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Sex-specific differences in adult cognition and neuroplasticity following repeated combinatory stress and TrkB receptor antagonism in adolescence

Idu Azogu, Isabelle Cossette, Joana Mukunzi, Ogechi Ibeke, Helene Plamondon*

Behavioral Neuroscience Group, School of Psychology, University of Ottawa, 136 Jean-Jacques Lussier, Ottawa, ON, Canada

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ABSTRACT

Evidence supports brain-derived neurotrophic factor (BDNF) and its primary receptor tyrosine-related kinase B (TrkB) as targets in the treatment of mood disorders. This study characterized the impact of a 10-day combinatory stress paradigm (alternating days of restraint stress and forced swim) and administration of the selective TrkB antagonist ANA-12 (0.5 mg/kg, i.p.) during adolescence in male and female Wistar rats on adulthood behavioral and neurochemical responses. The social interaction/preference (SIT/SP), and Y maze conditioned place preference (YMCPP) and passive avoidance tests (YMPAT), initiated on PND 62, served to determine sex-related behavioral responses. Results support reduced sociability in females in the SIT/SP, but no impact of ANA-12 to regulate sociability or social memory. Blockade of TrkB during adolescence facilitated YMCPP-related reward behavior in both sexes, and reduced YMPAT fear conditioning in females. Following behavioral testing, rats were exposed to 5-min acute forced swim and brains collected 2 h post swim to determine effects of adolescent TrkB blockade and stress exposure on neurochemical regulators of stress and plasticity. Findings show elevated glucocorticoid receptor (GR-) and TrkB-immunoreactivity (ir) in the amygdalar central nucleus, and GR-ir in the hypothalamic paraventricular nucleus of females compared to males. In the hippocampal CA1, BDNF-ir was lower in females versus males, and GR-ir was elevated in stress versus non-stress males. Together, we demonstrate that inherent sex-specific differences, which may modulate impact of adolescence stress exposure and TrkB inhibition, differentially affect male and female adulthood behavior and biochemical response profiles, suggesting that these responses are in part conditioned by prior experience.

1. Introduction

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis during adolescence can trigger the onset of anxiety/mood disorders and behavioral impairments in later life in both humans and rodents (Heim et al., 2004; Kessler and Magee, 1993; McCormick et al., 2010), due to a delayed shut-off and less centrally mediated feedback from limbic brain areas (Romeo et al., 2004). In the rat, adolescence (conservatively ranging from postnatal day (PND) 28 to 56 (O'Dell, 2009; Spear, 2000)) is a highly sensitive period of increased brain development. During this period, prolonged or chronic exposure to stress can have lasting programming effects on behavior and stress-responsive brain regions (e.g. prefrontal cortex, amygdala, paraventricular nucleus of the hypothalamus (PVN) and hippocampus) involved in plasticity, cognition and emotional processing. For instance, chronic stress exposure during adolescence in male rats enhanced adult reversal learning and impaired working memory performance (Chaby et al., 2015), increased open field anxiety and reduced social interactions (Green et al., 2013), and triggered poor avoidance learning behaviors (Tsoory and Richter-Levin, 2006). In female rats, exposure to 14 days of chronic variable stress in late adolescence induced a blunted HPA axis reactivity in adulthood when tested with a novel stressor and increased immobility in the forced swim (Wulsin et al., 2016). Adolescence thus appears as a critical time for the development of anxiety-related disorders and lasting

Abbreviations: ANA-12, N2-(2-{[(2-oxoazepan-3-yl) amino]carbonyl}phenyl) benzo[*b*]thiophene-2-carboxamide; ANS, ANA-12 + no stress; AS, ANA-12 + stress; BDNF, Brain-derived neurotrophic factor; CA1, Cornu ammonis area 1 of the hippocampus; CA3, Cornu ammonis area 3 of the hippocampus; CeA, Central nucleus of the Amygdala; GR, Glucocorticoid receptor; EC, Empty cup (in the social interaction test); PND, Postnatal day; PVN, Paraventricular nucleus of the hypothalamus; S1, Stranger 1 (in the social interaction test); S2, Stranger 2 (in the social novelty preference test); SIT/SP, Social interaction/social novelty preference test; TrkB, Tyrosine-related kinase B receptor; VNS, Vehicle + no stress; VS, Vehicle + stress; YMCPP, Y maze conditioned place preference; YMPAT, Y maze passive avoidance test

* Corresponding author at: University of Ottawa, School of Psychology, Behavioral Neuroscience Group, 136 Jean-Jacques Lussier, Vanier Building, Rm. 2082, Ottawa, ON K1N 6N5, Canada.

E-mail address: hplamond@uottawa.ca (H. Plamondon).

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changes in neuroendocrine function.

Earlier studies have predominantly used male subjects in spite of sex-specific differences in the prevalence of various disorders, including depression and anxiety (Dalla et al., 2005, 2011; Gobinath et al., 2014; Gutiérrez-Lobos et al., 2002; Kessler, 2003). However, evidence suggests that women subjectively experience more stress than men and display increased stress vulnerability (Kudielka and Kirschbaum, 2005). In rats, sex differences also exist in observed increases in social interaction behavior in adult males versus females (Carrier and Kabbaj, 2012; Johnston and File, 1991; Stack et al., 2010), lower shock-induced locomotor activity in males during a passive avoidance task (Heinsbroek et al., 1988), but no differences in acquisition of cocaine or food conditioned place preference (Hilderbrand and Lasek, 2014; Rubinow et al., 2009; O'Dell, 2009). There appears the need to include sex as a determinant variable in understanding predisposition to emotional disorders and the mechanisms that are involved in sex-specific modulation of stress responsivity.

Notably, one of the hallmarks of adolescence in rodents and humans is a period of pubertal transitions, which has an influence on the crosstalk between gonadal and adrenal axes (Bale and Epperson, 2015). For female Wistar rats, onset of puberty occurs around PND 34–36 (vaginal opening; estrogen surge), whereas for males, it occurs later at PND 40–42 (balanopreputial separation; increased androgen concentration) (Castellano et al., 2011; Fernández-Fernández et al., 2005; McCormick and Mathews, 2010). These sex hormones play a role in behavior and physiology, estrogen and androgen respectively exerting excitatory and inhibitory effects on HPA axis activation (Handa and Weiser, 2014; McCormick et al., 2002, McCormick et al., 2017). Thus, in part due to the actions of circulating estrogen, higher levels of glucocorticoids (GCs, corticosterone in rodents) are typically reported in female rats after acute and repeated stress exposure compared to males (Azogu et al., 2018; Figueiredo et al., 2007), suggestive of a more reactive HPA axis system (Burgess and Handa, 1992). Importantly, sex-specificity in basal and stress-induced states have been related to effects of ovarian hormones on brain-derived neurotrophic factor (BDNF) regulation (Marco et al., 2013). BDNF has similar mechanisms of action, targets and antidepressant effects as estrogen (Scharfman and MacLusky, 2006). In response to estrogen, BDNF expression and release is increased, while progesterone counteracts estrogen-induced BDNF increase (Bimonte-Nelson et al., 2004; Franklin and Perrot-Sinal, 2006; Sohrabji and Lewis, 2006). Furthermore, elevated levels of GCs are reported to impact the expression of BDNF and activation of its primary receptor tyrosine-related kinase B (TrkB), influencing downstream signaling cascades involved in neuronal growth, maturation and maintenance (Huang and Reichardt, 2001; Smith et al., 1995a; Smith et al., 1995b). For instance, observed increases in TrkB expression within the striatum of socially defeated adolescent male mice relative to defeated adult mice (Montagud-Romero et al., 2017) illustrate age-related changes in TrkB signaling, which may be enhanced by stress exposure.

Of interest would be the assessment of sex-related changes in TrkB signaling. Recently, we showed that blockade of TrkB receptors, using the small molecule TrkB antagonist ANA-12, is capable of reducing CORT elevations post stress in adolescent male and female rats (Azogu et al., 2018). Furthermore, using western blot, we showed down-regulated protein expression of TrkB.FL and TrkB.T1 in the adult hippocampus of stressed males treated with ANA-12 during adolescence, effects that were opposite to ANA-12 stimulatory effects on expression of both isoforms in stressed females. These findings support sex differences in specific cognitive and emotional processing signatures (Azogu et al., 2018). TrkB inhibition under basal and stress conditions therefore appears as a means of discerning how sex and stress might influence the developmental role of TrkB signaling, and may be particularly important for understanding the role of neurotrophic factors in psychiatric disorders. To date, very few studies have assessed the effect of blockade of BDNF/TrkB activation upon adolescence stress exposure on sex-specific later life behavioral and neurochemical changes, and none have addressed the role of female estrous cycles on such responses.

Considering that the BDNF-estrogen interplay likely contributes to incidence of BDNF-related cognitive and emotional impairments across sex and development, we treated adolescent male and female rats with ANA-12 prior to exposure to a combinatory stress paradigm, alternating days of 30 min restraint and 15 min forced swim. We demonstrate that adolescence stress and TrkB inhibition differentially impact adult male and female behavior (in sociability, reward experience and fear conditioning). Furthermore, our data indicate that inherent sex differences, following an acute stressor, led to divergent biochemical response profiles of BDNF, GR and TrkB in the stress and plasticity-related regions of the PVN, central nucleus of the amygdala and hippocampal CA1, but not CA3, in adulthood, which surprisingly were unaffected by prior ANA-12 treatment or stress exposure from adolescence. Together, our findings support the hypothesis that sex-specific modulation of behaviors and neurochemistry after prior life experience may contribute to the differential prevalence of males and females in developing anxiety and mood disorders.

