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Hypothalamic vasopressin systems are more sensitive to the long term effects of social defeat in males versus females

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Summary Vasopressin signaling has important effects on the regulation of social behaviors and stress responses, and is considered a promising pathway to target for new therapeutics of stress-induced psychiatric disorders. Although there is evidence for sex differences in the behavioral effects of arginine vasopressin (AVP), few data have directly compared the effects of stress on endogenous AVP signaling in males and females. We used California mice (*Peromyscus californicus*) to study the short and long term effects of social defeat stress on AVP immunoreactive cells in the paraventricular nucleus (PVN) and the posteromedial bed nucleus of the stria terminalis (BNSTmp). Acute exposure to defeat increased AVP/c-fos cells in the PVN and SON of both males and females. In contrast, there were sex differences in the long term effects of defeat. Males but not females exposed to defeat had less *avp* mRNA in the PVN, and in two experiments defeat reduced the number of AVP positive cells in the caudal PVN of males but not females. Interestingly, during relatively benign social encounters with a target mouse, there was a rapid decrease in AVP percent staining (including cell bodies and fibers) in the PVN of males but not females. Defeat reduced AVP percent staining in males, but did not block the socially induced decrease in percent staining. When mice were tested in resident—intruder tests, males exposed to defeat were no less aggressive than control males whereas aggression was abolished in females. However, bouts of aggression were positively correlated with the number

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of AVP neurons in the BNSTmp of control males but not stressed males, suggesting that different mechanisms mediate aggression in control and stressed males. These data show that while acute AVP responses to defeat are similar in males and females, the long term effects of defeat on AVP are stronger in males.

1. Introduction

Women are diagnosed with major depressive disorder at nearly twice the rate of men (Kessler et al., 1994). Interestingly, social stressors appear to be a more salient trigger for depression in women than men. Adolescent girls are more likely than boys to report interpersonal conflict before the onset of depression (Cyranowski et al., 2000) while a recent study demonstrated that in dizygotic opposite-sex twins, poor social support is a stronger risk factor for depression in sisters versus brothers (Kendler and Gardner, 2014). The neuropeptide arginine vasopressin (AVP) may play an important role in mediating sex differences in behavioral responses to stress. Intranasal administration of AVP enhances threat oriented neural responses to neutral faces in men but suppresses these responses in women (Thompson et al., 2006). Physiological responses to stressors (Wotjak et al., 1996) and social behaviors (Lim and Young, 2006) are regulated by AVP. Sex differences in the function of vasopressin V1a receptor (V1aR) have also been reported (Insel and Hulihan, 1995; Winslow et al., 1993). Although there is growing interest in targeting AVP signaling as a novel therapeutic approach (Hodgson et al., 2014; Lee et al., 2013), the impact of psychosocial stress on AVP signaling in males versus females is poorly understood. This is a critical question if AVP-based pharmaceuticals are to be used successfully in men and women.

Despite the strong female sex bias toward depression and anxiety in humans, only a few animal model-based studies employ female subjects (Beery and Zucker, 2011). The social defeat model is a widely used and ethologically valid method for examining depression-like behaviors in rodents (Krishnan et al., 2007). The paradigm relies on aggressive interactions, and the relatively low intra-female aggression levels of domestic mice have limited sex-based comparisons (Ter Horst et al., 2009) but see (Shimamoto et al., 2011; Solomon et al., 2007). The California mouse (*Peromyscus californicus*) is a monogamous species in which both males and females defend territories (Ribble and Salvioni, 1990). Unlike *Mus musculus*, female California mice are aggressive toward females in standard resident—intruder tests (Silva et al., 2010). It is also possible to titrate bouts of social defeat so that males and females are exposed to equivalent intensities of defeat stress (Trainor et al., 2013). One of the most widely reported effects of defeat stress is social aversion (Russo and Nestler, 2013). Three episodes of social defeat induce social aversion in female California mice to a greater extent than male California mice (Greenberg et al., 2014; Trainor et al., 2011), and this effect is independent of gonadal steroids (Trainor et al., 2013). Although male California mice may appear to be ''resilient'' to defeat stress in the social interaction test, behavior is strongly affected in other domains. In a spatial memory task, defeat stress induces deficits in a spatial reversal learning task in male but not female California mice (Laredo et al., 2014). Interestingly, the combination of social approach and inflexible behavior observed in stressed male California mice aligns well with previously described proactive coping strategies (Koolhaas et al., 1999). Proactive coping strategies in rodents and domestic animals have been linked to higher aggression levels (Koolhaas et al., 1999). Vasopressin is an important regulator of male aggression, and defeat stress impacts AVP signaling in males (Litvin et al., 2011; Wood et al., 2010). However, to our knowledge the effects of defeat on AVP systems have not been examined in females.

Here we took a multifaceted approach to examine how defeat stress impacts AVP signaling systems in male and female California mice. First, we used c-fos immunohistochemistry to examine how AVP neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) responded acutely to episodes of defeat stress. Vasopressin cells in these nuclei can release AVP both peripherally and within the brain (Landgraf and Neumann, 2004). Next we focused on the long term effects (2—10 weeks) of defeat stress on AVP signaling in different social contexts. An important property of AVP signaling is that the behavioral effects depend on whether the environment is familiar or unfamiliar (Marler et al., 2003). We examined the effects of defeat on AVP function in an unfamiliar environment. Focal mice were exposed to either an unfamiliar stimulus mouse confined to a wire cage or an empty wire cage. This allowed us to gain more insights into how defeat stress altered the response of AVP in social versus nonsocial contexts. We also examined the posteromedial bed nucleus of the stria terminalis (BNSTmp) and the anterior hypothalamus (AH). Vasopressin neurons in the BNSTmp are highly sensitive to social experience (Bester-Meredith and Marler, 2001; Ho et al., 2010) and AVP acting in the AH facilitates male aggression (Ferris and Potegal, 1988). Next we examined the impact of defeat stress on brain and behavior in a familiar environment using resident—intruder tests. The effects of defeat stress on male (McCann et al., 2014) and female (Solomon et al., 2007) behavior in the resident—intruder test are well described in Syrian hamsters. However, the effects of defeat on AVP signaling in males and females have never been directly compared. In this study we used a triple-label immunochemistry protocol to examine both AVP and oxytocin (OT) neurons in light of recent reports that OT signaling inhibits male aggression (Calcagnoli et al., 2013). We also distinguished between rostral and caudal portions of the PVN because recent work in male *Mus* reported that AVP neurons in the caudal PVN are more reactive to social cues than rostral AVP neurons (Ho et al., 2010). Overall we found that short term responses of AVP-ir neurons to defeat were similar in males and females whereas over the long term, more changes in AVP-ir were observed in males. These results

suggest that over the long term, AVP signaling systems are more sensitive to defeat stress in males than females.

