified; behaviors from the remaining animals (9 Cre- males, 5 Cre- females, 8 Cre+ males, 5 Cre+ females) were recorded with a digital video recorder, and scored by an observer using Noldus Observer software (XT 11). Behaviors measured include time spent in open and closed arms, open arm entries, and the number of risk assessment behaviors (stretch-attend posture, head-dips; Rodgers and Cole 1993). Three subjects (2 Cre- males, 1 Cre+ male) were removed from EPM data analysis because they fell off the maze during testing.

4.2.5.2 Social Behavior

Ultrasonic vocalizations (USV), urine marking, and social investigation were recorded in an acrylic three-chamber apparatus (Harvard Apparatus, Holliston, MA, USA; dimensions: 20.3 x 42 x 22 cm). Subjects were all acclimated to the apparatus over 2 days of exposure (2 mins) to the testing area and 3-chamber apparatus. During the 3 days of behavior testing, mice were first exposed to the apparatus with no stimuli in either chamber; the following 2 testing days subjects were exposed to male and female stimulus animals in separate tests. Instead of a solid floor, the apparatus was placed on absorbent paper (Nalgene Versi-dry paper, Thermo Fisher Scientific, Waltham, MA, USA) so as to accurately measure urine marking. During testing with stimulus animals, subjects had access to either a CD-1 stimulus animal in a cylindrical cage (8 cm (D), 18 cm (H); 3 mm diameter steel bars, 7.4 mm spacing) or an empty cage placed in the outer chambers of the apparatus. Subjects and stimulus animals had limited ability to directly contact each other; they were able to pass extremities (e.g. paws, tail) through the smaller cage bars during investigation. The location of stimulus and the "clean" cage were counterbalanced across animals. After placing the subject in the center of the middle chamber, we measured, across a 5-minute trial, close investigation of clean and stimulus cages, as well as USV and urine marking, as described below. After testing, the apparatus and cages were thoroughly cleaned with 70% ethanol and allowed to dry before further testing. The order of male and female stimuli presentation was counterbalanced across subjects.

4.2.5.3 Social Investigation and Ultrasonic Vocalizations

Close investigation of stimulus cages was defined as time spent sniffing within 2 cm of each cage. USV were detected using a condenser microphone connected to an amplifier (UltraSoundGate CM16/CMPA, 10 kHz - 200 kHz, frequency range) placed 4 cm inside the

apparatus and directly above the center compartment. USV were sampled at 200 kHz (16-bit) with target frequency set to 70 kHz (UltraSoundGate 116Hb, Avisoft Bioacoustics, Berlin, Germany). Recordings were then analyzed using a MATLAB (MATLAB, Mathworks, RRID:SCR_001622) plug-in that automates USV analysis (MUPET[179]). Using this program, sonograms were generated by calculating the power spectrum on Hamming-windowed data and then transformed into compact acoustic feature representations (Gammatone Filterbank). Each 200-millisecond window containing the maximum USV syllable duration was then clustered, via machine learning algorithms, into USV syllable types (repertoire units) based on time-frequency USV shape. Repertoire units that appeared as background noise were discarded. As the number of USV syllables produced is low in this strain, we limited our analysis to the total number of USV syllables produced during each test.

4.2.5.4 Urine Marking

Following testing, each substrate sheet was allowed to dry and was then sprayed with ninhydrin fixative (LC-NIN-16; Tritech Forensics Inc., Southport, NC, USA) to visualize urine marks. After twenty-four hours, sheets were imaged with a digital camera and then analyzed using imaging software (ImageJ, RRID:SCR_003070). Visualized marking was outlined and areas measured and summed. Urine marking was measured as the total area (pixels) of visualized ninhydrin urine marks in the entire arena. Urine marks that were larger than 6 cm2 and directed toward corners were counted as elimination ‘pools’ and were removed from analysis.

4.2.5.5 Copulatory and Aggressive Behavior

To measure copulatory behavior, the stimulus mouse was placed in the subject’s home cage for ninety minutes. The latency to mount, latency to intromit, number of mounts and intromissions, and percent of male ejaculations were recorded. To measure territorial aggression,

stimulus animals were placed in the subject's home cage and then removed after the subject's first offensive attack (biting) within a ten-minute period; the latency to this attack was recorded.

4.2.5.6 Odor Discrimination

At the end of experiment 1, a subset of subjects (9 Cre-, 5 Cre+) were tested for their olfactory ability using a habituation-discrimination approach that measured whether subjects could distinguish between social and non-social odors. Animals were repeatedly exposed (five times; two minute trials; 1 minute intertrial intervals) in their home cage to filter paper containing one odorant. Afterwards, the stimulus was replaced with a different odor which was then also repeatedly presented. Subjects were first exposed to distilled water, then to one of three non-social odors (lemon extract, almond extract, coconut extract, Kroger, Cincinnati OH, USA), then to a second non-social odors (counterbalanced), followed by a social odor (male or female urine), and then by the other social odor (female or male urine, counterbalanced). We compared the amount of time Cre+ and Cre- subjects spent investigating each odor between the first and last presentation of each odor and then between the last presentation of the familiar odor and the first presentation of the novel odor, using mixed-model ANOVA (investigation time as a within-subjects factor, genotype as a between-subjects factor)

4.2.6 Experiment 2: LPS-induced sickness behavior

At least one week after the behavioral tests in Experiment 1, subjects were tested for sickness behaviors. Subjects were weighed and injected intraperitoneally with either 0.5 mg/kg LPS (from e. coli 0111:B4, Sigma, St. Louis, MO, USA) or sterile saline one hour before dark phase (ZT11). As described in previous experiments, the open field test (OFT) was conducted three hours following LPS injections, and the elevated zero maze (EZM) test was conducted immediately following the OFT. Sucrose preference was then assessed in the home cage during

the following 24-hour period. The day after LPS or saline injections, subjects were tested during the dark phase in the three-chamber apparatus for social preference and novelty detection. Subjects were tested twice, first within ~24 hours after LPS/Saline, then again one week later after Saline/LPSin a counterbalanced manner. In all cases, animals were acclimated to the behavior testing suite for at least one hour before testing, and tests were done during the dark phase under dim red lighting.

4.2.6.1 Open-Field and Elevated Zero Maze

Three hours after LPS injections, animals were placed in a 43 cm x 43 cm x 30 cm open field chamber for 10 min and behavior was automatically tracked via infrared beam breaks (Med Associates, Fairfax, VT, USA). Distance traveled and time spent in the (anxiogenic) center area were analyzed as measures of locomotion and anxiety-like behavior, respectively. Immediately after OFT, subjects were tested on an elevated zero maze (EZM). This apparatus consists of a 5.5 cm wide circular platform (internal diameter 35 cm) raised 50 cm off the ground, with two equally spaced enclosed compartments covering half of the platform. Video was manually scored (Noldus Observer) by an observer blind to subject genotype for time spent in both open and closed arms as a measure of anxiety-like behavior, and for zone crosses (subject crossing from open to closed arm and vice versa) as a measure of activity.

4.2.6.2 Sucrose Preference

For at least 2 days before LPS/saline injections, subjects were acclimated to having two water bottles placed in their home cage. After OFT/EZM assessment, subjects were returned to their home cage, and bottles were replaced with pre-weighed bottles, one containing sucrose solution (2.5% in tap water) and the other tap water. Animals had access to both sucrose solution and water for the next 20 hours, until the start of the next day's testing. The bottles were then

removed and weighed to measure consumption. A control water bottle in a nearby empty cage showed a <1 g loss over the same period and room conditions. Sucrose consumption, water consumption and preference, calculated as the percentage of sucrose consumed of total consumption (Sucrose / (Sucrose + Water) *100%), were analyzed.

4.2.6.3 Social Preference and Social Novelty Preference

To measure social preference and social novelty, animals were tested in the three-chamber apparatus as described above, 26 hours after LPS injection. Subjects were placed in the apparatus for 5 min before testing to habituate the subjects to the environment and were then temporarily removed while stimulus animals/objects, contained within smaller cages (8 cm diameter, 18 cm height, 3-mm diameter bars, 7.4 mm spacing) were placed in the center of each of the two outer chambers. First, to test for social preference, a novel toy object (either a mouse, robot, or small car figurine) and a novel same-sex C57 stimulus animal were placed in opposite cages. Subjects and stimulus animals had limited ability to directly contact each other; they were able to pass extremities (e.g. paws, tail) through the smaller cage bars during investigation. The subjects were then returned to the apparatus and allowed 10 min to explore the apparatus. At the end of this test, the subjects were removed again, and the toy object replaced with a novel stimulus animal (from a different cage from the first stimulus) to test for recognition of social novelty. The subject was then placed into the center chamber again and given 10 min to explore the apparatus. The position of object and original animal was counterbalanced across trials but did not change between social preference and social novelty preference tests. Videos were manually scored (Noldus Observer) by an observer blind to subject genotype for outer chamber entries as a measure of activity, time spent in each chamber, and close investigation, defined by the subject's snout within 2 cm of the stimulus cage.