2. Methodology

2.1. Animals and housing

Male and female Wistar rats, aged postnatal day (PND) 21 (between 50 and 60 g upon arrival), were obtained from Charles River Laboratories (Rochefort, Quebec, Canada). As all animals were exposed to the same mode of transport, potential impact of stress during transport at a young age was similar across animals and represented a baseline for all. Rats were housed in same sex pairs in Plexiglas cages with litter flooring as nesting material and maintained on a 12 h L: 12 h D photoperiod (lights on at 0700) with room temperature (RT) of 21–23 °C with 60% relative humidity. Pair housing may improve social contact (Dean, 1999), as it adds to the social aspect of the natural species-specific behavior, and it has been shown to modulate the rats' responses to chronic stress in a sex-specific manner (Westenbroek et al., 2005). A plastic black tube was placed in each cage as a 'safe' area. Standard Purina rat chow and water were available ad libitum. After five days of acclimation to the animal vivarium at the Vanier Building of the University of Ottawa, animals were exposed to the 10-day combinatory repeated stress paradigm. All procedures were in accordance with the Canadian Council of Animal Care and approved by the University of Ottawa Animal Care Committee. Experimentation complied with the ARRIVE guidelines and was in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Drug/vehicle injection

Rats were randomly divided into 4 experimental groups: ANA-12 + stress (AS), ANA-12 + no stress (ANS), Vehicle + stress (VS), and Vehicle + no stress (VNS). The number of animals sampled for each behavioral test and immunohistochemistry are indicated in the respective sections. On PND 26, experimentally naïve Wistar rats received intraperitoneal (i.p.) injections of the selective TrkB receptor antagonist *N*-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl] benzo[*b*]thiophene-2-carboxamide (ANA-12, SML0209, Sigma-Aldrich; 0.5 mg/kg, i.p. at a volume of 10 ml/kg (Cazorla et al., 2011; Ma et al., 2016; Ren et al., 2015; Zhang et al., 2015a; Zhang et al., 2015b)) or vehicle (0.9% saline containing 20% (2-hydroxypropyl)-β-cyclodextrin; H107, Sigma-Aldrich) 30 min prior to the start of the combinatory stress (or control - no stress) sessions on days 1, 4, 7 and 10 of the stress paradigm. Dose selection was based on pharmacokinetics revealing active concentrations of ANA-12 in mice brain as early as 30 min (~400 nM), and up to 6 h after i.p. injection (~10 nM) (Cazorla et al., 2011). Dosing intervals were previously assessed in a pilot study in our

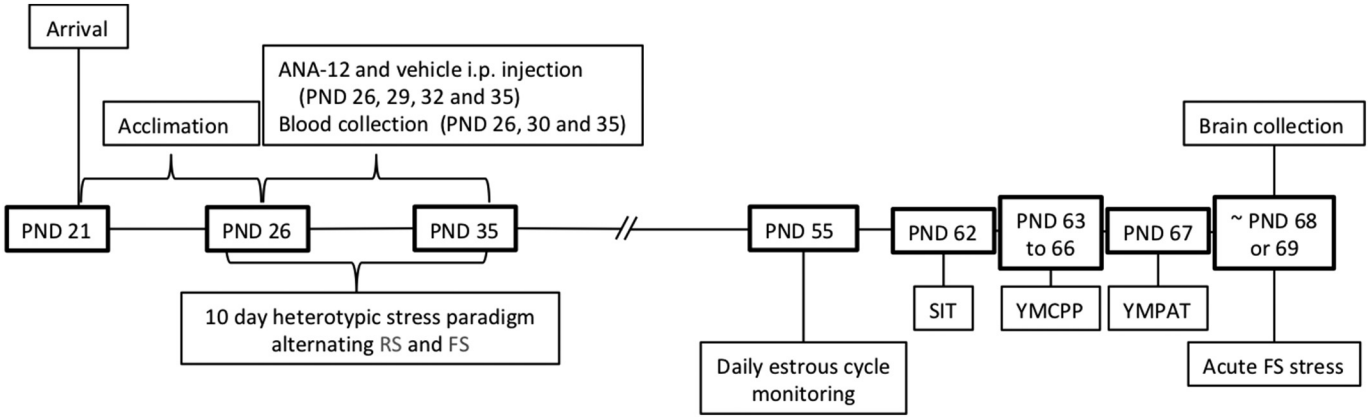


Fig. 1. Experimental timeline.
Upon arrival at the vivarium at PND 21, animals were allowed 5 days of acclimation and then were subjected to the 10-day combinatory stress paradigm. Only stressed animals were exposed to alternating days of 30 min RS and 15 min FS. All groups received 0.5 mg/kg, i.p. of either ANA-12 or vehicle solution at PND 26, 29, 32 and 35, corresponding to days 1, 4, 7 and 10 of the paradigm. Daily estrous cycle monitoring for females commenced at PND 55, and continued to the end of the study. Animals underwent behavioral testing for the SIT/SP (PND 62), YMCPP (PND 63 to 66) and YMPAT (PND 67). Following an acute FS on PND 68 or 69, animals were euthanized and brains collected. PND: postnatal day; RS: restraint stress; FS: forced swim; i.p.: intraperitoneal; SIT/SP: social interaction/ social preference test; YMCPP: Y maze conditioned place preference test; YMPAT: Y maze passive avoidance test.

lab. Timeline is represented in Fig. 1.

2.3. Combinatory stress paradigm

The alternating schedule in the 10-day combinatory stress paradigm is adapted from a 28-day combinatory stress paradigm consisting of restraint and forced swim stressors, which is shown to reduce habituation to repeated exposure to a single stressor, increase BDNF mRNA and protein in the PVN, and enhance activation of the HPA axis (Zamora-Gonzalez et al., 2013). This has been replicated in our lab (Azogu et al., 2018; Azogu and Plamondon, 2017a, 2017b). These stressors are also reported to attenuate BDNF mRNA levels in the hippocampus (see reviews (Duman and Monteggia, 2006; Smith, 1996)), and increase BDNF expression in the male rat hypothalamus (Rage et al., 2002). Stress groups (AS and VS rats) were subjected to alternating 30 min restraint (odd numbered days) and 15 min forced swim (even numbered days) stressors between 0930 and 1100 from PND 26 to 35. The restraint stressor consisted of a clear plastic container with an air hole to allow for easy breathing, and the forced swim stressor used a transparent cylindrical container (20.32 cm in diameter and 43.18 cm in height) filled with tap water (23 ± 2 °C) at a height of approximately 33 cm. Black cardboard pieces placed between each cylinder and at the extreme edges were used to block the rats' view of the other animals being tested at the same time. The cylinders were cleaned, and the water was replenished in between animals. Overhead lighting in the restraint stress room was 800–900 lx, and 650–700 lx in the forced swim stress room.

2.4. Vaginal cytology

Assessment of estrous cycle phases (lasting 4–5 days) was initiated on PND 55 up to the end of the study, between 0900 and 0930. Males were handled daily for equivalent periods, during weight assessments. Following vaginal lavage, fresh cells were immediately examined under a light microscope with 10× objective and cytology determined based on abundance and appearance. Cell description (Marcondes et al., 2002) were as follows: proestrus - round nucleated epithelial cells; estrus – anucleated, cornified epithelial cells; metestrus – similar proportion of epithelial cells, cornified cells and leukocytes; and diestrus – predominance of little round leukocytes. Female rats appeared to cycle normally within 4–5 days and became readily accustomed to the cell collection, suggesting that stimulation resulting from collecting cells from the vaginal canal did not induce pseudo-pregnancies (Goldman et al., 2007). For the social interaction/social preference test (SIT/SP), the number of animals in each phase of the estrous cycle (proestrus, estrus, metestrus, diestrus) were as follows: AS (4, 3, 3, 2), ANS (2, 4, 4, 2), VS (3, 2, 6, 1) and VNS (1, 3, 6, 2); for the Y maze conditioned place preference (YMCPP) habituation: AS (3, 5, 2, 3), ANS (3, 3, 3, 3), VS (4, 2, 3, 3) and VNS (8, 2, 1, 2); for the YMCPP test: AS (3, 3, 6, 1), ANS (4, 3, 4, 2), VS (3, 3, 5, 1) and VNS (2, 5, 2, 4); and for the Y maze passive avoidance test (YMPAT): AS (4, 2, 2, 5), ANS (3, 3, 4, 2), VS (4, 3, 1, 4) and VNS (6, 3, 3, 1). Due to low sample numbers of estrous stage in some groups, cycle stages were pooled to run analysis on behavioral measures as per Gray (1977) (i.e. proestrus + estrus = estrus; and metestrus + diestrus = non-estrus). Sample size used for the immunohistochemistry did not allow factoring of estrous cycle in the analysis.