2. Materials and methods

2.1. Animals

California mice (*P. californicus*) were raised in a vivarium at UC Davis. Mice were ear punched for identification and maintained in clear polypropylene cages provided with Carefresh or Sanichip bedding and cotton nestlets. Mice were provided with food (Harlan Teklad 2016) and water ad libitum. Mice were maintained on a 16 h light/8 h dark cycle (lights off 1400 PST). Unlike other species of *Peromyscus*, the California mouse reproductive system is not suppressed under winter-like short day light cycles (Steinman et al., 2012). All mice were 3-month-old adults and housed in cages of 2—3 same sex individuals. All behavioral tests were conducted during lights out (1430—1730) under dim red light (3 lux). All experiments were approved by the UC Davis Institutional Animal Care and Use Committee and animals were maintained according to the recommendations of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2. Experiment 1: short term effects of social defeat on AVP circuits

Males and females were randomly assigned to either social defeat or handling control. Mice assigned to social defeat were placed into a home cage with an aggressive same sex resident for 7 min or until the focal mouse was exposed to 10 offensive attacks (Trainor et al., 2013). Control mice were introduced into an empty cage for 7 min. The first set of mice was euthanized 1 h after a single episode of social defeat or control. The second set of mice was euthanized after a third episode of defeat or control. One hour after defeat or control, mice were anesthetized with isoflurane and rapidly decapitated. Brains were immersion fixed in 5% acrolein in 0.1 M phosphate buffered saline (PBS) overnight at 4 °C and cryoprotected in 20% sucrose in PBS for 48 h at 4 °C. Brain tissue from both sets of mice underwent AVP/c-fos double labeling to examine putative activation of AVP-ir neurons during social defeat (Section 2.5).

2.3. Experiment 2: Long term effects of defeat on brain and behavior in an unfamiliar environment: social interaction test

Three sets of male and female California mice were used to examine the long term effects of defeat stress on peripheral and central AVP function. All mice were exposed to 3 bouts of social defeat or handling control as in experiment 1. In experiment 2a mice were tested in an open field test, a habituation—dishabituation test, and a light dark box test 4—9 weeks post-defeat. These behavioral data have been published as experiment 2 of Trainor et al. (2011). One week after the light—dark box test, these mice were tested in the social interaction either in the presence or absence of a social stimulus. Social interaction testing was conducted using a large Plexiglas box (89 × 63 × 60 cm) as previously described (Greenberg et al., 2014). In the open field phase each mouse spent 3 min in the empty arena. During the acclimation phase a small wire cage was placed against a wall of the arena while the focal mouse was still inside the arena. Interaction with the cage was defined by the amount of time the focal mouse spent within 8 cm of the cage. During the interaction phase, half of the focal mice were exposed to an empty cage (target absent) and half of the mice were exposed to an unfamiliar target mouse (target present). This allowed us to determine the impact of social cues on AVP immunoreactivity. One hour after the interaction phase mice were anesthetized with isoflurane and rapidly decapitated. Brains were removed and fixed as described in experiment 1.

Mice in experiment 2b were used to study peripheral AVP levels. These mice were randomly assigned to control or defeat stress as described in experiment 1. Two weeks later all mice were tested with a target mouse during the interaction phase. Immediately after the interaction phase, mice were anesthetized with isoflurane and rapidly decapitated. Trunk blood was collected on ice in heparinzed tubes and then centrifuged. Plasma was frozen at −40 °C until being used for EIA (Section 2.6). Mice in experiment 2c were used to study *avp* mRNA in punch samples containing the PVN. Mice were assigned to control or defeat stress and then euthanized two weeks later during the light phase. Brains were rapidly removed and 2 mm slices were prepared using a brain matrix as previously described (Campi et al., 2014). A 1 mm punch tool was used to collect a sample of the hypothalamus containing the PVN. Samples were frozen on dry ice and stored at −40 °C for gene expression analysis (Section 2.7). These samples were not collected in the context of a social interaction test, and so should reflect a measurement of baseline *avp* mRNA expression.

2.4. Experiment 3: effects of defeat on brain and behavior in a familiar environment: resident—intruder test

One set of male and female mice were randomly assigned to 3 episodes of social defeat or control conditions as described above. Two weeks later each mouse was single housed for 3 days prior to resident—intruder testing. Single housing for a short period of time is a conventional approach for assessing aggression in rodents (Nelson and Trainor, 2007). At the time of testing the lid of the focal mouse's home cage was lifted and a group-housed virgin same-sex intruder was placed inside. After 10 min the intruder was removed. Residents and intruders did not significantly differ in weight. Behavioral observations were scored by an observer blind treatment and sex. We recorded time spent freezing, boxing, and escaping. We also quantified anogenital sniffing, a behavior that provides access to olfactory cues that in turn activate neural circuits modulating aggressive behaviors in rodents (Nelson and Trainor, 2007). In addition we recorded the frequency the focal mouse bit the intruder. Latency to first bite (attack latency) was also recorded and 600 s was assigned to mice that were not aggressive. A bout of freezing was defined as when a mouse had all four paws placed against a surface and did not move its head or any part

of its body for 2 s. A bout of boxing was defined as when the focal mouse stood on its hind legs and moved the forepaws toward the intruder. A bout of escape was defined as a stereotyped behavior wherein the focal mouse stood along the edge of the home cage and jumped side-to-side. A bout of anogenital sniffing was counted as time where the focal mouse had its nose in immediate proximity to the anogenital region of the intruder and followed the intruder if it moved. One hour after behavior testing each mouse was euthanized and brains fixed for immunostaining as in experiments 1 and 2. These brains were stained using triple-label IHC for OT, AVP and c-fos (Section 2.5).

2.5. Immunohistochemistry

2.5.1. Immunostaining procedure

Brains were cut at 40 μm and sections were then exposed to two 5 min PBS washes. Next, sections were incubated for 10 min in 0.1 M sodium borohydride in PBS and then blocked in 10% normal donkey serum (NDS) in PBS. Sections were incubated overnight at 4 °C in rabbit anti-c-fos (1:2500, PC38 T, EMD Millipore, Billerica, MA) diluted in PBS with 0.5% Triton X (PBS-Tx) with 2% NDS. The following day tissue was washed three times for 5 min each in PBS (3× 5 min-washes) and then incubated for 2 h at room temperature in Alexa Fluor 555 conjugated-donkey anti-rabbit IgG (1:500, A-31572, Invitrogen, Carlsbad, CA) in PBS-Tx with 2% NDS. Tissue was then washed in three 5-min PBS washes. Sections were then incubated in a solution of guinea pig anti-AVP (1:5000, T-5048.0050, Bachem, King of Prussia, PA) and mouse anti-oxytocin monoclonal (clone 4G11 MAB5296, Millipore) in PBS-TX with 2% NDS for 48 h at 4 °C. Tissue then underwent three 5-min washes and was then incubated in a CF488A-conjugated donkey anti-guinea pig secondary (1:500, 20165, Biotium, Hayward, CA) and a biotin-conjugated goat anti-rabbit secondary (1:250, 715-065-150, Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. After three 5 min PBS washes, sections were incubated in Alexa Fluor 350-conjugated streptavidin (1:250, S-11249, Invitrogen) in PBS-Tx. Just before mounting, tissue was washed in PBS for 5 min, then a 10 min PBS wash, followed by a 30 min wash in PBS 0.1% Tx and finally 5 min in PBS. Stained sections were mounted onto Superfrost plus slides (Fisher, Pittsburgh, PA, USA) and coverslipped using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). We tested antibody specificity by preadsorption with the OT and AVP antibodies with either OT or AVP recombinant peptide (Supplementary Fig. 1). Preadsorption with AVP peptide abolished AVP-immunostaining but not OT while preadsorption with OT peptide abolished OT immunostaining but not AVP.