4.2.7 Histology and In Situ Hybridization

Following all testing, subjects were killed via CO2 asphyxiation. Brains were extracted and flash-frozen via submersion in 2-methyl-2-butanol (Sigma, St. Louis, MO, USA) for 10-20 s and stored at −80°C until sectioned into 3 series of 20 μm. Tissue was sectioned and labeled for AVP mRNA (accession number NM_027106.4) via fluorescent in situ hybridization as described in detail in previous work[103,131]. Bilateral images of the SCN were taken at 20x magnification using a Zeiss Axio Imager.M2 microscope (Carl Zeiss Microimaging, Göttingen, Germany), and analyzed using ImageJ. The SCN was outlined, and fluorescent cells were manually counted in each hemisphere of the SCN. Cell counts and SCN area were summed and averaged across each animal to generate an overall SCN cell/area metric which we then compared across ablated and control subjects. Ablated subjects were included in the analysis if they had > 30% SCN AVP-expressing cell ablation overall and >50% deletion within at least one hemisphere.

4.2.8 Data Analysis

In Experiment 1, the EPM data were analyzed for effects of genotype using independent t-tests; urine marking, USV, sex behavior, and aggressive behavior were analyzed separately by sex for effects of genotype. Data that did not meet requirements for parametric testing were transformed using a Box-Cox procedure (urine marking) or analyzed using a nonparametric Kolmgorov-Smirnoff test (USV). Social investigation was analyzed using a mixed-model, four-way ANOVA, using stimulus identity (animal or empty cage) and stimulus sex (male or female trials) as within-subject variables, and genotype (Cre+, Cre-) and subject sex (Male, Female) as between-subject variables.

In Experiment 2, data from OFT, EZM, and sucrose preference testing was analyzed using a mixed-model, three-way ANOVA, using treatment (LPS, Saline) as within-subjects

variable and both subject sex and genotype as between-subject variables. Investigation in the Social Preference test was analyzed using a four-way mixed model ANOVA with the same parameters as the prior tests and inclusion of stimulus identity as a within-subjects variable.

Results with p-values equal to or less than 0.05 were considered statistically significant, and results with p-values of 0.05 to 0.09 were considered trending effects. Degrees of freedom were not adjusted in our mixed model ANOVA analyses as all data sets maintained sphericity (Mauchly's test of sphericity). Post-hoc t-tests were used to analyze genotype differences following significant ANOVA main effects and interactions. In addition, we calculated Pearson's correlation coefficients to analyze the relationship between SCN AVP cell loss and behavior, using the number of remaining SCN AVP cells/mm2 in Cre+ subjects. All statistical analysis was conducted using SPSS 27 (IBM)

4.3 Results

4.3.1 Fluorescent in situ hybridization and cell ablation

Viral expression of Cre-dependent caspase caused a marked AVP cell reduction in the SCN. Cre+ subjects were kept in the analysis if there was at least a 30% reduction of cell count/area as compared to Cre- controls. 4 Cre+ males and 6 Cre+ females were removed for not meeting this criteria. The average reduction in ablated Cre+ animals was 53.55 +- 3.75 % of controls. There was a significant difference between the cell count/area of Cre- and Cre+ subjects (t-test, $t = 7.003$, $p < 0.001$, $d = 2.19$). Cell reductions occurred throughout the anterior-posterior axis of the SCN, with no clear directional bias. Figure 1 shows representative photomicrographs of AVP labeling in a Cre- and Cre+ subject. No behavioral measures were significantly correlated with the number of remaining SCN AVP cells.

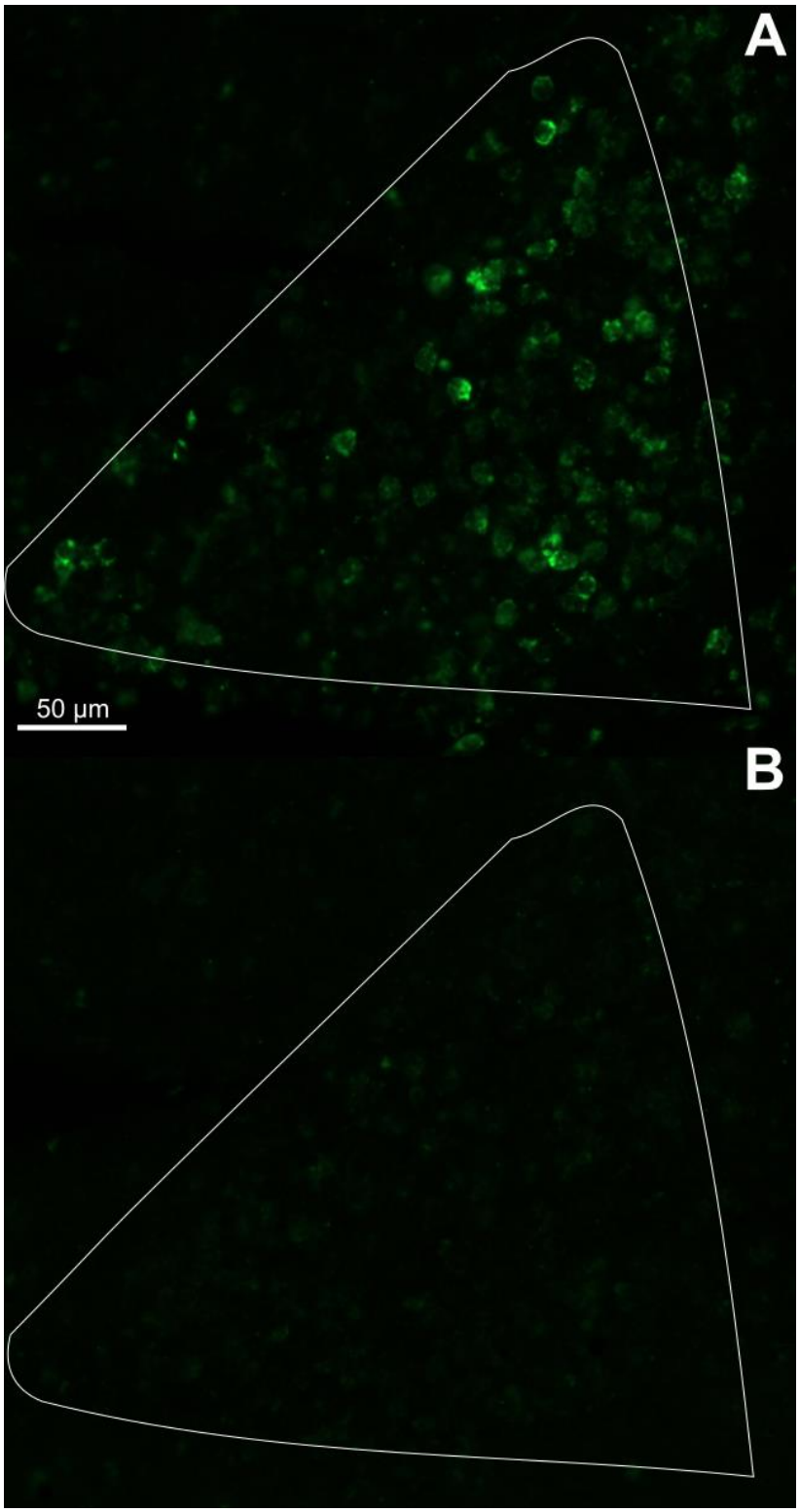

Figure 4-1

AVP fluorescent in situ hybridization. Example photomicrograph of one unilateral SCN (outlined) in both (A) a Cre- control animal and (B) a Cre+ animal with 52% AVP cell reduction.

4.3.2 Experiment 1

4.3.2.1 Elevated Plus Maze

Cre+ animals spent significantly less time in the open arms of the elevated plus maze (t = 2.67, p = 0.014, d = 1.09), and more time in the closed arms of the maze (t = 2.51, p = 0.023, d = 1.03). Cre+ animals produced fewer head dips (t = 2.74, p = 0.012, d = 1.12), but did not decrease the amount of stretch-attends compared to controls (t = -0.21, p = 0.83). The total number of entries into all zones (a measure of general activity) did not differ between genotypes (t = -.494, p = 0.63).