2.5. Behavioral testing

Male and female Wistar rats underwent behavioral testing in the social interaction test/social preference (SIT/SP; PND 62), Y maze conditioned place preference (YMCPP; PND 63–66) and Y maze passive avoidance test (YMPAT; PND 67). All behavioral testing occurred between 0930 and 1600. Overhead lighting in the behavioral testing room (SIT/SP and Y maze) was 800–900 lx. The behavioral tests were thoroughly cleaned in between animals with 70% ethanol.

2.5.1. Social interaction/social novelty preference test (SIT/SP)

The SIT/SP (n = 12 to 14 per group) is a three-chamber sociability paradigm used to assess social affiliation/motivation and social memory/preference associated with interaction with a novel conspecific (Crawley, 2004; Moy et al., 2004). Two clear Plexiglas walls with removable middle partitions divided a modified open field arena (LWH: 75 cm × 75 cm × 30 cm; standing on a table 90 cm above the floor) into three chambers. An overhead camera recorded each behavioral session. White curtains separated the experimenter's recording area. As adapted from Kaidanovich-Beilin et al. (2011), the experimental rat habituated 5 min to its surroundings following initial placement in the middle chamber, and explored the two outer chambers containing identical wire containment cups. The rat was then enclosed in the middle chamber while a stranger rat (S1) was placed under one of the empty wire cups. During the first 10 min session (social interaction test, SIT), the rat was presented with a choice between initiating direct

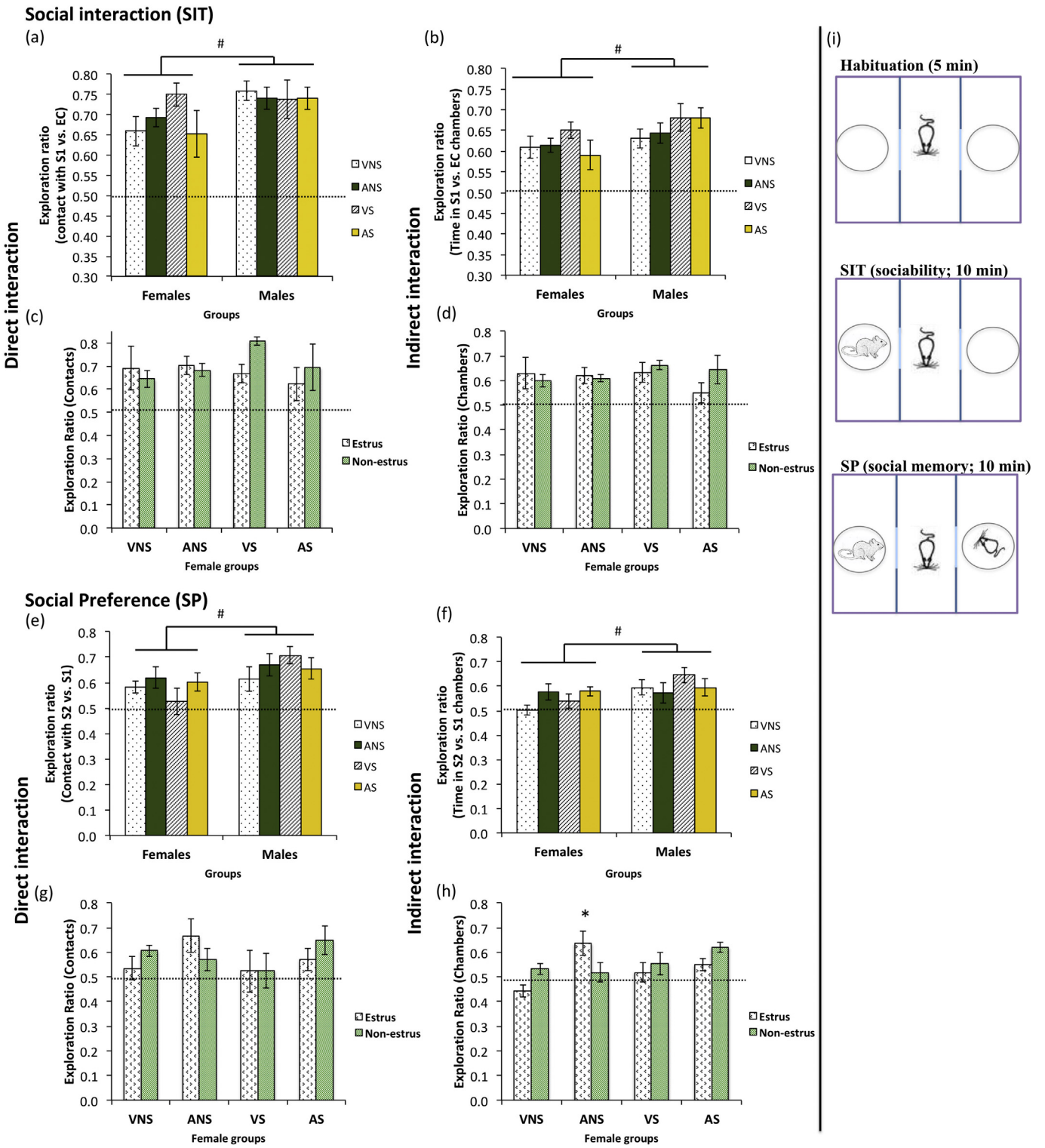


Fig. 2. Sociability and predilection for novel experiences in the social interaction/social preference test (SIT/SP) is attenuated in females.
(2a–h) Graphs represent exploration ratios for the direct (SIT: contact with S1 vs. EC; SP: contact with S2 vs. S1) and indirect (SIT: Time in S1 vs. EC chambers; SP: Time in S2 vs. S1 chambers) interactions. Overall, reduced social behavior was noted in females vs. males (#, $p < 0.05$). (2 h) Exploration ratio for time spent in chambers was significantly higher for ANS females in estrus compared to their veh-treated counterparts (*, $p < 0.05$). Dashed lines represent a chance score of 0.5 for exploration ratio. Exploration ratio above 0.5 is indicative of preference for sociability and/or social memory, while a ratio below 0.5 indicates aversion to social interaction and/or social preference. (2i) Schematic diagram of the SIT/SP protocol: Experimental rat is allowed to habituate to the apparatus for 5 mins, prior to a 10 min session to interact with S1 and EC, followed by another 10 min session to interact with S1 (now familiar rat) and S2 (novel rat). VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: vehicle + stress; AS: ANA-12 + stress; EC: empty cup; S1: stranger 1 rat; S2: stranger 2 rat. Female group numbers in pooled estrous cycles (estrus and non-estrus) were as follows: AS (7, 5), ANS (6, 6), VS (5, 7) and VNS (4, 8). Observed statistical power was insufficient in SP measures to allow for a correct statistical analysis of the influence of estrous cycle phases. Data are expressed as means ± SEM.

interaction with S1 under the wire cup in the first chamber or spending time with the empty cup (EC) in the second chamber. After this session, the experimental rat was again enclosed in the middle chamber and a novel conspecific (S2) was placed under the EC in the second chamber. In a subsequent 10 min session (social preference, SP), the experimental rat could freely engage in direct and indirect interaction with the rats in either chamber (See Fig. 2i for illustrated protocol). Placement of stranger rats was counterbalanced between trials. Measurements included the duration of direct interactions [i.e. time spent on direct contacts between S1 and the EC in session 1, and between S1 (now-familiar rat) and S2 in session 2], and duration of indirect interactions in each chamber [i.e. time spent in the respective chambers doing other behaviors such as walking, grooming and freezing that are not observable as direct contacts with the EC or conspecifics] (Kaidanovich-Beilin et al., 2011). The exploration ratio in the SIT was calculated as $[T_{S1} / (T_{S1} + T_{EC})]$, thus direct or indirect interactions with S1 was deemed higher relative to the EC when the exploration ratio > 0.5 (i.e. indicative of normal sociability, motivation and affiliation), while an exploration ratio < 0.5 indicated reduced interactions with S1 relative to the EC. In the SP, the exploration ratio was defined as $[T_{S2} / (T_{S2} + T_{S1})]$, an exploration ratio > 0.5 indicating increased preference for S2 relative to S1 (i.e. social memory and predilection for novelty). The dashed line in the resulting graphs indicates a chance level of exploration (i.e. ratio of 0.5).