2.5.2. Immunohistochemical analysis

Sections were stained every 120 μm beginning from approximately Bregma level 0.14 (anterior BNST) through (mouse Bregma level −1.46). A monochromatic Axiocam MRm camera (Carl Zeiss Meditec) connected to a Zeiss Axioimager (Carl Zeiss Meditec, Inc., Dublin, CA) was used to image slides. Using ImageJ software (NIH, Bethesda, MD) a box was placed in each region to be quantified. The box size was 0.35 × 0.43 mm for PVN, 0.26 × 0.28 mm for BNSTmp, 0.36 × 0.36 mm for AH and SON. Cell counts were converted to cells/mm^2 due to the different box sizes used for each nucleus. For the AVP/c-fos double label, the number of AVP-ir neurons, c-fos-ir cells and c-fos/AVP colocalizations were manually counted by an observer blind to treatment group. For experiment 3, the number of OT neurons and OT/c-fos colocalizations were counted as well. In the PVN, magnocellular and parvocellular neurons were not distinguished because the distribution of these cells is overlaping in California mice (de Jong et al., 2009). For experiment 2a, the same size box was used to measure the percentage of AVP staining in the combined rostral and caudal PVN. Briefly, immunopositive pixels within the imageJ-derived box were converted to black by manually establishing the threshold level that obtained the best signal-to-noise ratio for immunoreactive cell bodies and fibers. Percentage staining translates to the percentage of the region of interest occupied by black pixels.

2.6. Enzyme-Linked Immunoassay (EIA)

Plasma AVP concentrations were measured with an enzyme immunoassay kit following the manufacturer's instructions (Enzo Life Science, Farmingdale, NY, USA). Samples were not extracted prior to running the assay. This assay has a sensitivity of 3.4 pg/mL. Slopes of regression lines generated from AVP-spiked and unspiked serial dilutions of California mouse plasma did not differ from each other nor did they differ from that of the standards. The intra-assay coefficient of variation was 17.4%. Concentrations were calculated using a 4-parameter logistic curve fit as recommended by the manufacturer. Data were log transformed for statistical analysis.

2.7. Quantitative real-time PCR analysis

RNA was extracted from punch samples using RNAqueous kits (Life Technologies) and reverse transcribed using iScript (BioRad). All sequences were amplified using SYBR green chemistry on an Applied Biosystems ViiA7 instrument. Specific forward (AGTGTCGCGAGGGTTTTC) and reverse (GGGCTTGGCAGAATCCAC) primers for *avp* mRNA were designed based on California mouse sequence (Genbank accession JX977025.1). In preliminary analyses, expression of GAPDH and β-actin were found to be higher in females than males, similar to a previous report (Perrot-Sinal et al., 2001). Thus we designed *Peromyscus*-specific forward (TCTAGTGGGAGGTCCTGTGG) and reverse (TGCGTTAGACCAGCAGAAGG) primers for *β-2 microglobulin* (*b2m*, Genbank Acession XM006995122.1). There were no differences in cycle thresholds between groups for *b2m*. For each sample *avp* mRNA was normalized to *b2m* expression.

2.8. Statistical analyses

Data was assessed for normality using Q—Q plots and homogeneity of variance using Levene's test. Cell count data were square root transformed for analysis and percent colocalization data were square root arc-sin transformed to achieve normality and homogeneity of variance. For experiment 2a, we used three-way ANOVA on immunohistochemistry data (sex, stress, and exposure to a target mouse). For experiment 2b we used two-way ANOVA to examine effects of

sex, social defeat stress and the interaction. In experiment 2b gene expression data were normalized to male controls and then analyzed with Mann–Whitney *U* tests due to heterogeneous variance between groups. In experiment 3 a χ^2-test was used to analyze the probability of engaging in aggression. Attack latency data were analyzed with Mann–Whitney *U* tests. Two-way ANOVA was used to analyze behaviors in the resident–intruder test and immunohistochemistry data using sex and stress as variables. For ANOVA analyses planned comparisons were used to test the effect of defeat stress in males and females. For a few variables, an interaction between stress and sex was not detected, but planned comparisons reported different results in males and females. In these cases we also report Cohen's *d* as a measure of effect size.

3. Results

3.1. Experiment 1: short term effects of social defeat on AVP circuits

Defeat stress increased AVP/c-fos colocalizations in the hypothalamus of both males and females. Specifically, defeat increased AVP/c-fos colocalizations 2-fold in the PVN after a single episode of defeat (Fig. 1A, $F_{1,27} = 14.12$, $P = 0.001$) or after a third episode of defeat (Fig. 1A, $F_{1,28} = 11.71$, $P < 0.01$). Interestingly, there was a significant sex × stress interaction on AVP/c-fos colocalizations in the SON after one episode of defeat (Fig. 1B, $F_{1,25} = 16.4$, $P < 0.001$) with only males showing increased AVP/c-fos colocalizations (Fig. 1B). However, after three episodes of defeat both males and females had increased AVP/c-fos colocalizations compared to controls (Fig. 1B, $F_{1,25} = 8.37$, $P < 0.01$). In contrast, other regions containing AVP-ir cell bodies were not responsive to social defeat stress after a third episode of defeat stress (Supplementary Table 1). These included the rostral and caudal BNSTmp and the anterior hypothalamus (Data not shown, all *P*'s > 0.05). There was a main effect of defeat stress on the number of c-fos cells in the central nucleus of the amygdala (Supplementary Table 1, CeA, $F_{1,27} = 4.3$, $P < 0.05$).

3.2. Experiment 2: Long term effects of social defeat on AVP brain circuits and blood levels

Mice in experiment 2a were tested 10 weeks after defeat, yet we observed a consistent pattern of male biased