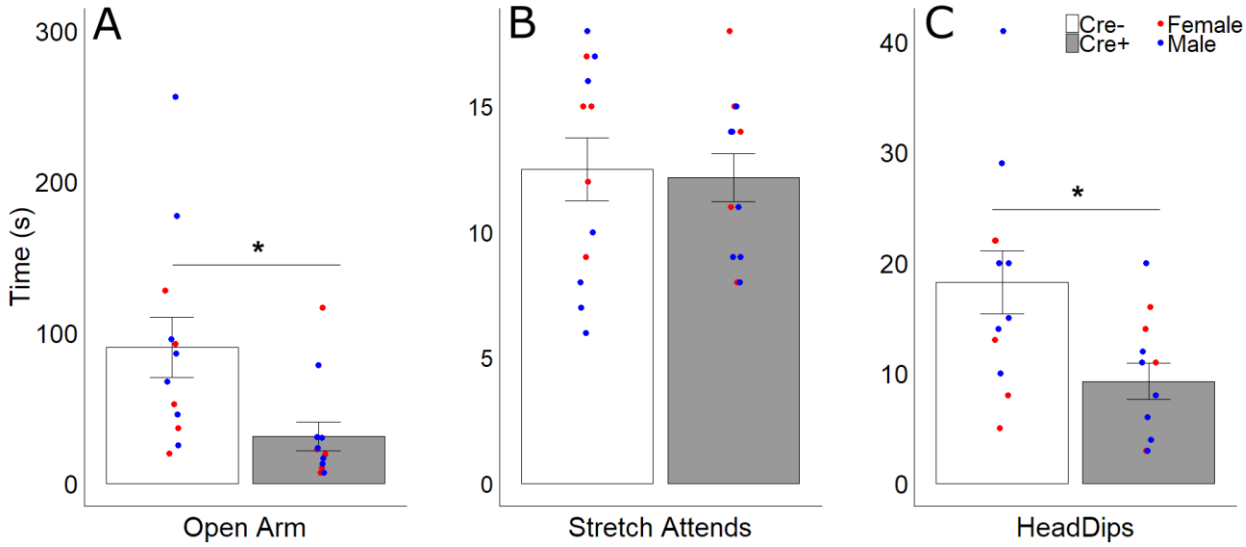

Figure 4-2
Anxiety-like behaviors in the elevated plus maze. Mean ± SEM of (A) time spent in the open arms, (B) number of stretch attend postures, and (C) number of head dips for Cre- (white bars) and Cre+ (gray bars) subjects. Points represent individual data from males (blue points) and females (red points); asterisks represent significant (p < 0.05) genotype differences.

4.3.2.2 Social Investigation

For all subjects, there was a significant difference between investigation of the empty cage and the stimulus cage. All subjects spent more time investigating social stimuli than the empty chamber (F = 45.395, p < 0.001), with a trending interaction of subject sex (F = 3.922, p =

0.055). There were no other significant main effects or interactions of subject genotype or stimulus sex. Neither sex ($F = 0.64$, $p = 0.43$) nor genotype ($F = 1.14$, $p = 0.29$) altered total investigation time of empty chambers during the initial no-stimulus trial.

As mouse communication behavior varies significantly based on the sex of both the subject and stimulus[103,131], urine marking and USV were analyzed seperately for both sexes and each trial (no stimuli, male stimulus, female stimulus). In response to the clean environment, Cre+ males deposited more urine than Cre- males ($t = 2.583$, $p = 0.02$); there was, however, no genotype differences between female subjects in urine deposition ($t = -.314$, $p = 0.759$). Urine marking to social cues (male or female stimuli) did not differ according to genotype in male or female subjects. Genotype did not alter urine marking of either sex during investigation of male (Male subjects $t = -0.405$, $p = 0.689$; Female subjects $t = -0.008$, $p = 0.994$) or female (Male subjects $t = -1.121$, $p = 0.273$; Female subjects $t = 0.571$, p - 0.779) stimluls trials.

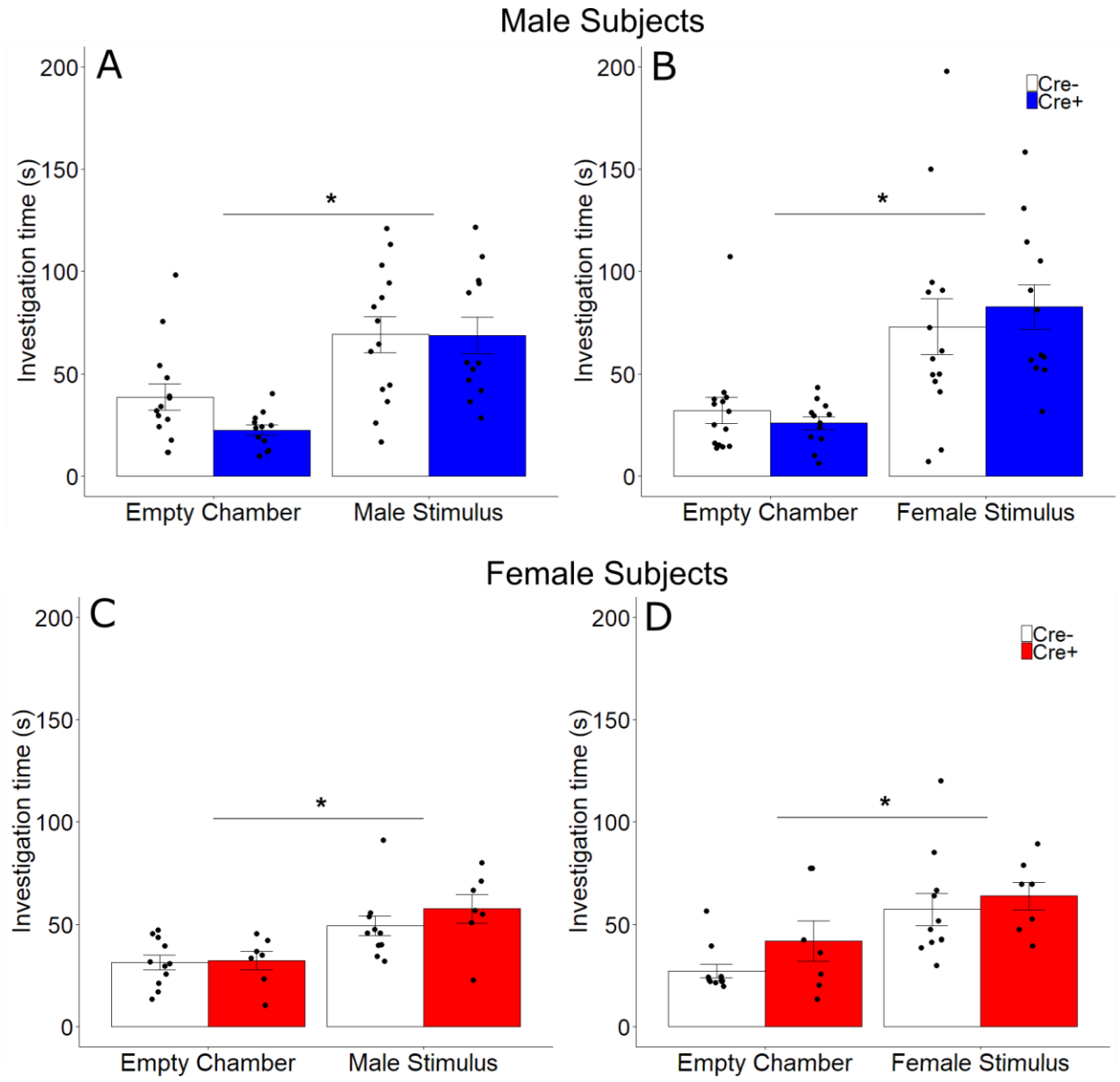

Figure 4-3

Investigation of male and female stimuli. Mean ± SEM of investigation time of male (A, C) and female (B, D) stimulus animals compared to a clean cage by male (A, B) and female (C, D) subjects in both Cre- (white bars) and Cre+ (filled bars) subjects. Points represent individual data and asterisks represent significant ($p < 0.05$) investigation of stimulus animals.