2.5.2. Y maze conditioned place preference (YMCPP)

On the following day, conditioned place preference ($n = 13$ per group) assessed associative learning for valuation between food reward and environmental context. As previously described (Azogu and Plamondon, 2017b), animals were tested in a modified Y-shaped Plexiglas structure (LWH: 35.5 cm × 15 cm × 30 cm) with three arms and Plexiglas sliding doors (START arm – grey door, left and right arms – white doors). The place conditioning involved a biased protocol in which food reward was paired with the initially non-preferred side of the Y maze apparatus. Floor texture was similar in all three arms, however visual cues (horizontal vs. vertical black and white striped walls) were utilized for visual discrimination. Steel-caged grates atop each arm prevented the rat from jumping out of the maze during testing. The apparatus rested on a table (90 cm above the floor) with an overhead camera and separated by white curtains from the experimenter's area. For the habituation session (1st day, 10 min), a rat was placed in the designated "START" arm for 2 min, after which the grey sliding door was raised, and the rat could freely explore the maze. An experimenter blind to the groups scored the videos, labeling one arm as the "least preferred" based on time spent in each arm. For the conditioning sessions (2nd and 3rd days), rats underwent four counterbalanced forced choice run sessions (i.e. a total of 5 min in each arm × 2 sessions × 2 days) in which they were allowed access to either the least preferred arm (now paired with Honey Nut Cheerios®), or the other arm (non-food paired) for 5 min each. Briefly, following placement in the START arm for 2 min, the rat was only allowed entry to the food-paired arm and a sliding Plexiglas door was lowered, preventing exit. After a 5 min session, the animal was returned to the START arm for 2 min after which only the non-food paired arm was available for entry for 5 min. Following a 30-min interval, the rat was exposed to an additional conditioning session. On the 4th day (test session; 10 min), free access was allowed for all arms. A rat was inside one arm if both front paws had crossed the threshold of the arm (See Fig. 3f for illustrated protocol). Calculated measures include time spent in the food and non-food arms, the percent change in time spent in the initially least preferred arm [(test − habituation) / habituation) × 100] (Millot et al., 2014), and the preference ratio (time in food arm/(time in food arm + time in non-food arm)) during the test session. A preference ratio > 0.5 indicated a preference for the food arm, a ratio of 0.5 indicated no arm preference, and a ratio < 0.5 indicated a preference for the non-food arm (Yates et al., 2013). Development of a conditioned place preference is characterized by increased time spent in the initially least preferred arm during the test compared to the habituation session (Huston et al., 2013). Rats were placed on an overnight restricted diet (with access to 4–5 food pellets) following the 1st day habituation session and ending after the test session on the 4th day, such that the positive reinforcement of the food reward would be more effective in distinguishing the arms. Percent change in body weight was calculated to determine changes prior to and after diet restriction. Percent values (reported as Means ± SD) did not significantly differ between the groups [Females: AS (2.27% ± 2.29); ANS (1.82% ± 1.46); VS (3.22% ± 2.83); VNS (2.61% ± 1.71). Males: AS (1.70% ± 1.38); ANS (3.57% ± 1.03); VS (2.58% ± 1.44); VNS (1.66% ± 1.84)].

2.5.3. Y maze passive avoidance task (YMPAT)

The YMPAT ($n = 13$ per group) assesses learning and memory by measuring the latency to re-enter the arm paired with an aversive stimulus (e.g. air-puffs (Moriarty et al., 2012)) on a subsequent maze exposure (Azogu et al., 2015; Azogu and Plamondon, 2017b). Briefly, a rat was placed in the enclosed tail end of the maze (START arm) for 2 min, after which all three Plexiglas doors were raised, allowing free exploration of each arm. For Latency 1 (L1) session, a sliding door was gently closed behind the rat as soon as it entered an arm with its four paws. The rat then received small jets of compressed air every 15 s over a 60 s period prior to removal from the maze. After a 5-min interval, the rat was re-exposed to the maze. For Latency 2 (L2) session, the arm paired with the aversive stimulus was the only one available for re-entry (See Fig. 4f for illustrated protocol). Latency to re-enter this arm was measured in *sec*, assessing inhibitory avoidance. Number of risk assessments and the length of time spent in the aversive arm during the L2 session were also scored. Risk assessment was designated as the number of times the rat approached the arm without entering it. Immobility in the aversive arm (in sec) assessed anxiety-related expectation. Maximal latency for re-entry into the aversive arm was 5 min.

2.6. Brain tissue collection

At PND 69 and 2 h following a 5 min acute forced swim, rats were deeply anesthetized with isofluorane (3% in oxygen) and quickly decapitated. Collected fresh brains were frozen on dry ice and stored at −80 °C, prior to sectioning into 14 µm polarized slices (Fisherbrand Superfrost® Plus Microscope Slides) using a cryostat (Leica CM1900, Leica Microsystems, Germany). Three tissues were mounted per slide, every 9th section at 252 µm intervals, and stored at −80 °C in preparation for immunohistochemical detection.

2.7. Immunohistochemistry (IHC)

A subset of animals ($n = 7$ to 8 per group, depending on integrity of brain slice) were used for immunohistochemical detection of polyclonal mouse anti-GR (1:400, CAT #: sc-56,851, RRID: AB_2267526, Santa Cruz; raised against a peptide fragment of a well conserved region of human GR), polyclonal rabbit anti-TrkB (1:400, CAT #: sc-8316, RRID: AB_2155274, Santa Cruz; epitope corresponding to amino acids 160–340 mapping within the extracellular domain of TrkB of human origin), and polyclonal rabbit anti-BDNF (1:400, CAT #: sc-546, RRID: AB_630940, Santa Cruz; epitope mapping within an internal region of BDNF of human origin). According to the Paxinos and Watson Rat Brain Atlas (1998), brain regions of interest included the PVN and CeA (Bregma −0.92 mm to −2.12 mm) which were dual-labeled for TrkB and GR, while sections of the dorsal hippocampus CA1 and CA3 pyramidal layers (Bregma −3.14 mm to −4.16 mm) were dual-labeled for BDNF and GR. Briefly, brain sections were post fixed with 4% paraformaldehyde containing 2% picric acid in 0.1 M phosphate buffered saline (PBS, pH = 7.4) for 5 min, then rinsed 3 × 5 min in PBS. For double labeling of TrkB and GR, sections were blocked with PBS-T-BSA (i.e. 0.1 g of bovine serum albumin (BSA) in 0.1 M PBS containing 0.3%

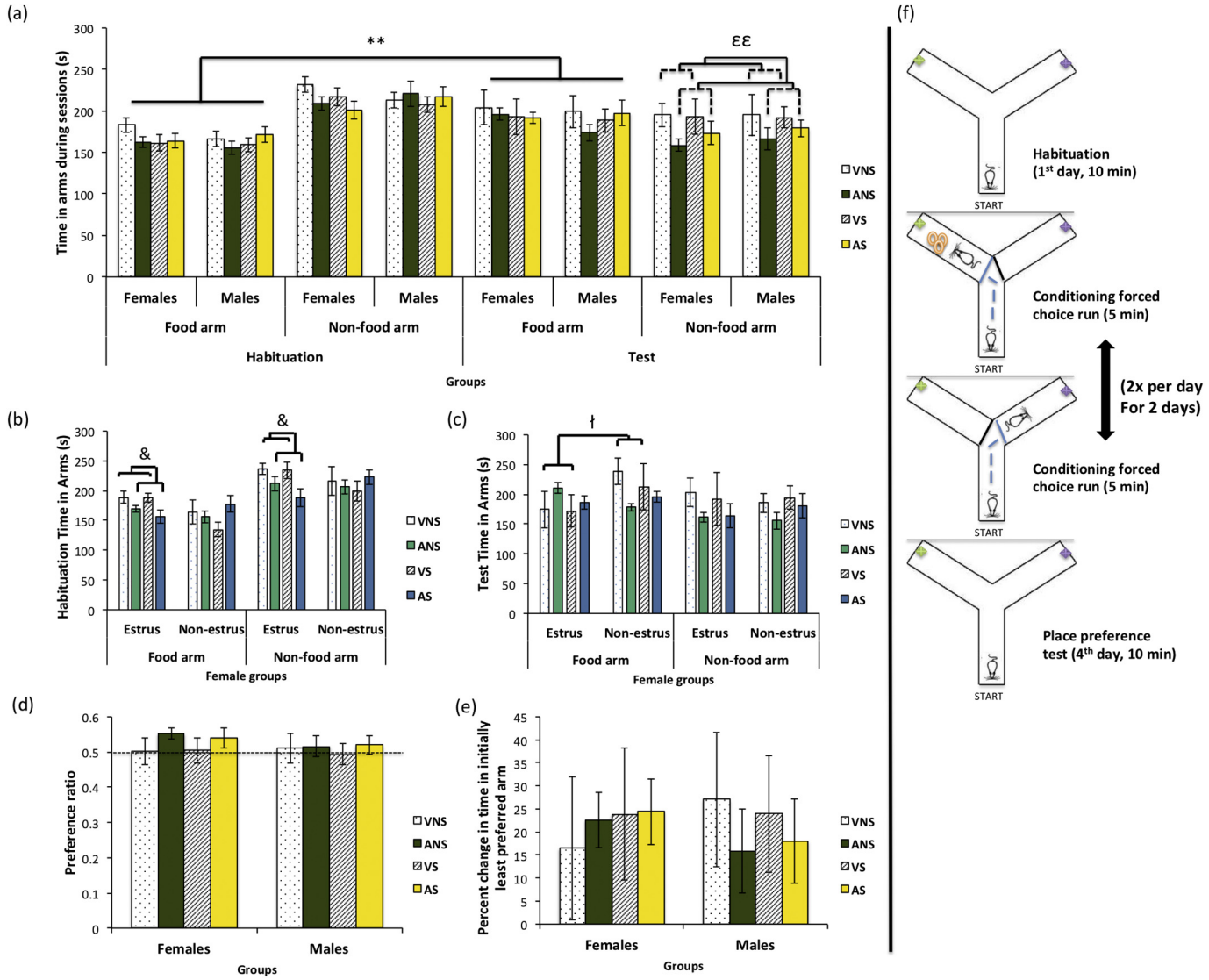


Fig. 3. Animals exhibit place preference post-conditioning and ANA-12 treatment reduced time spent in non-food paired arm for the Y maze conditioned place preference test (YMCPP).