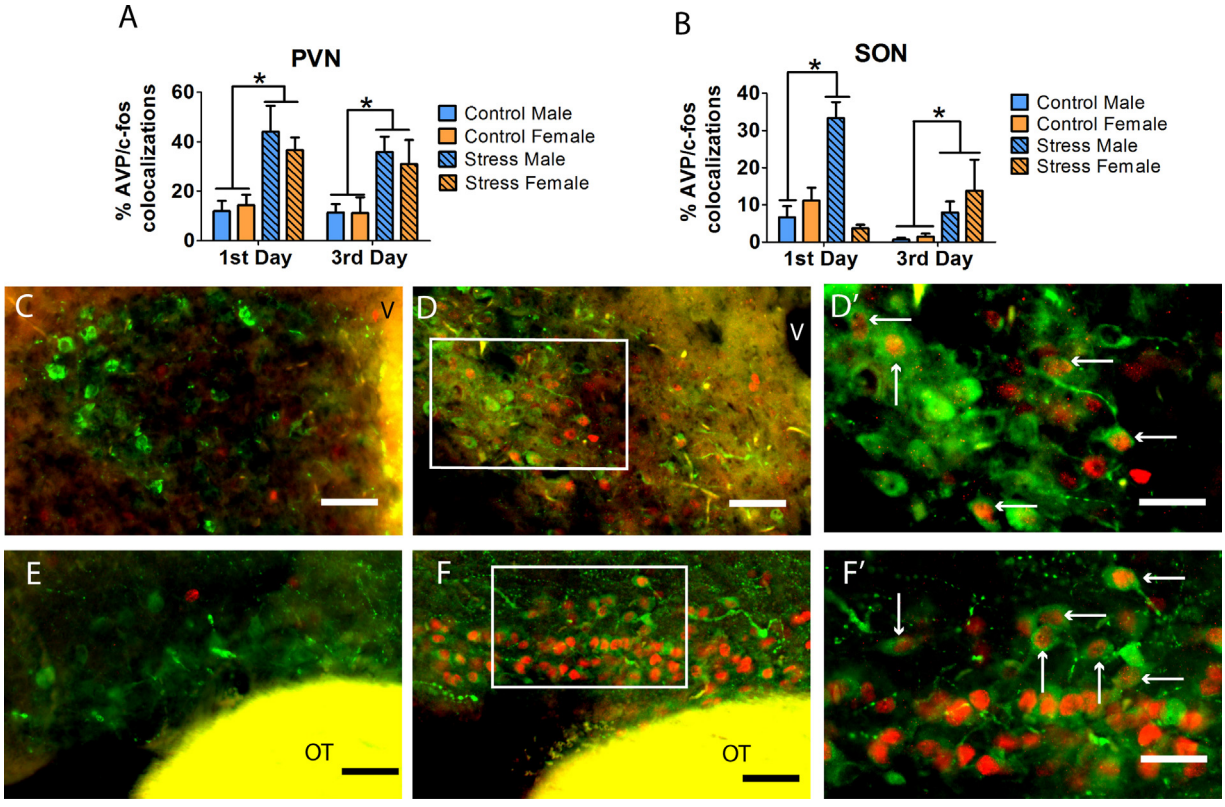

Figure 1 Acute effects of defeat stress on AVP/c-fos percent colocalizations in the paraventricular nucleus (A, PVN; $n = 7–9$ per group) and supraoptic nucleus (B, SON; $n = 6–9$ per group) after one or three episodes of defeat in experiment 1. In both regions defeat stress increased the percentage of AVP cells expressing c-fos. Data are shown as mean ± SE. *$P < 0.01$ versus control. Representative images of c-fos (red) and AVP (green) expression in the PVN of females exposed to a third episode of control (C) or defeat (D) conditions. Representative images of the SON of in a control (E) and stressed (F) male at the same time point; scale bars = 50 μm. (D' and F') higher magnification images of area enclosed in the white box in the corresponding panel; scale bars = 30 μm. White arrows designate c-fos-immunoreactive nuclei within AVP-immunoreactive cell bodies. V, third ventricle. OT, optic tract. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

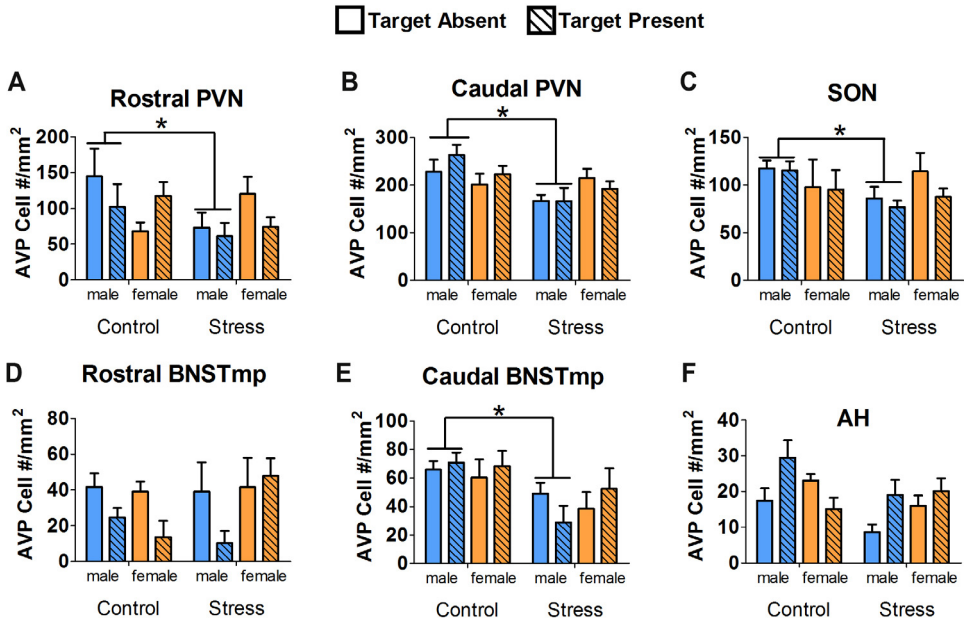

Figure 2 Vasopressin-immunoreactive cells in PVN, posteromedial bed nucleus of the stria terminalis (BNSTmp), supraoptic nucleus (SON), and anterior hypothalamus (AH) ten weeks after social defeat (experiment 2a). During the interaction phase of the social interaction test, mice were tested with either an empty cage (target absent, open bars) or an unfamiliar same sex mouse in the cage (target present, hashed bars). For males, defeat stress reduced the number of AVP-immunoreactive cells in the rostral (A) and caudal (B) portions of the PVN, the supraoptic nucleus (C), and the caudal part of the BNSTmp (E). There were no differences in AVP cell number in the (D) rostral BNSTmp or the AH (F) ($n = 4–5$ per group). Data are shown as mean ± SE. *$P < 0.05$, effect of stress.

reductions in the number of AVP-ir cell bodies across several nuclei. For the number of AVP-ir cell bodies, the sex × stress interaction was significant in the caudal PVN (Fig. 2B $F_{1,31} = 5.98$, $P < 0.05$) and SON (Fig. 2C, $F_{1,30} = 4.23$, $P < 0.05$). Planned comparisons indicated that defeat reduced the number of AVP-ir cell bodies in male but not female mice (Fig. 2B and C). There was also a nonsignificant trend for a sex × stress interaction in the rostral PVN (Fig. 2A, $F_{1,31} = 3.46$, $P = 0.07$). In the caudal BNSTmp there was a main effect of stress on the number of AVP neurons (Fig. 2E, $F_{1,31} = 5.6$, $P < 0.05$). Planned comparisons showed a significant decrease in AVP-ir cell body numbers in males (Fig. 2E, $P < 0.05$, effect size, $d = 0.52$) but not females ($P = 0.48$, $d = 0.11$). In the rostral BNSTmp (Fig. 2D) and AH (Fig. 2F), there were no differences in AVP neuron counts (all P's > 0.05). All ANOVA analyses of cell counts included the presence or absence of a social stimulus as a factor. Interestingly, this factor had no impact on AVP cell counts and did not interact with stress or sex. In general, behavior in the social interaction test was consistent with our previous studies (Trainor et al., 2013), although the mean time spent interacting with a novel mouse was higher than expected for stressed females (Supplementary Table 2). While the presence of a social stimulus had no impact on AVP cell counts, measures of percent staining (which includes both cell bodies and immunoreactive fibers) were strongly impacted by the presence of a social stimulus.