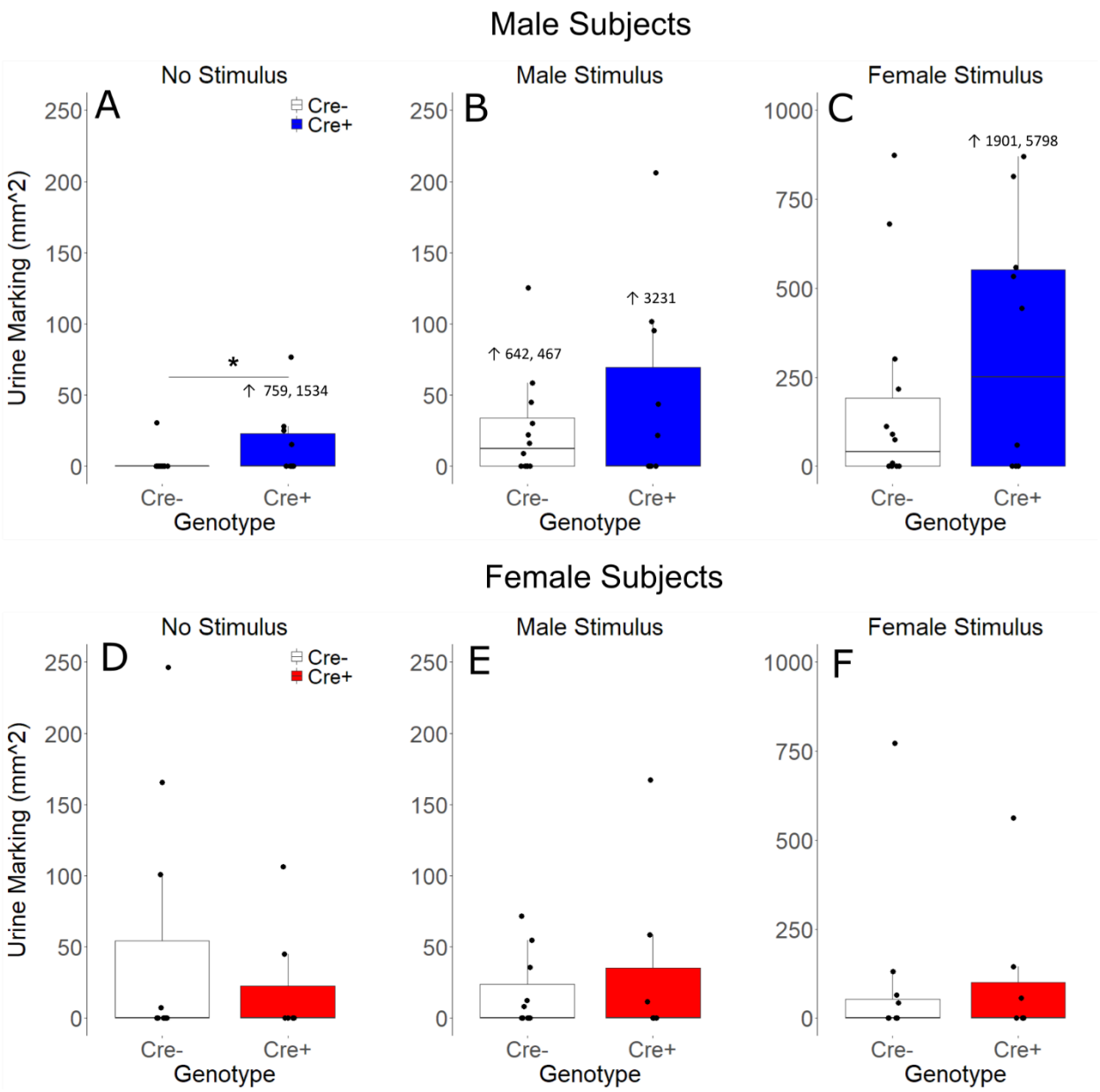

Figure 4-4

Urine marking. Median and interquartile range of urine marking area (mm2) when subjects were presented with no stimuli (A, D), male stimuli (B, E), and female stimuli (C, F), for both male (A, B, C) and Female (D, E, F) subjects. Points represent individual subjects. Outliers that are greater than the scale of boxplots are represented as numbers rather than data points.

4.3.2.3 Sex, Aggression, Odor Discrimination

There were no significant differences between genotypes of either sex in copulatory behavior (number or latency of mounts, intromissions, or ejaculations) or offensive aggression (latency to attack).

All subjects showed habituation in their investigation to repeated exposures to both non-social (first non-social odor test: $F = 8.55$, $p = 0.012$; second non-social odor test: $F = 50.781$, $p < 0.01$) and social odors (first social odor test: $F = 81.12$, $p < 0.01$; second social odor test: $F = 51.891$, $p < 0.00$), with no significant main effect of genotype. All subjects also discriminated between non-social odors ($F = 12.95$, $p = 0.004$), between social odors ($F = 42.219$, $p < 0.001$), and between a non-social and social odor ($F = 160.00$, $p < 0.001$), with no main effect of genotype, indicating that reduction in SCN AVP cells did not cause significant deficits in basic odor processing.

4.3.3 Experiment 2

4.3.3.1 Open Field (OFT) and Elevated Zero Maze (EZM)

In the OFT, treatment with LPS reduced time spent in the anxiogenic center zone by all subjects ($F=17.786$, $p < 0.001$); two subjects did not move from their initial placement in the testing environment and so were removed as outliers (both were >3 standard deviations above the mean). There was a trend towards a sex difference, with males spending slightly more time in the center than females ($F = 3.870$, $p = 0.056$), but no other main effects or interactions were detected in the OFT. LPS treatment also reduced distance traveled, a measure of overall activity ($F = 731.515$, $p < 0.001$), with no other significant main effects or interactions. Unexpectedly, treatment with LPS did not alter time spent in the open arms in the EZM; however, LPS treatment did decrease the number of entries into the open arm ($F = 90.81$, $p < 0.001$), with a

trending interaction of sex (F = 3.849, p = 0.057). There were no other significant main effects or interactions in EZM test metrics.

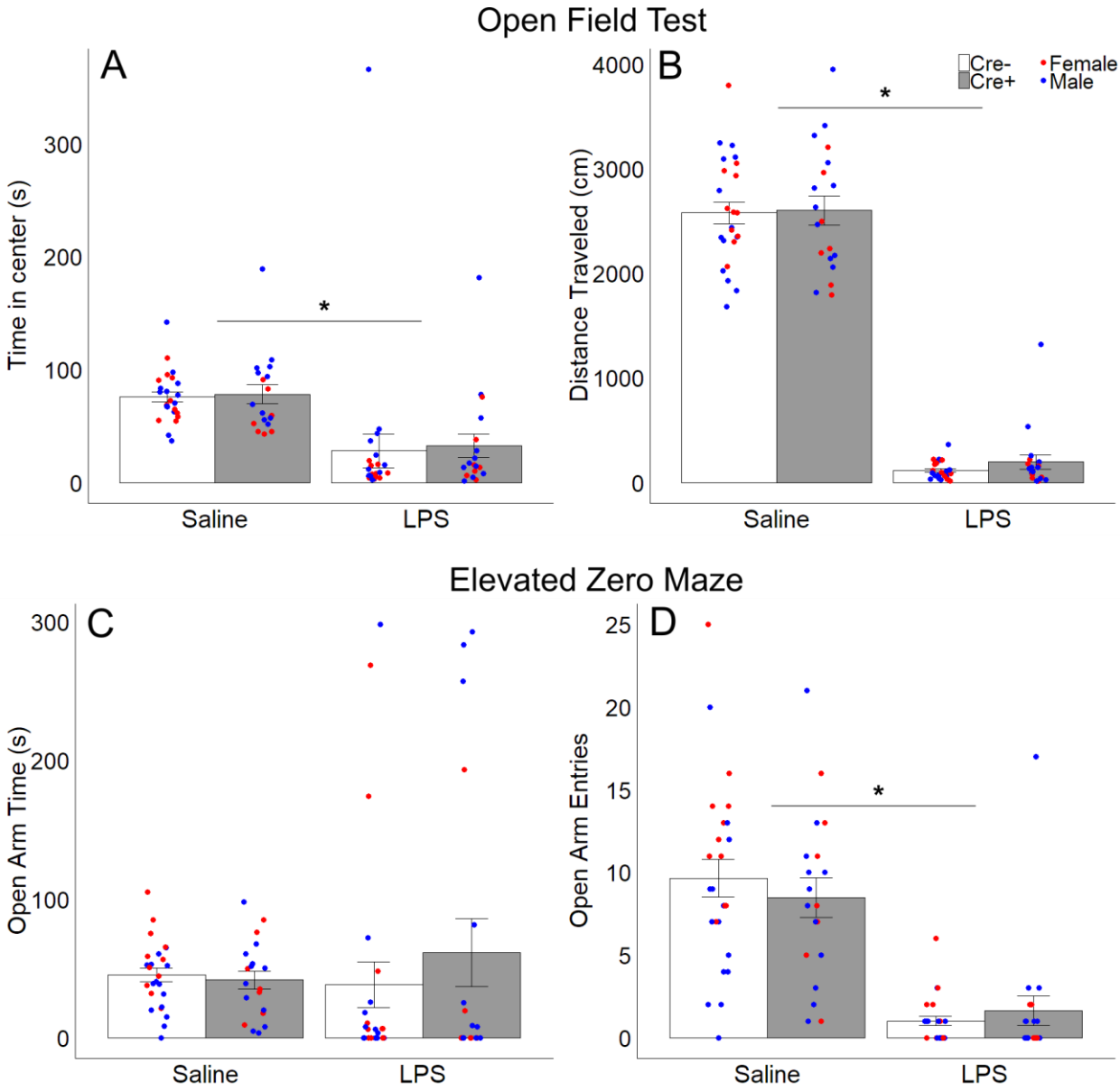

Figure 4-5

Anxiety-like behavior and locomotion in the open field test and elevated zero maze. Mean ± SEM of (A) time in the center zone and (B) distance traveled in the OFT; (C) time in the open arms, and (D) number of entries into the open arms in the EZM for Cre- (white bars) and Cre+ (gray bars) subjects after LPS and saline treatment. Points represent individual data from males (blue points) and females (red points); asterisks represent significant ($p < 0.05$) treatment differences.