(3a). In both sexes, time spent in the initially least preferred arm was significantly increased post-conditioning (**, $p < 0.01$). In the non-food paired arm during the test session, ANA-12 treatment attenuated time spent in this arm relative to vehicle treatment (εε, $p < 0.01$). (3b) In females, ANA-12-treated animals in estrus spent less time in the food and non-food arms during the habituation session compared to their veh-treated counterparts in estrus (&, $p < 0.05$). (3c) Veh-treated females in non-estrus spent more time in the food arm during the test session than their counterparts in estrus (ł, $p < 0.05$). (3d) Groups did not significantly differ for preference ratio. Dashed line represents a chance score of 0.5 for preference ratio indicating no arm preference. (3e) Groups did not differ significantly for percent change in initially least preferred arm. (3f) Schematic diagram of the YMCPP protocol: 1st day – 10 min habituation session to explore all 3 arms; 2nd and 3rd days – 5 min forced choice run conditioning sessions of food pairing in the initially least preferred arm and no food pairing in the most preferred arm, twice a day; 4th day – 10 min session to explore all 3 arms and determine place preference. VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: vehicle + stress; AS: ANA-12 + stress; YMCPP: Y maze conditioned place preference test. Female group numbers in pooled estrous cycles (estrus and non-estrus) for the YMCPP habituation were as follows: AS (8, 5), ANS (6, 6), VS (6, 6) and VNS (10, 3). For the YMCPP test, female group numbers in pooled estrous cycles (estrus and non-estrus) were as follows: AS (6, 7), ANS (7, 6), VS (6, 6) and VNS (7, 6). Observed statistical power was insufficient in YMCPP measures to allow for a correct statistical analysis of the influence of estrous cycle phases. Data are expressed as means ± SEM.

Triton (T)) for 30 min at RT, followed by antibody incubation for 24 h at 4 °C in PBS-T-BSA. For BDNF and GR dual labeling, slides were incubated for 50 min at RT with blocking buffer, PBS-T-BSA (containing 0.02% T). Tissues were then incubated for 24 h at RT with the antibodies mixed in PBS-T-BSA. Subsequently, slides were rinsed 3 × 5 min and incubated with the corresponding secondary antibody: Alexa 488-conjugated donkey anti-mouse (1:500, Invitrogen) and Alexa 594-conjugated donkey anti-rabbit (1:500, Invitrogen) in the dark for 2 h at RT. Following rinses, sections were incubated for 10 min in 1 µg/ml Hoechst nucleic acid stain (Hoechst 33342, Invitrogen) at RT in the dark to label binding at the AT regions of DNA. Following a last series of rinses, an anti-fade medium containing 0.1% *p*-phenylenediamine in phosphate buffered glycerol was applied, and the slides cover-slipped and sealed with nail polish. Special controls were run to test for antibody specificity by incubation of tissue slices in PBS-T-BSA, omitting the primary antibody prior to secondary antibody incubation.

2.8. Quantification of immunoreactivity

Digital images (photomicrographs) of fluorescence immunolabeling were obtained using an Olympus DX51 microscope (Center Valley, PA, USA) and the ProgRes Capture Pro 2.7.6 software under a 20×

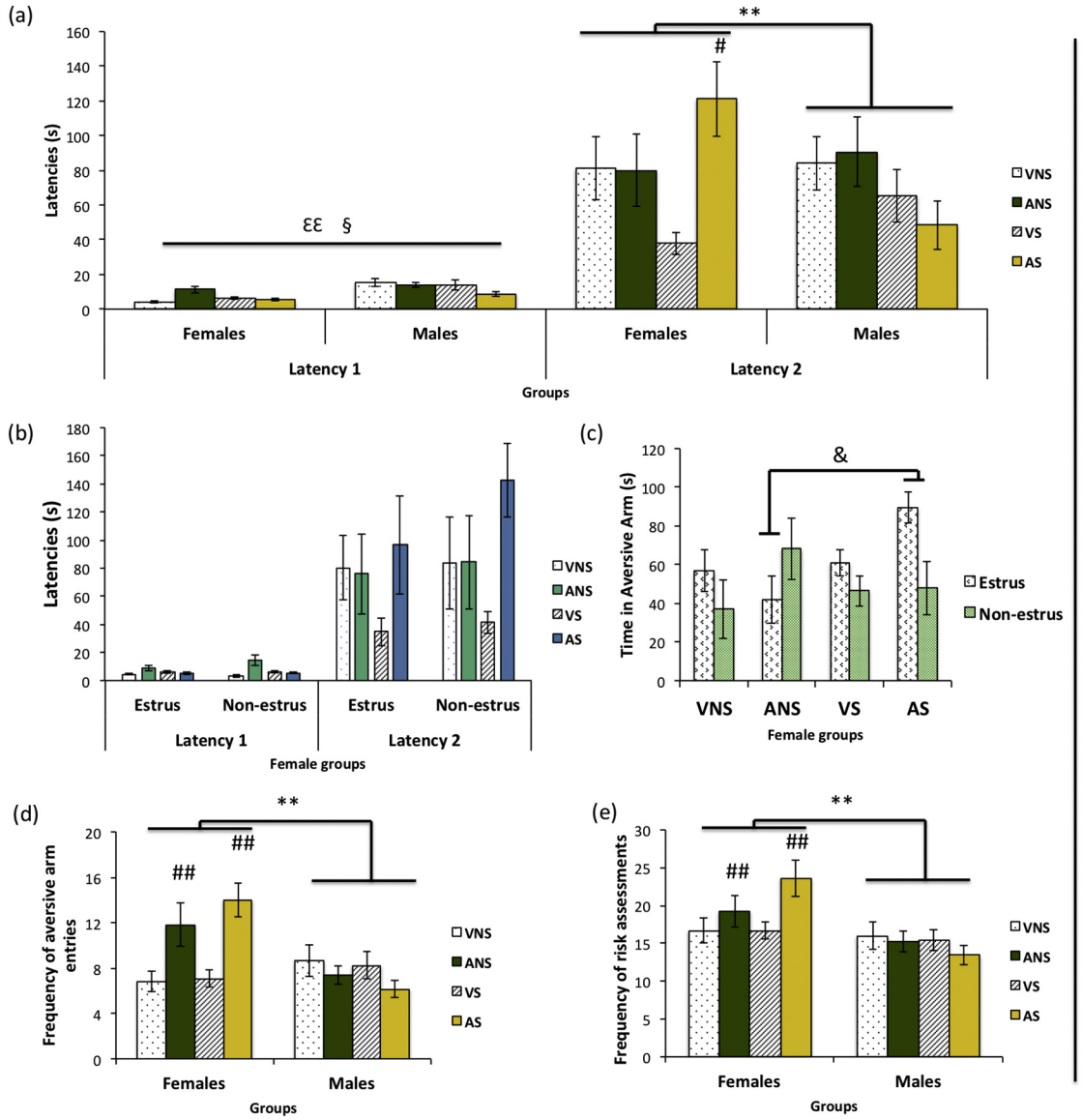


Fig. 4. Females exhibit improved memory performance, following ANA-12 treatment, in the Y maze passive avoidance test (YMPAT).
(4a) Latency 1: Increased latency to enter arm upon 1st exposure is noted in males vs. females (εε, $p < 0.01$) and in non-stress vs. stress animals (§, $p < 0.05$). Latency 2: Increased latency to re-enter aversive arm during 2nd exposure is noted in females vs. males (**, $p < 0.01$). Notably, ANA-12 vs. vehicle treatment in females increased latency to re-enter aversive arm, mainly in stressed females treated with ANA-12 (#, $p < 0.05$). (4b) Estrous stage assessment of latencies was not statistically different. (4c) ANA-12-treated stress females in estrus spent more time in the aversive arm relative to their non-stress counterparts in estrus (&, $p < 0.05$). (4d) Females made more entries in the aversive arm compared to males (**, $p < 0.01$), an effect that is heightened in ANA-12 treated females relative to ANA-12-treated males and veh-treated females (##, $p < 0.01$). (4e) Frequency of risk assessment during second exposure is increased in females compared to males (**, $p < 0.01$), notably enhanced in ANA-12-treated females relative to ANA-12-treated males and veh-treated females (##, $p < 0.01$). (4f) Schematic diagram of the YMPAT protocol: for latency 1 determination, animals have up to 5 min to enter either of the experimental arms from the START arm. Once inside, they are sequestered and exposed to 1 min of aversive air puffs at 15 s intervals. For latency 2 determination, animals have up to 5 min to reenter this aversive arm, the other arm is closed off. VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: vehicle + stress; AS: ANA-12 + stress; YMPAT: Y maze passive avoidance test. Female group numbers in pooled estrous cycles (estrus and non-estrus) were as follows: AS (6, 7), ANS (6, 6), VS (7, 5) and VNS (9, 4). Observed statistical power was insufficient in the YMPAT measures to allow for a correct statistical analysis of the influence of estrous cycle phases. Data are expressed as means ± SEM.

objective lens (eyepiece 10×; numerical aperture 0.75). Immunoreactivity was quantified with the ImageJ software (ImageJ, National Institutes of Health, Bethesda, MD) and the method described by Hayes et al. (2005). Percentages of optical densities (mean grey values; estimates of staining intensity) from fluorescence photomicrographs (200×) were obtained following manual selection of a brain region of interest (in square pixels) relative to a subthreshold background. This consisted of the subtraction of background staining within each brain area, using an auto-threshold method (e.g. Moments, an algorithm presented in the ImageJ software) prior to signal intensity analysis to get a valid optical density of tissue protein expression. Four anatomically matched pictures of both hemispheres were assessed per animal per group to produce an average immunoreactive score (i.e. the mean grey values) and the data presented as background corrected standardized image densities for each brain region. Representative photomicrographs were taken using an Olympus BX51 microscope (Center Valley, PA, USA) with a 20× oil lens magnification and FV100 Fluoview software.