Interestingly there was a significant sex × social stimulus interaction for AVP-percent staining across the entire PVN (Fig. 3A, $F_{1,31} = 7.7$, $P = 0.01$). Exposure to a novel target mouse induced an almost 2-fold decrease in percent staining in males (Fig. 3D and E) but not females. There was also a trend for a sex × stress interaction ($F_{1,31} = 3.52$, $P = 0.07$). Stress reduced AVP-percent staining in males ($F_{1,16} = 6.9$, $P < 0.05$) but not females ($F_{1,15} = 0.1$, ns). The reduced AVP immunoreactivity in stressed males was consistent with results from experiment 2b which examined plasma AVP immediately after a social interaction test. There was a trend for a sex × stress interaction (Fig. 3B, $F_{1,25} = 4.2$, $P = 0.05$) and planned comparisons confirmed that control males had higher circulating levels of AVP than males exposed to defeat while there was no difference in females (Fig. 3B). In experiment 2c, defeat stress significantly reduced *avp* mRNA in PVN samples in males (Fig. 3C, Mann–Whitney, $P < 0.05$). Although it appeared that mean *avp* mRNA was elevated in control females, this group was not significantly different from control males or stressed females due to high variability.

Interestingly there was a sex × stress interaction for the total number of c-fos cells in the AH ($F_{1,30} = 8.7$, $P < 0.01$), a brain region important for the control of male aggression. Stressed males had fewer c-fos positive cells than control males whereas there was no significant effect of stress in females (Supplementary Table 1). There was also a main effect of stress on total number of c-fos in the caudal BNSTmp (Supplementary Table 1, $F_{1,30} = 5.73$, $P < 0.05$). No differences in c-fos expression were observed in any subregions of the amygdala that were examined (Supplementary Table 1).

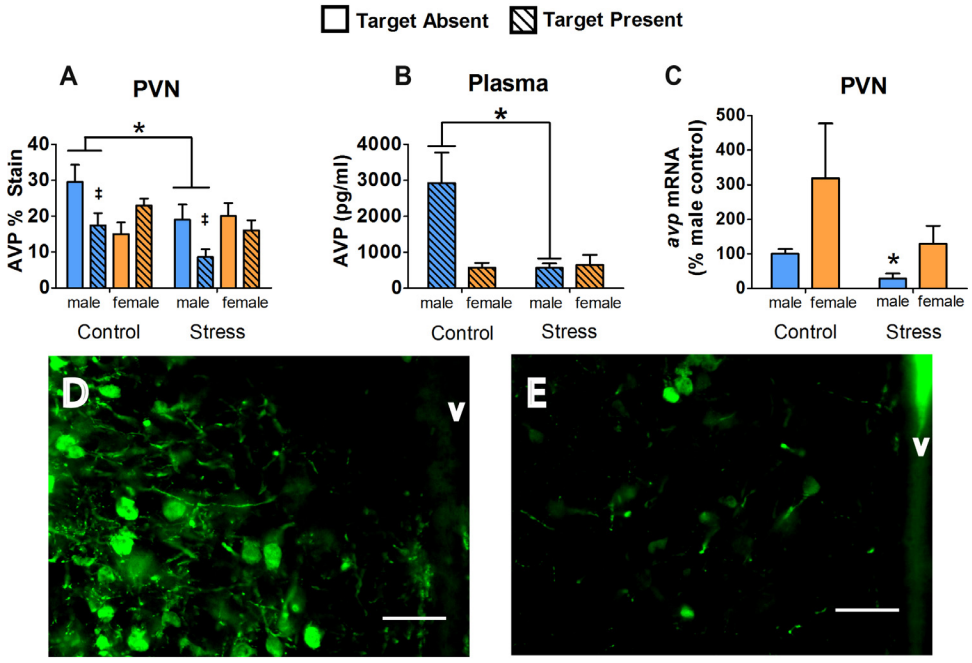

Figure 3 Effects of defeat stress on three cohorts of mice examining AVP percent staining (A, experiment 2a, $n = 5$ per group), plasma AVP (B, experiment 2b, $n = 6–9$ per group), or *avp* mRNA (C, experiment 2, $n = 5–12$ per group). Ten weeks following social defeat, AVP percent staining was reduced in males but not females (A). Males that were tested with a target present (hashed bars) had significantly less AVP percent staining than males tested with without a target mouse (open bars). Two weeks following social defeat, and immediately following a social interaction test with a target mouse present, control males had significantly higher plasma AVP levels compared to stressed males (B). Two weeks after defeat, stressed males also had reduced *avp* mRNA in punch samples containing the PVN (C). Representative photomicrographs demonstrating AVP-immunostaining in the PVN of control male mice in the absence (D) or presence (E) of a target mouse. Data are shown as mean ± SE. * $P < 0.05$ effect of defeat stress, ‡ $P < 0.05$ effect of target mouse. V, third ventricle. Scale bars = 50 μm.

3.3. Experiment 3: Long term effects of defeat on brain and behavior in the resident–intruder test

Defeat stress affected the behavior of both males and females in the resident–intruder test, but there were also important sex differences. The most striking sex difference was in offensive attacks. Defeat stress eliminated aggression in females (Fig. 4A, χ^2-test, $P = 0.042$) whereas most stressed males still attacked intruders. Defeat increased attack latency in females (Fig. 4B, Mann–Whitney, $P < 0.05$) but not males. There was also a main effect of stress on chasing ($F_{1,37} = 4.2$, $P < 0.05$). Planned comparisons showed that the effect of defeat was significant in females (Fig. 4C, effect size, $d = 0.33$) but not in males ($d = 0.09$). However, it is an oversimplification to describe the behavior of stressed male California mice as ''resilient'' in a resident–intruder test. In rodents, the detection of pheromones by anogenital sniffing is an initiating factor in territorial aggression (Nakamura et al., 2007). Defeat stress reduced anogenital sniffing ($F_{1,37} = 5.34$, $P = 0.026$), and planned comparisons showed that this effect was significant in males (Fig. 4F, $P < 0.05$, effect size $d = 0.27$), but not did not reach significance in females ($P = 0.18$, $d = 0.20$). Thus although aggression was present in stressed male California mice, it was not associated with normal levels of social investigation. There were no differences in boxing behavior or time spent in defensive postures (data not shown). Fear related behaviors were more similar in stressed males and females. Defeat stress increased freezing behavior ($F_{1,37} = 10.28$, $P < 0.01$), and planned comparisons showed that this effect was significant in both males and females (Fig. 4D). In contrast time spent attempting to escape the home cage showed a significant main effect of stress ($F_{1,37} = 4.7$, $P < 0.05$) and a trend for sex × stress interaction ($F_{1,37} = 2.90$, $P = 0.09$). Planned comparisons showed defeat stress significantly increased escape behavior in males (Fig. 4E, $P < 0.05$, $d = 0.38$) but not females ($P = 0.74$, $d = 0.05$).