4.3.3.2 Sucrose Preference Test

Treatment with LPS reduced preference for sucrose compared to water ($F = 31.805$, $p < 0.001$), with no effect of genotype or sex. Both sucrose consumption ($F = 210.618$, $p < 0.001$) and water consumption ($F = 12.623$, $p = 0.001$) were reduced by LPS injections. However, only the consumption of sucrose showed a significant effect of genotype ($F = 5.540$, $p = 0.024$) and a trend toward a treatment by genotype interaction ($F = 3.753$, $p = 0.06$). Cre+ animals consumed more sucrose than Cre- controls, although this did not affect the overall preference percentage. There were no significant effects of genotype on water consumption, and no main effects or interactions with sex on any measure. Additionally, there was no genotype effect on body weight ($F = 0.001$, $p = 0.973$), although there was an expected sex difference ($F = 47.293$, $p < 0.001$).

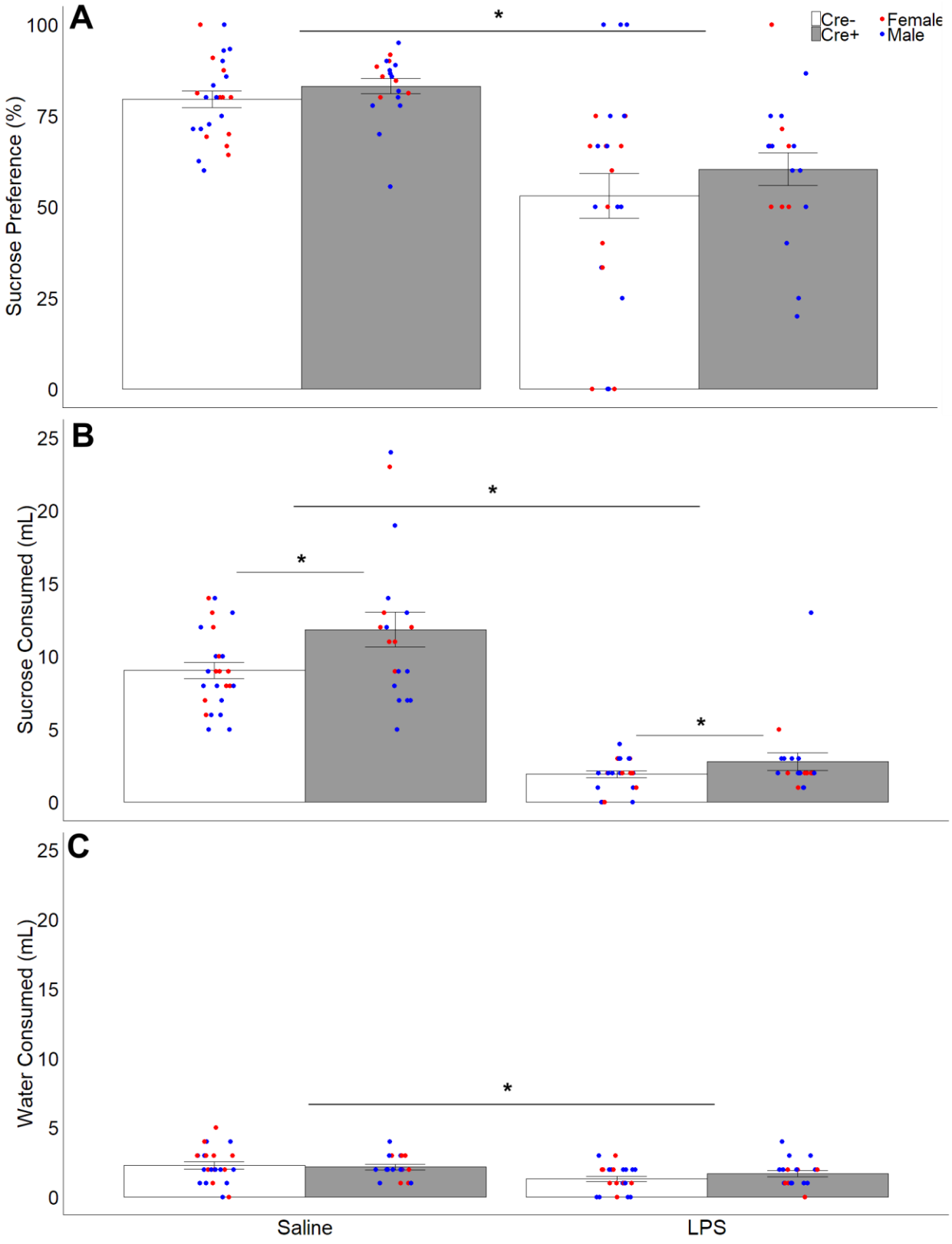
A
B
C
Cre-
Cre+
Female
Male
Sucrose Preference (%)
Sucrose Consumed (mL)
Water Consumed (mL)
Saline
LPS
*

Figure 4-6.

Fluid Consumption and Sucrose Preference. Mean ± SEM of (A) sucrose preference percentage, (B) sucrose solution consumption, and (C) water consumption during the sucrose preference test for Cre- (white bars) and Cre+ (gray bars) subjects after LPS and saline treatment. Points represent individual data from male (blue points) and female (red points) subjects; asterisks represent significant ($p < 0.05$) treatment differences in all three measures, and significant genotype differences in sucrose consumption.

4.3.3.3 Social Preference tests

In the social preference phase of testing, subjects showed a preference for investigating the animal stimulus over the object stimulus ($F = 49.549$, $p < 0.001$), with a significant interaction between treatment and stimulus identity ($F = 6.656$, $p = 0.014$). Subjects investigated the animal stimulus over the object in both saline ($t = 6.183$, $p < 0.001$) and LPS ($t = 6.826$, $p < 0.001$) conditions. In the social novelty phase of testing, subjects investigated the novel stimulus animal more than the familiar animal ($F = 12,744$, $p = 0.001$), with a significant effect of LPS treatment ($F = 8.055$, $p = 0.007$), and an interaction between treatment and stimulus ($F = 5.179$, $p = 0.028$). Subjects spent more time investigating the novel stimulus animal than the original stimulus animal in both saline ($t = -2.472$, $p = 0.018$) and LPS ($t = 3.308$, $p = 0.002$) conditions. LPS treatment did significantly decrease the number of chamber entries, a measure of general activity, across tests ($F = 153.823$, $p < 0.001$).