2.9. Statistical analysis

Using SPSS software (version 23), three-way ANOVA was performed to assess sex (male vs. female), treatment (ANA-12 vs. vehicle) and stress (stress vs. no stress) main effects and interactions on the separate dependent variables of each behavioral test (SIT/SP, YMCPP and YMPAT) or neurochemical changes (TrkB, GR and BDNF). Three-way repeated measures ANOVA assessed the three independent variables on latencies (Latency 1 and 2) in the YMPAT and preference in the YMCPP. One-sample *t*-test assessed differences from the chance population score of 0.5 (i.e. the dashed line indicating an equal exploration or preference) for individual exploration ratios in the SIT/SP and the preference ratio in the YMCPP. In females, three-way ANOVA was conducted to assess impact of estrous stage (estrus vs. non-estrus), treatment and stress on behavioral responding across groups. The assumptions of homogeneity of variance and normality were verified, along with Mauchly's test of sphericity. Estimates of effect size were calculated and the partial eta squared (η_p^2) proportion of variance accounted for throughout the respective analyses. Cohen's benchmark values for η_p^2 were utilized: 0.0099, 0.0588, and 0.1379 for small, medium and large effects, respectively (Richardson, 2011). Observed power (1-β) was determined, and is only reported for main effects and interactions for estrous stage. In some behavioral tests (SP, YMCPP and YMPAT), statistical power was insufficient to allow for a correct statistical analysis of the influence of estrous cycle phases. For post-hoc analyses, Bonferroni correction was applied to determine pairwise comparisons. Data are presented as mean ± SEM ($p < 0.05$), unless otherwise stated.

3. Behavioral results

3.1. Effect of adolescent stress and ANA-12 on sex-specific differences in sociability in the social interaction test (SIT) during adulthood

3.1.1. SIT - direct interaction

Three-way ANOVA revealed similar contact with stranger 1 (S1) across all groups ($p > 0.05$), however females had increased contact with the empty cup (EC) compared to male groups ($F(1,88) = 4.27$, $p = 0.042$, $\eta_p^2 = 0.05$) (Fig. not shown). Analysis also revealed lower exploration ratio for direct contact with S1 vs. EC ($F(1,88) = 4.81$, $p = 0.031$, $\eta_p^2 = 0.05$) in female versus male groups (Fig. 2a). All groups had exploration ratios above the chance score of 0.5 ($p < 0.05$).

Estrous cycles: Three-way ANOVA on contact with S1 for combined estrous cycle stages (i.e. proestrus + estrus = estrus; and metestrus + diestrus = non-estrus) indicated a stress × estrous cycle interaction ($F(1,40) = 8.60$, $p = 0.006$, $\eta_p^2 = 0.18$, 1-β = 0.82). Stress females in non-estrus made more contacts with S1 than their non-stress counterparts ($p = 0.035$, $\eta_p^2 = 0.11$) and stress females in estrus ($p = 0.014$, $\eta_p^2 = 0.14$) (Fig. not shown). However, estrous cycle stage had no significant impact on exploration ratio for direct contact with S1 vs. EC ($p > 0.05$) (Fig. 2c).

3.1.2. SIT - indirect interaction

Three-way ANOVAs revealed that relative to males, indirect interaction time (i.e. doing other behaviors such as grooming and walking that did not involve direct contact with the conspecific) was reduced in female groups in the S1-paired chamber ($F(1,88) = 5.13$, $p = 0.026$, $\eta_p^2 = 0.06$), and females rather spent more time in the EC-paired chamber ($F(1,88) = 4.13$, $p = 0.045$, $\eta_p^2 = 0.05$) (Fig. not shown). Analysis also revealed lower exploration ratio for the S1- vs. EC-paired chamber in female versus male groups ($F(1,88) = 5.23$, $p = 0.024$, $\eta_p^2 = 0.06$) (Fig. 2b). All groups had exploration ratios above the chance score of 0.5 ($p < 0.05$).

Estrous cycles: Three-way ANOVA of combined estrous cycle stages for exploration ratio for indirect contact in the S1 vs. EC-paired chamber did not differ ($p > 0.05$) (Fig. 2d).

3.2. Effect of adolescent stress and ANA-12 on sex-specific differences in preference for social novelty in the social preference test (SP) during adulthood

3.2.1. SP - direct interaction

Three-way ANOVA for direct contact with stranger 1 (S1, now familiar rat) revealed a treatment × sex interaction ($F(1,88) = 4.28$, $p = 0.041$, $\eta_p^2 = 0.05$) and a main effect of sex ($F(1,88) = 5.54$, $p = 0.021$, $\eta_p^2 = 0.06$), as reflected in increased contact with S1 made by females relative to males, most notable in veh-treated groups ($p = 0.002$, $\eta_p^2 = 0.10$) (Fig. not shown). Although direct contact with stranger 2 (S2, novel conspecific) was comparable across groups ($p > 0.05$), exploration ratio for direct interaction with S2 vs. S1 was reduced in female relative to male groups, as indicated by a main effect of sex ($F(1,88) = 7.45$, $p = 0.008$, $\eta_p^2 = 0.08$) (Fig. 2e). Exploration ratios were above the chance score of 0.5 ($p < 0.05$) for all groups except for the veh-treated stress females.

Estrous cycles: Three-way ANOVA of combined estrous cycle stages yielded no significant effects for exploration ratio for direct contact with S2 vs. S1 ($p > 0.05$) (Fig. 2g).

3.2.2. SP - indirect interaction

Three-way ANOVA revealed a treatment × sex interaction ($F(1,88) = 6.31$, $p = 0.014$, $\eta_p^2 = 0.07$) for indirect interaction in which veh-treated females spent more time in the S1-paired chamber compared to ANA-12-treated females ($p = 0.047$, $\eta_p^2 = 0.04$) and veh-treated males ($p = 0.003$, $\eta_p^2 = 0.10$) (Fig. not shown). In the S2-paired chamber, a main effect of sex ($F(1,88) = 6.19$, $p = 0.015$, $\eta_p^2 = 0.07$) was due to reduced time spent in the chamber by females relative to males. Analysis for exploration ratio revealed a treatment × sex interaction ($F(1,88) = 4.39$, $p = 0.039$, $\eta_p^2 = 0.05$) and a main effect of sex ($F(1,88) = 5.55$, $p = 0.021$, $\eta_p^2 = 0.06$), which confirmed significantly lower exploration ratios for time spent in the S2- vs. S1-paired chamber by females compared to males, most notable in veh-treated groups ($p = 0.002$, $\eta_p^2 = 0.10$) (Fig. 2f). All groups had exploration ratios above the chance score of 0.5 ($p < 0.05$) except for both veh-treated female groups and the ANA-12-treated non-stress males.

Estrous cycles: Three-way ANOVA of combined estrous cycle stages revealed a treatment × stress × estrous cycle interaction ($F(1,40) = 5.23$, $p = 0.028$, $\eta_p^2 = 0.12$, 1-β = 0.61) for reduced time spent in the S1-paired chamber by the ANA-12-treated non-stress females in estrus compared to their veh-treated counterparts ($p = 0.001$, $\eta_p^2 = 0.24$) (Fig. not shown). Furthermore, treatment × stress × estrous cycle interactions supported increased time spent in S2-paired chamber ($F(1,40) = 4.21$, $p = 0.047$, $\eta_p^2 = 0.10$) (Fig. not shown) and higher exploration ratio for S2- vs. S1-paired chamber ($F(1,40) = 5.56$, $p = 0.023$, $\eta_p^2 = 0.12$, 1-β = 0.63) (Fig. 2h) by ANA-12-treated non-stress females in estrus compared to their veh-treated counterparts [Pairwise comparisons (time in S2 chamber: $p = 0.018$, $\eta_p^2 = 0.13$) and (exploration ratio: $p = 0.001$, $\eta_p^2 = 0.23$)].

3.3. Effect of adolescent stress and ANA-12 on sex-specific differences in the Y maze conditioned place preference test (YMCPP) during adulthood

3.3.1. YMCPP – preference, preference ratio and percent change in preference for the initially food paired arm

Three-way repeated measures ANOVA revealed a main effect of preference in the YMCPP ($F(1,96) = 18.98$, $p < 0.001$, $\eta_p^2 = 0.17$) (Fig. 3a), supporting a learned association by all animals, i.e. they spent increased time in the initially least preferred arm during the post conditioning test vs. the preconditioning habituation session (Mean ± SD: 192.86 ± 54.42 vs. 165.36 ± 30.84 s). There were no observable sex-specific differences for arm choice during the habituation session, neither did analysis of time in the food arm yield significance during the test session ($p > 0.05$). Notably, rats treated with ANA-12, independent of sex and stress exposure, spent less time in the non-food

arm during the test session relative to veh-treated rats, as revealed by a significant main effect of treatment ($F(1,96) = 4.93$, $p = 0.029$, $\eta_p^2 = 0.05$) (Fig. 3a). Analysis for preference ratio (Fig. 3d) and percent change in preference for time in the initially least preferred arm during the test session (Fig. 3e) were not significant ($p > 0.05$).