As in experiment 2a, defeat stress interacted with sex on AVP-ir cell body number in the caudal PVN ($F_{1,29} = 5.9$, $P < 0.05$). Planned comparisons showed that stress reduced the number of AVP positive neurons in males but not females (Fig. 5A). In contrast there was a main effect of defeat stress on the percentage of AVP/c-fos cells in the caudal PVN ($F_{1,26} = 5.9$, $P < 0.05$). Planned comparisons showed that stress increased AVP/c-fos colocalizations in both males and females (Fig. 5B). The effects of stress on colocalizations appeared to be impacted by changes in AVP cell number, as there was no significant effect of stress on the total number of AVP/c-fos colocalizations (Supplementary Table 3). Although stress had no effect on AVP cell number or c-fos colocalizations in the rostral PVN, there was a significant main effect of stress on the percentage of c-fos/OT colocalizations ($F_{1,33} = 4.8$, $P < 0.05$). Planned comparisons revealed that the main effect was driven by an increase in OT/c-fos colocalizations in females (Fig. 5C,

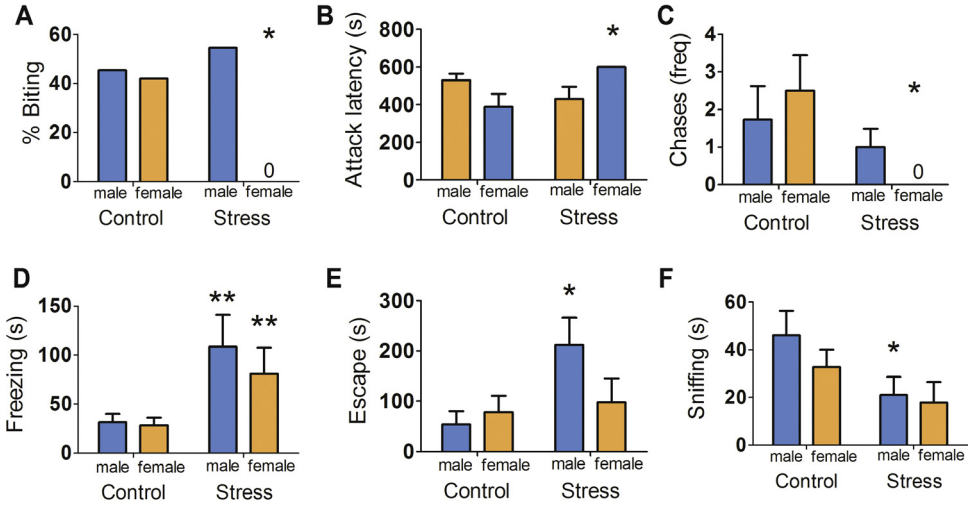

Figure 4 Effects of social defeat on behavior during resident—intruder tests conducted two weeks later ($n = 8$—11 per group). Social defeat decreased biting (A), increased attack latency (B), and decreased chases (C) in females but not males. In contrast, defeat decreased sniffing (F) and increased escape behavior (E) in males. Defeat increased freezing behavior in both males and females (D). Data are shown as mean ± SE. *$P < 0.05$, Mann—Whitney U test for 2B, planned comparisons for 2C—F, **$P = 0.01$, versus control.

$P < 0.05$, $d = 0.45$) but not males ($P = 0.45$, $d = 0.12$). Here, analyses of the total number of OT/c-fos colocalizations produced similar results as percent colocalizations (Supplementary Table 3). In experiment 3, there were no differences in AVP or OT cell number in the rostral PVN, the SON or either portion of the BNSTmp (Supplementary Table 3, all P's > 0.05). An interesting correlation emerged between the number of AVP-ir cell bodies in the caudal BNSTmp and number of bites during the resident—intruder test. There was a significant positive correlation between AVP-ir neuron number and number of bites for control males (Fig. 5E, $r = 0.99$, $P < 0.001$) while in control females this correlation was negative (Fig. 5G, $r = -0.82$, $P < 0.05$). Defeat abolished these relationships (Fig. 5F and H). There were no correlations between bites and the number of AVP-ir neurons in the PVN (all P's > 0.2). Finally, defeat stress increased the number of c-fos labeled cells in the CEA ($F_{1,12} = 7.9$, $P < 0.05$) and this effect was observed in both males and

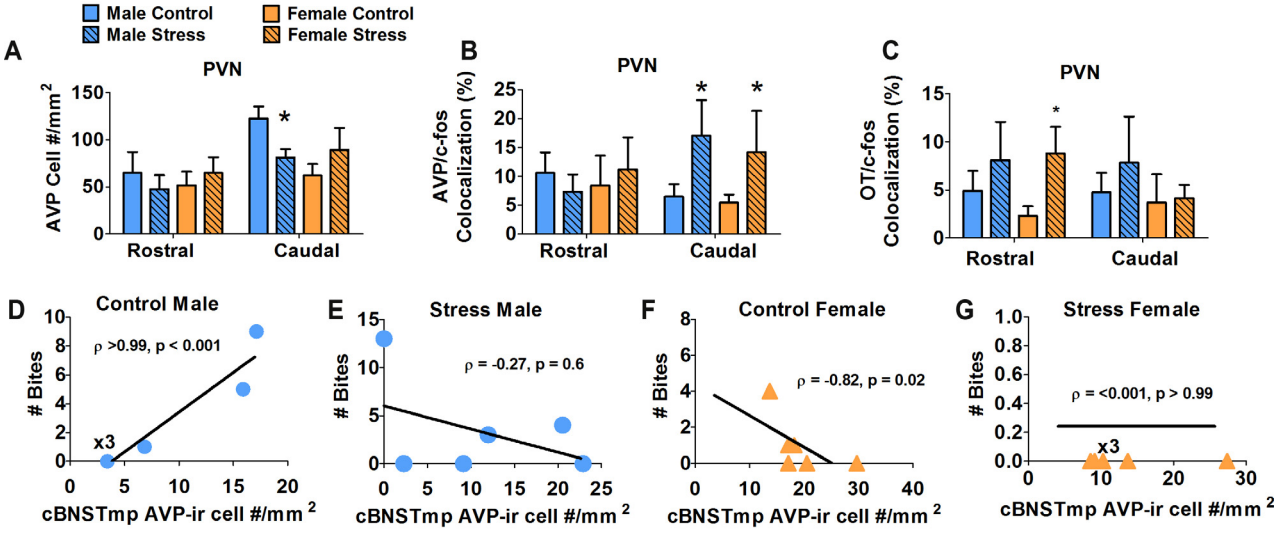

Figure 5 Immunohistochemistry analyses of mice tested in resident—intruder tests (experiment 3, $n = 7$—11 per group). Males exposed to defeat had fewer AVP-cells in the caudal PVN (A). Defeat stress increased the percentage of AVP cells expressing c-fos in caudal PVN of both males and females (B). In the rostral PVN stress increased the percentage of OT cells expressing c-fos in females but not males (C). Data are shown as mean ± SE. *$P < 0.05$ versus control. Spearman rank correlations showed that in control males (D) but not stressed males (E) the number of AVP positive cells was positively correlated with bites directed toward the intruder. In control females (F) but not stressed females (G) there was a negative correlation between the number of AVP positive cells and bites.