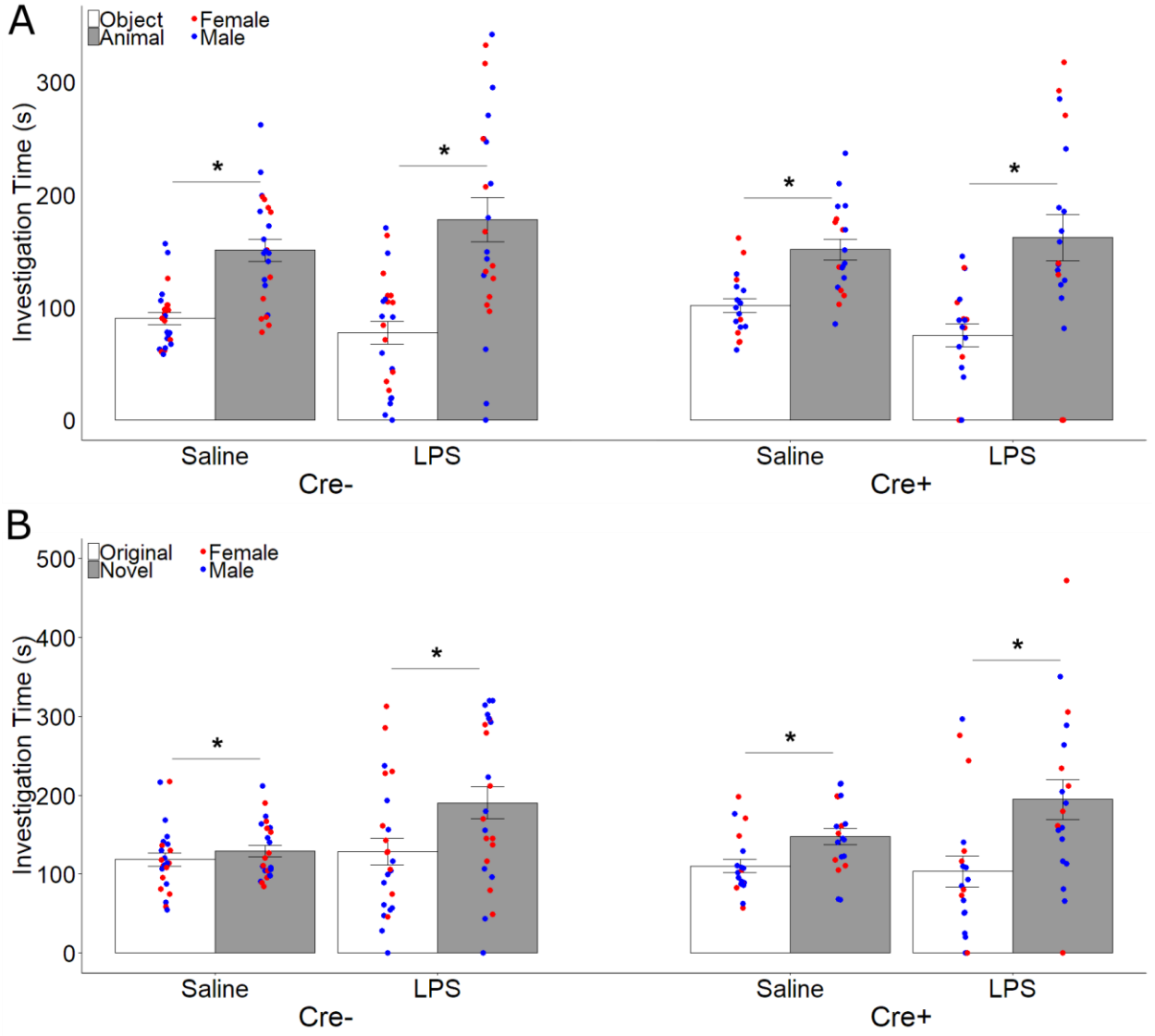

Figure 4-7.

Close investigation in social preference tests. (A) Mean ± SEM of time spent investigating the object stimulus (white bars) and animal stimulus (gray bars) in the social preference test for both Cre- and Cre+ subjects after LPS and saline treatment. (B) Mean ± SEM of time spent investigating the original animal stimulus (white bars) and novel animal stimulus (gray bars) in the social preference test for both Cre- and Cre+ subjects after LPS and saline treatment. Points represent individual data from male (blue points) and female (red points) subjects; asterisks represent significant ($p < 0.05$) differences in stimulus investigation.

4.4 Discussion

Substantial reduction in the number of SCN AVP cells caused an increase in anxiety-like behavior and sucrose consumption in both sexes, and increased urination in male mice only. Reductions of AVP cells in SCN did not have measurable effects on social behaviors, social communication, odor discrimination or intensity of sickness behaviors. These results highlight a specific role for this cell population in modulating anxiety and perhaps in regulation of water balance.

AVP cells within the SCN were significantly reduced by our manipulation, but not entirely eliminated. The remaining AVP cells may be sufficient to maintain the function of this cell population and so we cannot eliminate possible contributions of SCN AVP to social behavior or sickness regulation. SCN AVP cells are primarily GABAergic[180–182]. Therefore, the effects we observed after SCN AVP cell reduction may be due to loss of inhibition or other neuropeptides co-released from these cells rather than the loss of AVP signaling; more specific targeting of AVP would be required to confirm its function within the SCN.

AVP within the SCN serves to coordinate its cellular clocks[77,78], and so behavioral effects of SCN AVP cell reduction may be due to altered circadian rhythms. Studies comparing anxiety-like behavior in the elevated plus maze (EPM) during active (dark) and inactive (light) phases have found effects of cycle phase on measures of general activity, such as arm entries but not the time spent in open or closed arms[183,184]. Indeed, ablation of SCN AVP cells did not alter overall activity, suggesting that the observed effects on anxiety-like behavior were likely not due to a lesion-induced shift in phase. Moreover, circadian shifts in SCN AVP cellular rhythms only alter behavioral activity rhythms in free running conditions[77,171], rather than in the consistent 12:12

light cycle conditions under which our subjects were housed. This consistent light acts as a strong zeitgeber, which would have prevented substantial shifts in activity patterns.

Reduction of SCN AVP cells caused a significant increase in anxiety-like behaviors (decreased time in open arm, reduced exploratory head dips) in the EPM, but not when later tested in the open-field test (OFT) or the elevated zero-maze (EZM). However, both of these tests were conducted after the EPM, and so subjects were exposed to considerably more handling and testing prior to OFT/EZM testing. As handling, injections, and repeated testing in anxiogenic environments can all reduce subsequent measures of anxiety[185–187], it is possible that this habituation effect reduced our ability to detect changes in anxiety-like behavior in these later tests.

The increase in anxiety-like behavior after SCN AVP ablation may be driven by its projections to the dorsomedial hypothalamus (DMH) and/or subnuclei of the paraventricular nucleus (PVN)[57,170]. These projections, particularly those targeting inhibitory interneurons surrounding the PVN and those in DMH, could directly or indirectly alter PVN and HPA axis activity[170]. For example, changes in SCN AVP can both increase or decrease AVP content within the PVN, depending on PVN cell type; reducing AVP in SCN increased AVP expression in magnocellular neurons but decreased it in parvocellular neurons[188]. Consequently, reducing SCN AVP may increase anxiety through activation of the HPA axis, or from reductions in neural projections from parvocellular AVP cells. Notably, Cre-dependent ablations of PVN AVP cells caused similar increases in anxiety in the EPM as observed in the present study[83,103], suggesting that SCN AVP projections may work via AVP cells in PVN to regulate anxiety.

Reduction of SCN AVP cells in both males and female mice increased their consumption of sucrose in the sucrose preference test but did not change their sucrose preference or water

consumption. Increased sucrose consumption may indicate an increase in caloric drive, as SCN AVP inhibits food intake[80]. However, there was no weight gain after SCN AVP ablation, which would be expected if food intake was increased overall. This suggests that reducing SCN AVP increases the hedonic value of sucrose and, therefore, increases the motivation to consume sucrose[189]. Both water intake and sucrose consumption follow a circadian pattern[190], with higher drinking during the active phase (dark phase, ZT 12-24 in mice) than in inactive (light) phase. As SCN AVP expression is high during the day and low during the night phase[166,191,192], lower AVP during the active phase may contribute to increased intake of sucrose and would be consistent with our results. However, SCN AVP release is responsible for an increase in drinking at the end of the active phase; prevention of this AVP signal eliminates this anticipatory drinking and results in dehydration after rest[81,172]. While loss of SCN AVP decreases fluid consumption during this specific period, our measurement of sucrose intake aggregated consumption over a 24-hour period and observed increased fluid consumption. It is, therefore, possible that loss of anticipatory drinking causes slight dehydration followed by a compensatory increase in overall fluid intake. Alternatively, our results may reflect differences in neural regulation of motivation to consume sucrose or other palatable substances independent from anticipatory thirst.

While sucrose intake was increased in both sexes, increased urination was only seen in males after SCN AVP ablation; these males increased production of urine marks in an empty, clean environment compared to control animals without a significant increase in urine marking to male or female stimuli. A sex difference in urination is unsurpsing, given that males mark more than female in previous studies using this paradigm[103,131] and that sex effects urination[193,194]. However, there is typically minimal urination in a no-stimulus/clean environment[103,131]; suggesting that increased urination observed is not indicative of alterations in

social communication. It may be that a disruption in fluid balance, or disruptions in peripheral AVP release may be responsible for this increased urination; AVP rhythm disruptions contribute to conditions such as nocturnal enuresis in humans[195,196]. Increased urination after SCN AVP ablation does not indicate diabetes insipidus, as seen in examples of whole-body AVP loss [197,198], although it does suggest some role for SCN AVP in regulating fluid homeostasis.

SCN AVP has been studied for its roles in regulating circadian biology, both in maintaining cellular rhythmicity in the SCN and in maintaining circadian rhythms in output regions. Our results highlight an additional role for SCN AVP in regulating anxiety behaviors, potentially through regulation of PVN AVP. Additionally, these results indicate that SCN AVP contributes to sucrose consumption; however, it is not clear whether these effects are due to changes in reward processing or alterations in circadian drinking behavior.

5 CONCLUSIONS

Targeted ablation of three distinct AVP cell populations provides insight into the roles of these cells in regulating anxiety and social behaviors, modulating motivational changes in sickness, and contributing to hedonic drive. PVN AVP cells regulate anxiety behavior and regulate social preference and sucrose preference during sickness. BNST AVP cells regulate male social behavior, as multiple lines of evidence suggest; additionally, BNST AVP cell regulate sucrose consumption in both sexes, suggesting a role for these cells in non-social motivation. SCN AVP cells regulate anxiety-like behaviors and sucrose consumption in both sexes, and contribute to male urination. AVP is well known for its regulation anxiety and social behaviors, and these data further support this role of AVP; these data also suggest a role for AVP in non-social motivation by regulating sucrose consumption.