Estrous cycles: Three-way ANOVA of combined estrous cycle stages revealed treatment × estrous cycle interactions for habituation time in food ($F(1,43) = 6.84$, $p = 0.012$, $\eta_p^2 = 0.14$, 1-β = 0.72) and non-food arms ($F(1,43) = 4.23$, $p = 0.046$, $\eta_p^2 = 0.09$, 1-β = 0.52), related to reduced time spent in either arm during the habituation session by ANA-12-treated females in estrus compared to their veh-treated counterparts [Pairwise comparisons (time in food arm: $p = 0.017$, $\eta_p^2 = 0.13$) and (time in non-food arm: $p = 0.009$, $\eta_p^2 = 0.15$)] (Fig. 3b). During the test session, a treatment × estrous cycle interaction ($F(1,43) = 4.10$, $p = 0.049$, $\eta_p^2 = 0.09$, 1-β = 0.51) was related to reduced time spent in the food arm by veh-treated females in estrus compared to veh-treated females in non-estrus ($p = 0.024$, $\eta_p^2 = 0.11$) (Fig. 3c).

3.4. Effect of adolescent stress and ANA-12 on sex-specific differences in the Y maze passive avoidance test (YMPAT) during adulthood

3.4.1. YMPAT - latencies

Three-way repeated measures ANOVA revealed a treatment × stress × sex interaction ($F(1,96) = 4.41$, $p = 0.038$, $\eta_p^2 = 0.04$) (Fig. 4a) for latencies, related to increased latency to enter an arm during initial exposure (latency 1) exhibited by ANA-12-treated stress males compared to ANA-12-treated stress females ($p = 0.038$, $\eta_p^2 = 0.17$), whereas latency to re-enter the arm after exposure to aversive air puffs (latency 2) was heightened in females relative to males ($p = 0.009$, $\eta_p^2 = 0.25$), influenced by the ANA-12-treated stress females. Main effects of sex [males > females; ($F(1,96) = 26.34$, $p < 0.001$, $\eta_p^2 = 0.22$)] and stress [non-stress > stress; ($F(1,96) = 4.27$, $p = 0.041$, $\eta_p^2 = 0.04$)] were found for latency 1. Analysis for latencies indicated improved memory of an aversive stimulus by all groups as, compared to latency 1, latency 2 was highly increased ($F(1,96) = 124.83$, $p < 0.001$, $\eta_p^2 = 0.57$). Post-hoc assessment of a treatment × sex interaction ($F(1,96) = 4.49$, $p = 0.037$, $\eta_p^2 = 0.05$) indicated increased differences in latency to re-enter the aversive arm during second exposure (latency 2) compared to initial exposure (latency 1) between ANA-12 vs. veh-treated females ($p = 0.013$, $\eta_p^2 = 0.06$), mainly impacted by the stress females treated with ANA-12. Time in START arm did not differ for these measures ($p > 0.05$) (Fig. not shown).

Estrous cycles: Three-way ANOVA of combined estrous cycle stages yielded no significant effects for latencies ($p > 0.05$) (Fig. 4b).

3.4.2. YMPAT - Aversive arm entries and risk assessments

Three-way ANOVA for time in the aversive arm was comparable across groups ($p > 0.05$) (Fig. not shown). Post hoc analysis for a treatment × sex interaction for frequency of entries into the aversive arm during latency 2 ($F(1,96) = 19.52$, $p < 0.001$, $\eta_p^2 = 0.17$) revealed that ANA-12-treated females exhibited higher frequency of entries compared to ANA-12-treated males ($p < 0.001$, $\eta_p^2 = 0.21$) and veh-treated females ($p < 0.001$, $\eta_p^2 = 0.20$) (Fig. 4d). Furthermore, significant main effects were noted for frequency of aversive arm entries [females > males; ($F(1,96) = 7.34$, $p = 0.008$, $\eta_p^2 = 0.07$)] and [ANA-12 > vehicle; ($F(1,96) = 6.25$, $p = 0.014$, $\eta_p^2 = 0.06$)].

Similar to frequency of aversive arm entries, three-way ANOVA revealed a treatment × sex interaction ($F(1,96) = 6.61$, $p = 0.012$, $\eta_p^2 = 0.06$) for elevated frequency of risk assessments during second maze exposure (latency 2) by ANA-12-treated females relative to their male counterparts ($p < 0.001$, $\eta_p^2 = 0.16$) and veh-treated females ($p = 0.006$, $\eta_p^2 = 0.08$) (Fig. 4e). A main effect of sex ($F(1,96) = 11.33$, $p = 0.001$, $\eta_p^2 = 0.11$) was related to an overall increase in frequency of risk assessments by female compared to male groups. Immobility/freezing in the aversive arm was not significant ($p > 0.05$).

Estrous cycles: Three-way ANOVA of combined estrous cycle stages on time in the aversive arm upon re-exposure revealed treatment × stress × estrous cycle interactions indicating that following ANA-12 treatment, stress females in estrus spent more time in the aversive arm upon re-exposure ($F(1,44) = 4.75$, $p = 0.035$, $\eta_p^2 = 0.10$, 1-β = 0.57) (Fig. 4c) and less time in the START arm ($F(1,44) = 4.78$, $p = 0.034$, $\eta_p^2 = 0.10$, 1-β = 0.57) (Fig. not shown) relative to non-stress females in estrus [Pairwise comparisons (aversive arm time: $p = 0.006$, $\eta_p^2 = 0.16$) and (START arm time: $p = 0.005$, $\eta_p^2 = 0.16$)]. Estrous stage influence on frequencies of aversive arm entries and risk assessments were not significant ($p > 0.05$).

4. Immunohistochemical results

4.1. Effect of adolescent stress and ANA-12 on sex-specific differences in GR- and TrkB-ir in the PVN following acute forced swim stress in adulthood

For GR-ir in the PVN, an area that can be activated by physiological changes such as stress, three-way ANOVA revealed a sex × stress interaction ($F(1,43) = 10.33$, $p = 0.002$, $\eta_p^2 = 0.19$), attributed to reduced PVN GR-ir in stressed males compared to stress females ($p < 0.001$, $\eta_p^2 = 0.32$) and non-stress males ($p < 0.001$, $\eta_p^2 = 0.27$). Main effects of sex [females > males; $F(1,43) = 10.83$, $p = 0.002$, $\eta_p^2 = 0.20$)] and stress [non-stress > stress; $F(1,43) = 5.46$, $p = 0.024$, $\eta_p^2 = 0.11$)] were also observed (Fig. 5b). PVN TrkB-ir analyses yielded no significant results ($p > 0.05$) (Fig. 5c).

4.2. Effect of adolescent stress and ANA-12 on sex-specific differences in GR- and TrkB-ir in the CeA following acute forced swim stress in adulthood

We examined levels of GR and TrkB in the CeA, a site implicated in anxiety and fear. Three-way ANOVA revealed main effects of sex for increased GR-ir ($F(1,42) = 7.73$, $p = 0.008$, $\eta_p^2 = 0.16$) and TrkB-ir ($F(1,41) = 8.46$, $p = 0.006$, $\eta_p^2 = 0.17$) in females compared to males (Fig. 6b and 6c, respectively).

4.3. Effect of adolescent stress and ANA-12 on sex-specific differences in GR- and BDNF-ir in the hippocampal CA1 and CA3 layers following acute forced swim stress in adulthood

GR and BDNF expression were examined in the dorsal hippocampus. In the CA1 pyramidal layer, three-way ANOVA revealed a sex × stress interaction ($F(1,40) = 4.88$, $p = 0.033$, $\eta_p^2 = 0.11$), which was attributed to increased GR-ir in stress vs. non-stress males ($p = 0.050$, $\eta_p^2 = 0.09$) (Fig. 7b). BDNF-ir was elevated in males compared to females ($F(1,40) = 7.59$, $p = 0.009$, $\eta_p^2 = 0.16$) (Fig. 7c). In the CA3 pyramidal layer, analyses of GR- and BDNF-ir (Fig. 7d and 7e) were not significant ($p > 0.05$).

5. Discussion

To our knowledge, this study is the first to compare sex-specific differences, and influence of combinatory stress and TrkB blockade during adolescence, on conditioned learning, social and fear-related behaviors, and expression of GR, TrkB, and BDNF in stress-responsive brain regions of adult male and female Wistar rats.