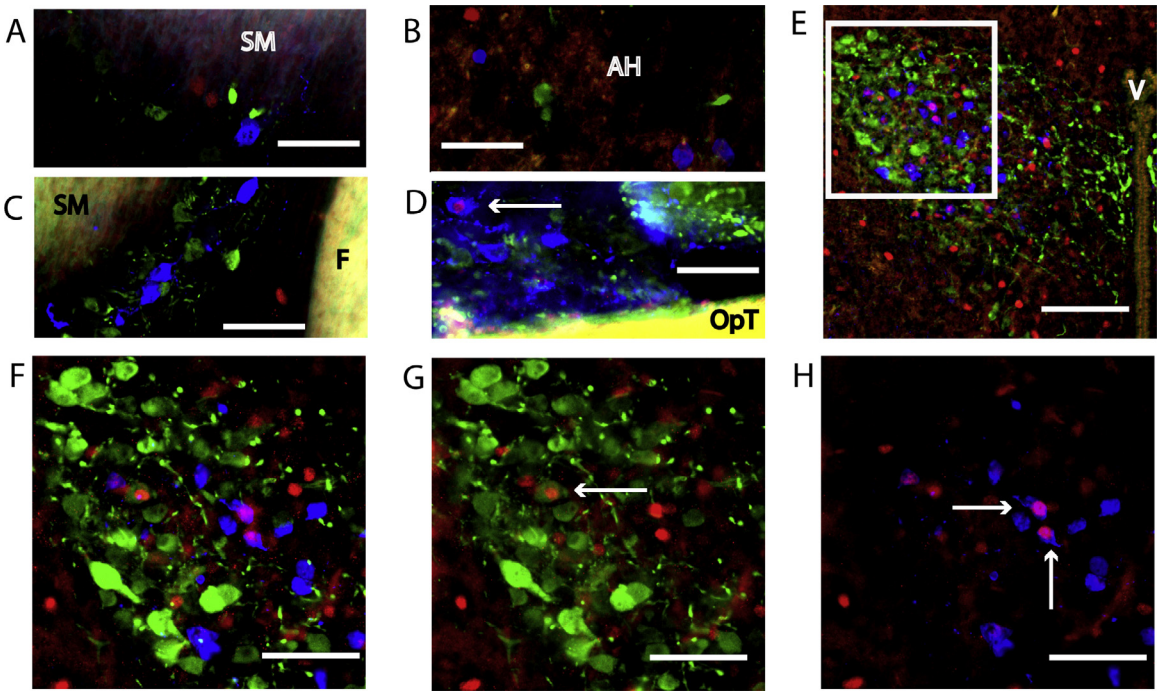

Figure 6 Representative photomicrographs depicting triple-label immunostaining for AVP (green), OT (blue) and c-fos (red) in the rostral (A) and caudal (C) posteromedial bed nucleus of the stria terminalis. Staining in the anterior hypothalamus (B), supraoptic nucleus (D), and the paraventricular nucleus (E). Higher magnification images of the boxed area from panel (E) with staining for all three antigens (F), AVP and c-fos (G), and OT and c-fos (H). AH, anterior hypothalamus; F, fornix; OT, optic tract; SM, stria meduallaris of the thalamus; V, third ventricle. (A—D & F—H) Scale bars = 30 μm; (E) scale bar = 100 μm. White arrows show c-fos/neuropeptide colocalization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

females (Supplementary Table 1). See Fig. 6. for examples of staining.

4. Discussion

Vasopressin signaling systems are important modulators of social behavior and are sensitive to psychosocial stress (Neumann and Landgraf, 2012). Our results show that the short term effects of defeat on AVP neurons are similar in both males and females. However, the long term effects of defeat on AVP neurons appear to be stronger in males than females. Males exposed to defeat had fewer AVP neurons in the PVN, most likely mediated by decreased expression of *avp* mRNA. Interestingly, we observed evidence that exposure to social cues in an unfamiliar environment may lead to rapid release of AVP in males but not females. Although AVP is generally considered to have more important effects on male aggressive behavior in familiar environments, our results suggest that AVP may have important effects on behavior in unfamiliar environments as well. Finally, defeat stress reduced aggressive behavior in females but not males. Intriguingly the positive relationship between AVP and aggression in control males was absent in stressed males, suggesting that a different circuit may be used to control aggression in stressed males. These data suggest that future studies will need to consider experience, sex and context when evaluating AVP-based pharmaceuticals.

4.1. Effects of social defeat on AVP-ir neurons in PVN of male California mice

In two experiments conducted at different time points and under different behavioral contexts, we found that social defeat stress reduced the number of AVP-ir cell bodies in the caudal PVN of males. In experiment 2a, brains were collected ten weeks after defeat stress and these animals had been behaviorally phenotyped (Trainor et al., 2011). Behavioral testing itself can impact brain and behavior (Crawley, 2007), and could potentially have impacted AVP immunoreactivity. However the mice in experiment 3 were tested in a single resident—intruder test two weeks after the last episode of defeat, and again defeat reduce AVP cell counts in males but not females. This suggests that increased sensitivity of caudal PVN AVP cells to defeat stress is a robust, long lasting phenomenon in male California mice and not an artifact of behavioral testing. The decrease in the number of AVP-ir cells could be due to increased AVP release or decreased AVP synthesis. On the one hand, ten days of chronic mild stress increased *avp* mRNA in the PVN of male California mice (De Jong et al., 2013) and social defeat increased *avp* mRNA in the PVN of male *M. musculus* (Erhardt

et al., 2009; Keeney et al., 2006). However, qPCR analysis revealed that stress induced an almost 3-fold decrease in baseline *avp* mRNA in male California mice two weeks after defeat stress. This observation supports the hypothesis that a long term effect of defeat stress is a reduction in PVN AVP synthesis. A previous study of subordination stress in *M. musculus* reported no difference in *avp* mRNA in the PVN from controls after 10 days, but a significant decrease after 20 days (Reber et al., 2007). This suggests that effects of social stress on *avp* expression in the PVN may be biphasic. Currently it is not clear why *avp* mRNA levels were so variable in control females, but it is possible that variability in gonadal hormones may have been a factor. Still, the qPCR data are consistent with analyses of AVP percent staining, which suggest a reduced capacity for AVP release in stressed males.

While defeat stress had long term effects on the number of AVP cells in the caudal PVN, we also observed that social encounters had important effects on the amount of immunoreactive AVP in the PVN. Unlike cell counts, measurements of percent staining are affected by immunoreactive AVP fibers and cell bodies. Males exposed to a novel target mouse showed an almost two-fold decrease in AVP percent staining in the PVN compared to males exposed to an empty cage. This effect was observed in both control and stressed males, even though stressed males had significantly less AVP-ir staining than control males. Currently, the mechanism leading to rapid decreases in AVP-ir staining is unclear. One possible explanation for these results is that encounters with an unfamiliar individual induce a rapid release of AVP. Our measurements of plasma AVP suggest at least some of the immunoreactive AVP in the PVN may be released via the median eminence in to blood during a social encounter. Levels of AVP in plasma were almost three times higher in control males than either stressed males or females (control or stressed). We did not measure baseline AVP levels, so it is possible that baseline plasma AVP levels are elevated in control males. However previous analyses of plasma AVP in prairie voles (Bales et al., 2011) and rats (Rhodes et al., 2001) in nonsocial contexts reported no sex differences plasma AVP. Furthermore, in rats plasma AVP is not elevated by immobilization or forced swim (Lang et al., 1983), suggesting that elevated levels of plasma AVP in male California mice were not due to exposure to the novel arena used for social interaction testing. This suggests that the presence of an unfamiliar individual was the major factor contributing to elevated AVP plasma levels in control male California mice. Further study of the long term effects of defeat stress on baseline AVP as well as AVP release within the brain will be needed to fully understand the long term effects of social stress on AVP release. Intriguingly, deficits in basal urinary AVP levels have been linked to increased severity of post-traumatic stress disorder in men but not women (Marshall, 2013). Currently, it's unclear whether the absence of a robust downregulation of AVP expression in female California mice is an important factor contributing to sex differences in behavioral responses to defeat. Further investigation of how endogenous AVP signaling systems respond to psychosocial stress in men and women will be important for evaluating the therapeutic potential of new pharmaceuticals targeting neuropeptide receptors.