In all of the direct manipulations of AVP cells, entire cells were deleted, including co-neurotransmitters, such as glutamate[199], GABA[180,182,199], galanin[104,157,158,200], and other co-factors expressed in AVP cells. Our results may be due to the loss of these co-factors and changes in excitatory or inhibitory tone in addition to AVP; further work, targeting AVP specifically would be needed to confirm AVP as necessary for these changes. These manipulations also could have triggered compensatory mechanisms, both from other cells in the targeted nuclei, and other AVP populations with shared projection regions, resulting in reduced efficacy of cell deletion. For example, peripheral release from an AVP source such as the SON may have maintained some functions of AVP and obscured effects of cell ablations. Targeting multiple AVP populations may result in stronger behavior effects; for example, ablating both PVN and SCN AVP cells may

cause more robust increases in anxiety behaviors than seen in the data from individual targeting of these regions.

LPS reliably induced sickness behavior, but the robust behavioral changes may have introduced ceiling effects, obscuring potential roles for AVP in sickness behaviors. If loss of AVP enhances sickness behavior, this may have gone undetected in instances where LPS greatly reduced behaviors such as locomotion. Preliminary data (unpublished) suggest that AVP mRNA expression may be altered in the PVN and SCN by chronic, low-grade inflammation; this may indicate a role for AVP outside of responding to immediate, acute illness, but needs further study.

5.1 Neuroanatomical Subdivisions of AVP Functions

5.1.1 Paraventricular Nucleus AVP Cells

The results from PVN cell ablation support a role for PVN AVP in tempering sickness after LPS challenge. In these experiments, PVN AVP removal enhanced the effects of sickness, primarily on anhedonia, but also on social preference, predominantly in males. PVN AVP is produced broadly in two populations of cells, releasing AVP peripherally through the pituitary or centrally through PVN afferents to other neuronal structures[63,112]. Unlike the PVN of the rat, the mouse PVN does not have clear morphological or anatomical divisions of these cell types, and both projections may contribute to the behavior seen[112]. AVP, in conjunction with corticotropin releasing factor (CRF) upregulate glucocorticoids[42,93], which inhibit pro-inflammatory cytokine production and suppress immune responding[56]. Loss of AVP from this system may suppress the typical increase of glucocorticoids after inflammatory challenge, and thus exacerbate the sickness behaviors seen here[201,202]. Further studies of physiological factors, such as serum levels of glucocorticoids and cytokines, and markers of increased neuroinflammation, would show the

role of PVN AVP in regulating inflammation. While general increased inflammation may be responsible for the enhanced effects of sickness behaviors seen after PVN AVP ablation, they may also be caused by changes in central projections. While the BNST is the main source of AVP fibers to the septal nuclei, a critical social behavior node[122], the PVN does contribute to AVP release in the lateral septum (LS)[57]. This projection may be a route for altering social behaviors, specifically in stressful contexts such as sickness.

PVN AVP reductions in sucrose preference support a role for these cells in depression and hedonic processing. Increases in PVN AVP is associated with depression in humans, contributing to HPA axis dysfunction[42,94,203]; our results suggest that PVN actions during inflammation may oppose inflammation-induced anhedonia. Anhedonia and depressive like behavior can be induced through multiple models, such as inflammation, chronic stress, social defeat stress, and early-life manipulations[204]; PVN AVP effects on anhedonia may be dependent on the contexts of depression-inducing stressor. PVN AVP may also effect sucrose preference through actions on reward circuitry; PVN AVP projects to the nucleus accumbens (NAc), the reward processing center of the brain[57,117]; and AVP acting in the NAc has been implicated in drug reward, although its function has not been thoroughly defined[118–120]. This may indicate a role for PVN AVP in regulating hedonic reward, and specifically may increase motivation to consume sucrose in opposition to other inflammatory mechanisms causing anhedonia. Targeting of AVP functions in these projection sites may confirm the role of PVN AVP and what circuitry is responsible for these behaviors

5.1.2 Bed Nucleus of the Stria Terminalis AVP Cells

The effects of BNST cell ablation do not support the hypothesis of these cells' involvement in sickness behavior. For the majority of behaviors measured, LPS clearly caused

sickness behavior, with minimal changes under BNST cell ablation. This may suggest that BNST AVP actions on sickness are limited to physiology, such as fever[38]. Data showing BNST AVP regulation of male fever are from studies in rats; it is also possible that mouse AVP does not play the same roles in inflammatory responding. Studies replicating the physiological effects of AVP in mouse fever would be needed to show a role for these cells in physiological regulation of sickness. Apart from sickness, BNST AVP cell ablation shows a role for these cells in regulating social behavior and sucrose consumption. Normal social behaviors in males are dependent on BNST AVP, and our results are in line with the known functions of hormone-sensitive AVP[46,65,67], in that removal of AVP impairs social recognition by reducing preference to investigate a novel conspecific. Additionally, ablation of AVP BNST cells increased sucrose consumption, while not altering other aspects of fluid or energy balance in this or a prior study[131]. Other subnuclei of the BNST respond to sucrose reward[161] and may provide a path for BNST AVP to regulate sucrose consumption. Unlike most effects of BNST AVP manipulation, this effect was seen in females as well as males, and may be independent of testosterone dependent BNST AVP expression.

5.1.3 Suprachiasmatic Nucleus AVP Cells

SCN AVP ablation did not alter sickness behaviors in this study, and our results do not support the hypothesis that SCN AVP regulates sickness behaviors. While the SCN as a whole is involved in balancing inflammatory responding[69,71], reduction of AVP cells specifically did not enhance sickness behaviors. This indicates that other SCN populations are critical for sickness responding, more AVP cell loss is needed for behavioral effects, or SCN AVP is not involved in behavioral sickness. Studies measuring pro-inflammatory cytokines, in both serum and neural tissue, may reveal roles of AVP cells in physiological immune regulation. As SCN AVP is

important for clock coordination[77], experiments in which animals are allowed to free-run in constant lighting conditions may be necessary to detect some effects of AVP cell deletion, especially those driven by circadian disruptions. Ablation of these cells did alter some behaviors even under a consistent light cycle, highlighting functions of SCN AVP. AVP cell loss increased anxiety-like behavior in the EPM; this may be due to projections to the PVN[57,205] and SCN regulation of PVN AVP[188]. Stressors increase SCN AVP release[206,207], but the behavioral action of this AVP release is not known; this study suggests SCN AVP reduces anxiety-like behaviors. SCN AVP ablation also increased sucrose intake and male urine output. These effects may be related to circadian control of fluid balance via SCN projections to the vascular organ of the lamina terminalis (OVLT)[57,172]; however, in contrast to our results, other studies suggest AVP increases water intake[81]. Clock genes, specifically *Per*, have been implicated in circadian patterns of sucrose intake and expression of clock genes in striatum and cortex[190]; AVP coordination of the clock, or clock gene regulation of SCN AVP rhythms[208], may regulate the relationship between SCN AVP and reward consumption. Further experiments that focus on AVP actions on water and sucrose intake throughout the cycle, instead of at one specific time point or summed over 24 hours, would be necessary to better understand the relationships between AVP, clock genes, thirst, and sucrose reward.

5.2 Behavioral Consequences of AVP Cell Deletion

While each of these experiments highlight distinct roles of AVP in separate nuclei, they also provide evidence for central AVP as a regulator of social behavior, anxiety-like behavior, and hedonic consumption. These may be due to shared projection nuclei and actions on vasopressin receptors, for instances where ablation of different cell populations causes similar effects. In contrast, AVP actions at different nuclei may also contribute to different behavioral

functions or possibly cause opposing behavioral effects. While targeting of AVP receptors or specific AVP terminals would be necessary to determine the behavioral roles of each AVPergic circuit, the results of cell ablation indicate promising targets for control of the behaviors measured in these experiments.

5.2.1 Social Behavior

BNST and PVN ablations both altered male social behavior, but in slightly different manners. BNST AVP is expressed primarily in males, while PVN AVP is equally expressed in both sexes; therefore it is not surprising to see male-biased effects from BNST manipulations, but male biased effects from PVN AVP ablation point to sex-differences in AVP projection sites. AVP actions in the LS are important for typical male social behavior[65,67,156,209]; while the majority of AVP afferents to the LS are from sexually dimorphic populations such as the BNST and medial amygdala (MeA), the PVN does provide sparse projections to the LS[57]. While ablation of both BNST and PVN AVP cells altered social behavior, changes were on different qualities of social preference. BNST cell ablation eliminated the normal preference to investigate a novel animal stimulus, providing further evidence for these cells in social motivation and recognition. In contrast, PVN cell ablation caused elimination of the normal preference to investigate a novel social stimulus over a non-social object, but only during sickness. This suggests that PVN AVP role in social behavior may depend on stressful contexts. Due to the shared projection site of the LS, it is a prime target for manipulating AVP receptors and terminals to better clarify the roles that BNST and PVN each contribute to social behavior.

5.2.2 Anxiety-like Behavior

AVP regulates anxiety-like behaviors via both PVN and SCN AVP. In a prior study with PVN ablated animals, AVP cell ablation caused increased anxiety in the elevated plus maze[103],

similar to the effects seen after SCN AVP ablation. However, for ablations of both nuclei, later tests of anxiety-like behavior, the open field test and elevated zero maze, did not show a baseline increase in anxiety after cell ablation, other than a remaining correlation between extent of PVN cell ablation and reduced time in the center of the OFT. This suggests that while AVP ablation increases anxiety, this effect is reduced with repeated testing and experience. Repeated testing and handling can reduce anxiety[185–187], and is likely driving the loss of effects on later tests of anxiety-like behaviors.

SCN and PVN AVP are not independent, as both populations influence AVP expression in the other. SCN AVP projects to the PVN[57], contributes to the circadian rhythm of the HPA axis[170], and influences PVN AVP expression. In rats, SCN AVP reductions cause increased AVP in magnocellular PVN cells, and decreased AVP in parvocellular PVN cells[188]. Conversely, glucocorticoids, regulated by HPA axis activity, can increase AVP expression in the SCN[210,211]. In these AVP cell ablation studies, both PVN and SCN cell ablations caused similar increases in anxiety-like behavior and suggest a shared system of action. This is at odds with studies that show a positive correlation between PVN AVP and anxiety like behavior, for example, high anxiety trait mice expressing greater AVP than low anxiety trait mice[48,49]. This discrepancy may be due to variations in AVP cell populations, much as how SCN AVP induces opposite effects on different PVN AVP cell populations. While both pituitary- and centrally-projecting PVN AVP cells were deleted[83,103], our results suggest that reductions in centrally-projecting PVN AVP, whether from loss of PVN AVP cells or as a result of reduced SCN AVP inputs, increase anxiety-like behaviors. More specific targeting of the SCN to parvocellular-like PVN cells and their central output would be necessary to test this hypothesis.

5.2.3 Sucrose Consumption

AVP cell ablation in all three experiments caused changes in sucrose consumption, indicating a widespread role for AVP in regulating sucrose consumption. AVP deletion in the PVN reduces sucrose preference during sickness, while AVP deletion from both the BNST and SCN increases sucrose consumption in healthy animals. Sucrose preference has been used as a model of anhedonia in models of depression, chronic stress, and sickness behavior, and reductions in sucrose preference are reversible by antidepressant actions on dopamine systems[6,212–215]. Hedonic aspects of "liking" and "wanting" may be separated in analysis of sucrose preference data, with preference percentage used as a measure of liking, while consumption of sucrose indicates wanting[189,216], which would implicate SCN and BNST AVP in motivation to consume sucrose. V1aR antagonism opposes stress-induced reductions in sucrose preference and consumption[217]; this aligns with increased sucrose consumption after AVP cell deletion. However, PVN AVP deletion further reduced sucrose preference during sickness, showing that AVP actions on sucrose preference are not entirely consistent. AVP actions in the ventral pallidum, a salience-processing nucleus[218], have been implicated in social reward and motivation[219]; this role may also extend to regulating non-social motivation. Research into AVP actions on drug-seeking and reinforcement may better clarify how AVP interacts with dopaminergic reward circuitry.

While there is limited work on AVP's role in stimulant drug taking, Godino and Renard[220] have developed a preliminary hypothesis on AVP actions in reward circuitry that is consistent with our data on sucrose consumption. They suggest that AVP acts directly in the NAc to reinforce drug seeking, while AVP actions outside the NAc opposes dopamine increases to the NAc during drug-taking. Cocaine and amphetamine administration increases AVP expression in

the NAc, and blocking AVP receptors inhibits conditioned cocaine responses[119,120]. In contrast, amphetamines decrease AVP release in the LS[221], increasing LS AVP inhibits amphetamine-induced place preference[222], and intracerebroventricular AVP inhibits cocaine self-administration[223]. While sucrose reward is not similar to abused stimulants, our results align with the hypothesis of AVP actions on reward. Loss of PVN AVP, which projects to the NAc, reduces sucrose preference and consumption, and while this is detected solely during sickness, still suggests a role of PVN AVP in motivating sucrose consumption. Conversely, loss of AVP from the BNST and SCN increased sucrose consumption, indicating that AVP release from these cells may act to reduce motivation to consume sucrose, similarly to how AVP outside the NAc may reduce rewarding salience of drugs of abuse.

5.3 Future Directions

There are many opportunities to further expand on the roles of AVP discussed here in future experiments. These can involve studies into physiological sickness and other models of inflammation such as chronic, low grade inflammation. Specific actions of AVP can be better understood by more precise targeting, such as reducing AVP gene expression or AVP receptors and terminals. Finally, much more work is needed to better understand a role of AVP in regulation of non-social motivation and reward.

Overall, our observations found minimal effects of AVP cell deletion on acute sickness behaviors; however, these cells may have influence over other aspects of inflammatory response. To avoid undue stress during behavioral testing, we did not examine serum factors or temperature throughout the inflammatory response, which could be altered after AVP ablations. Examining AVP-mediated effects on cytokine expression, glucocorticoid release, body temperature, and microglia activation in the 24-hours following LPS administration would

provide a picture of how AVP may influence sickness physiology. Of the cell populations studied, the PVN is most likely to regulate physiological factors, due to its regulation of the HPA axis, and that we observed effects of PVN AVP cell deletion on behavioral sickness. BNST and SCN may both be involved in fever and temperature regulation, and studies examining these, and potentially projections to the POA, may show a role for AVP cells in fever.

Other models of sickness may also reveal different functions of AVP cells. Preliminary data (unpublished), suggests that AVP expression in the PVN and SCN changes during low-grade, chronic inflammation, which has also been used in studies of sickness behavior[224–226] and is more similar to depression-inducing illness in humans than a single, acute bacterial infection. AVP may have a role in regulating longer term changes or recovery from sickness, and less influence on immediate responses. Additionally, other ways of stimulating the immune system, such as activating viral-sensing immune cells or direct administration of pro-inflammatory cytokines, may reveal functions of AVP not seen after acute LPS administration. Less intense sickness behavior may also avoid the potential ceiling effects discussed previously.

Experiments in our lab are targeting AVP gene expression and direct output sites using a variety of modern techniques, and these will provide essential data to understanding the specific functions of AVP circuits. Targeting AVP expression directly, using shRNA, allows replication of cell ablation results while eliminating potential effects of co-released factors (pilot data shows knockdown of BNST AVP using shRNA, Rigney). Optogenetic terminal stimulation[227,228], and application of AVP agonists and antagonists[229] can provide detail on AVP actions in specific projection sites. Manipulations of AVP receptors using similar methodologies will also be useful in examining functional consequences of AVP actions as they relate to anxiety-like behaviors, social behaviors, and sucrose consumption.

Future research is also needed to better understand the role of AVP in hedonic motivation. AVP effects on sucrose preference indicate some role of these cells in motivation, and may indicate a role for AVP in motivation to consume sucrose[189]. Other behavioral paradigms, such as placement preference, and tasks designed to determine willingness to work for a reward, would be needed in concert with AVP manipulations to better understand how AVP regulates non-social motivation. Uses of modern techniques, such as the ones our laboratory is using to study the roles of AVP in social communication, may be applied to tests of non-social motivation. Characterization of how AVP agonism and antagonism influence both liking and wanting of rewards would be necessary as a foundation before in-depth circuit manipulations.

5.4 Conclusion

Ablations of AVP cell populations have provided insights into the functions of these cells in regulating a range of behaviors, from expected alterations in social behavior to providing new evidence for AVP actions in hedonic consumption. PVN AVP specifically is involved in modulating motivation during sickness; while BNST and SCN AVP cell ablation did not alter sickness expression, further research into physiology and other forms of sickness are needed to determine what role, if any, these cell populations play in regulating sickness. BNST, and to a lesser extent PVN, AVP cells regulate male social behavior, while SCN and PVN AVP cells regulate anxiety-like behaviors. AVP cell deletion in the PVN, BNST, and SCN alter sucrose consumption, highlighting an important role for AVP in non-social, hedonic motivation; this is an interesting and understudied function of AVP, and provides a target for better understanding of how this neuropeptide maintains behavioral states.

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