4.2. Sex differences in resident—intruder tests: proactive versus reactive coping strategies

The most striking difference in the effects of defeat stress in the resident—intruder test was the elimination of aggression in females but not males. Although this result contrasts with the effects of social defeat in Syrian hamsters (Solomon et al., 2007), it is consistent with other results from California mouse studies. In the social interaction test stressed male California mice generally approach unfamiliar social stimuli (Greenberg et al., 2014; Trainor et al., 2013) and in tests of spatial memory stressed males show more deficits (Laredo et al., 2014). Aggression and inflexible behavior have been linked to proactive coping strategies in both rodents and domestic animals (Koolhaas et al., 1999). Compared to females, male California mice have reduced corticosterone responses both to defeat stress (Trainor et al., 2013) and also when engaging in aggression as a resident (Trainor et al., 2010). Reduced glucocorticoid reactivity is also consistent with previously described proactive coping strategies (Koolhaas et al., 1999). Although defeat stress did not reduce aggression in males, other behavioral domains were clearly affected. Like stressed females, stressed males showed increased freezing behavior and a corresponding increase in c-fos expression in the CEA. Most importantly, defeat stress reduced anogenital sniffing behavior in males. In male rodents, pheromones detected via anogenital sniffing are an important cue triggering offensive aggressive behavior (Nelson and Trainor, 2007). Despite reduced access to pheromones from intruders, stressed male California mice engaged in aggression at levels equivalent to control males. These results suggest that different mechanisms facilitate aggression in control and stressed males. This hypothesis was supported by analyses of AVP-immunoreactivity in the BNSTmp.

In California mice, increased AVP-ir has been linked to increased male aggression. In control males, we observed that the number of AVP-ir cells in the BNSTmp was positively correlated with the number of bites, consistent with a previous study (Laredo et al., 2013). Male California mice that are cross fostered by the less aggressive white-footed mouse (*P. leucopus*) are less aggressive and have reduced AVP-ir staining in the BNSTmp (Bester-Meredith and Marler, 2001). However, infusion of synthetic AVP in to the principal nucleus of the BNST (which is anterior and dorsal to the BNSTmp) inhibited aggression in Wistar rats (Veenema et al., 2010). Although it is possible that AVP acting in different subregions of the BNST have different effects on aggression, a more likely explanation for these contrasting observations is that AVP neurons in BNSTmp may project to the lateral septum. Infusions of AVP into the lateral septum have been reported to increase male aggression (Veenema et al., 2010). In stressed male California mice, the positive association between AVP neurons in the BNSTmp and aggression was eliminated. Defensive aggression is mediated by different molecular mechanisms than offensive aggression, and is often linked to increased fear responses (Chen et al., 1994). Our results suggest that defeat stress may shift control of aggressive behavior from an AVP-dependent circuit to a circuit less dependent on AVP. Intriguingly, the number of AVP neurons in the BNSTmp was negatively correlated with bites in control females. Relatively little is known about the

neural mechanisms controlling female aggression outside of the context of maternal defense. Studies in rats indicate that AVP acting in the PVN, principal nucleus of the BNST and CeA facilitate maternal aggression (Bosch, 2013). To our knowledge, no study has compared mechanisms controlling female aggression in maternal and non-maternal individuals. While the negative correlation in non-stressed females provides direction for further studies, it must be treated with caution due to the small sample size.

It is possible that single housing during resident–intruder tests may have had an impact on the results. In female prairie voles two months of social isolation increased plasma AVP responses to social conflict (Grippo et al., 2007a) whereas male and female prairie voles exposed to one hour of isolation per day for four weeks also exhibited increased AVP reactivity (Pournajafi-Nazarloo et al., 2013). The period of single housing we used for resident–intruder levels was much shorter (3 days) than periods typically used to study social isolation in rodents. The effects of defeat stress that we observed on AVP cell number in mice tested in resident–intruder tests were similar to results in experiment 2a, in which mice were socially housed. It appears that the three-day period of single housing we used did not have a major impact on our immunostaining results. Based on studies of social isolation, we would expect that a longer period of social isolation before resident–intruder testing would increase the activity of hypothalamic AVP systems, most likely in the PVN.

4.3. OT activity during conditioned defeat

While effects of social defeat stress may be larger in the AVP system of males, the OT system of females appears to show higher sensitivity. Stressed females had more OT/c-fos colocalizations in the rostral PVN following resident–intruder testing whereas this effect was not significant in males. Other forms of stress, such as social isolation, have been reported to increase baseline OT release into blood, primarily in females (Grippo et al., 2007b; Pournajafi-Nazarloo et al., 2013). Interestingly social isolation induced a 3-fold increase in OT/c-fos colocalizations in females and about a 1.5-fold increase in OT/c-fos colocalizations in males following a resident–intruder test (Grippo et al., 2007b). Future studies will need to follow up on whether defeat stress increases peripheral OT release, and whether any effects occur in a sex specific way. Our results suggest that, at least in the context of a resident–intruder test, the OT system may be more sensitive to the effects of defeat stress in females versus males.

4.4. Conclusions

In our previous studies, defeat stress generally did not decrease male social interaction, suggesting that male California mice might be similar to ''unsuceptible'' phenotypes described in *M. musculus* (Krishnan et al., 2007). Here, we observed that defeat stress had important effects on AVP and behavior in male California mice. This suggests that the resident–intruder test could reveal important behavioral changes in ''unsuceptible'' phenotypes identified with the social interaction test in other species. It has been hypothesized that AVP and OT signaling may modulate a balance between proactive and passive coping, with OT primarily mediating proactive coping and AVP primarily mediating passive coping (Neumann and Landgraf, 2012). Our current results suggest that defeat stress may reduce the capacity of the PVN to release AVP in male California mice, which might contribute to the general pattern of proactive coping behaviors seen in stressed males. Psychosocial stress can also induce changes in vasopressin receptors (V1aR, V1bR) or oxytocin receptor expression (OTR) (Peters et al., 2014). These receptors are somewhat promiscuous, as AVP and OT are capable of activating V1aR and OTR (Manning et al., 2012). Thus the adoption of alternative coping strategies may be modulated by a balance of both neuropeptide release and receptor expression.

Contributors

M.Q.S. and B.C.T. performed literature searches, designed the studies, analyzed the data. M.Q.S. and B.C.T. wrote the manuscript. M.Q.S., S.A.L., E.M.L., C.E.M., R.C.H., I.E.D., K.L.C., A.E.F. and J.K.K. performed studies and collected data. All authors contributed to and approved the final manuscript.

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Funding agencies and grants solely supported research costs and provided no additional role in the design, collection, analysis and interpretation of the data, in the writing of the report, and in the decision to submit this article for publication.

Conflict of interest

The authors have no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2014.09.009.

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