5.1. Behavioral assessments

5.1.1. Sociability and social novelty responses are sex-specific

Concordant with sex differences reported in the SIT (Johnston and File, 1991; Stack et al., 2010), alterations in social investigation and social memory were characterized by reduced conspecific interactions in female compared to male rats. This is consistent with reduced social

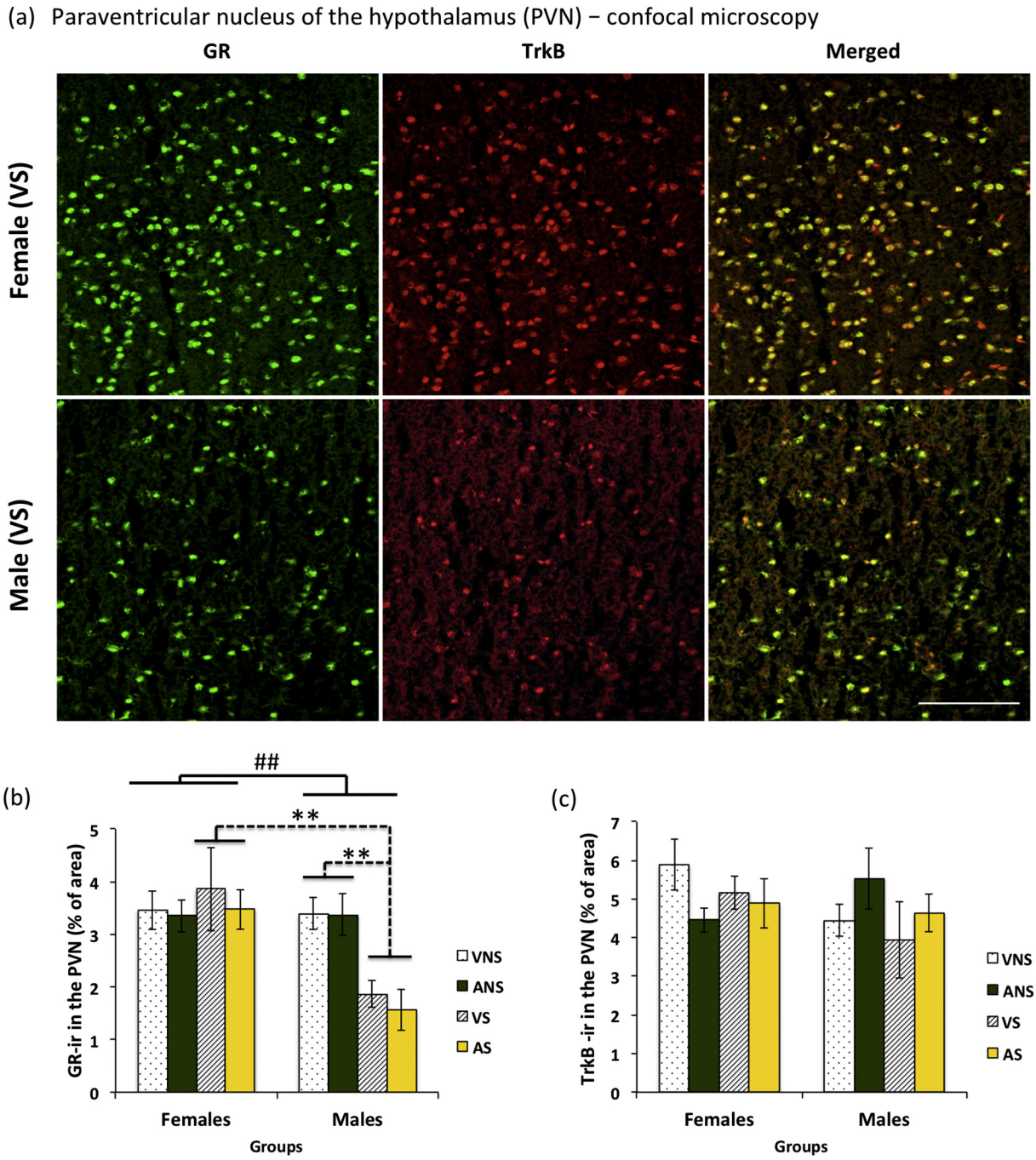


Fig. 5. GR-ir is reduced in stressed males in the paraventricular nucleus of the hypothalamus (PVN).
(5a) Representative confocal photomicrographs (20× oil objective) show glucocorticoid receptor (GR) – ir (green), tyrosine-related kinase B (TrkB) –ir (red), and merged images in the PVN of a female and male rat from the VS groups. Scale bar 100 μm. (5b) Stress males showed reduced GR-ir relative to stress females and non-stress males (**, $p < 0.01$). An effect of sex to increase GR-ir was noted in females relative to males (##, $p < 0.01$). (5c) Graph represents TrkB-ir in the PVN. No significant effects were noted. VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: vehicle + stress; AS: ANA-12 + stress; PVN: Paraventricular nucleus of the hypothalamus. Data are expressed as means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

preference being related to diminished TrkB expression at the PFC in mutant mice (Kaminitz et al., 2014). Using western blot, we recently showed lower expression of both TrkB isoforms (TrkB. FL and TrkB.T1) in the PFC of adult females compared to males (Azogu et al., 2018), which further support the sex differences observed in the SIT/SP. In addition, females reportedly have reduced expression of extracellular signal-regulated kinase 2 in the mPFC, which has been implicated in the mediation of social anxiety-like behaviors (Carrier and Kabbaj, 2012). It is noteworthy to state that none of the animals showed social avoidance. For instance, all exploration ratios in the SIT were significantly above chance level, indicating high discrimination of the conspecific versus the empty cup by both sexes and engagement in social affiliative behavior with the conspecific. Furthermore, although most groups were also above chance level in the SP, the VS female group failed to significantly show social novelty discrimination between the familiar and novel conspecifics, and between their chambers, which could be related to habituation of olfactory responses directed towards the novel conspecific (Moy et al., 2004) or social memory deficit in the

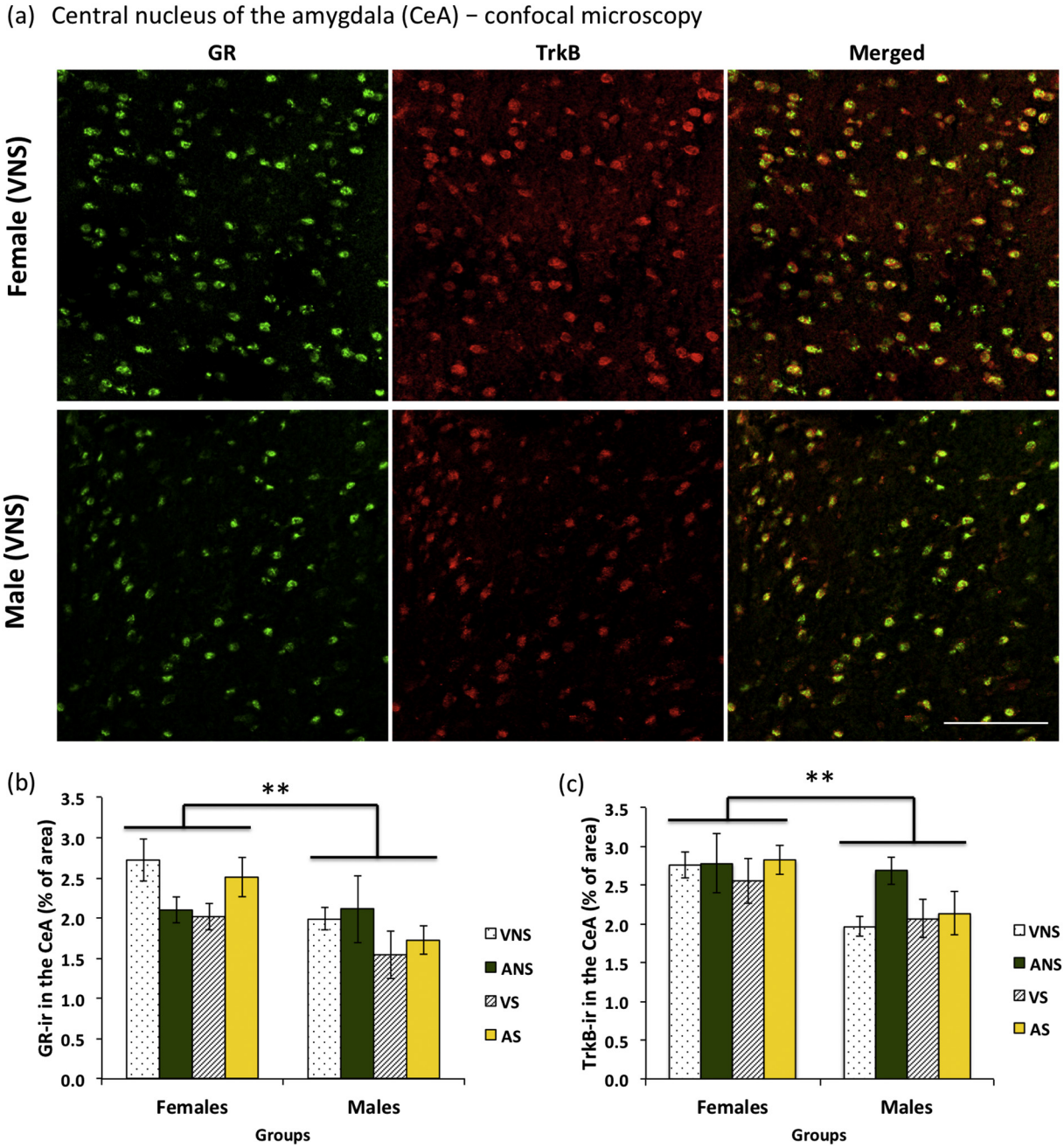
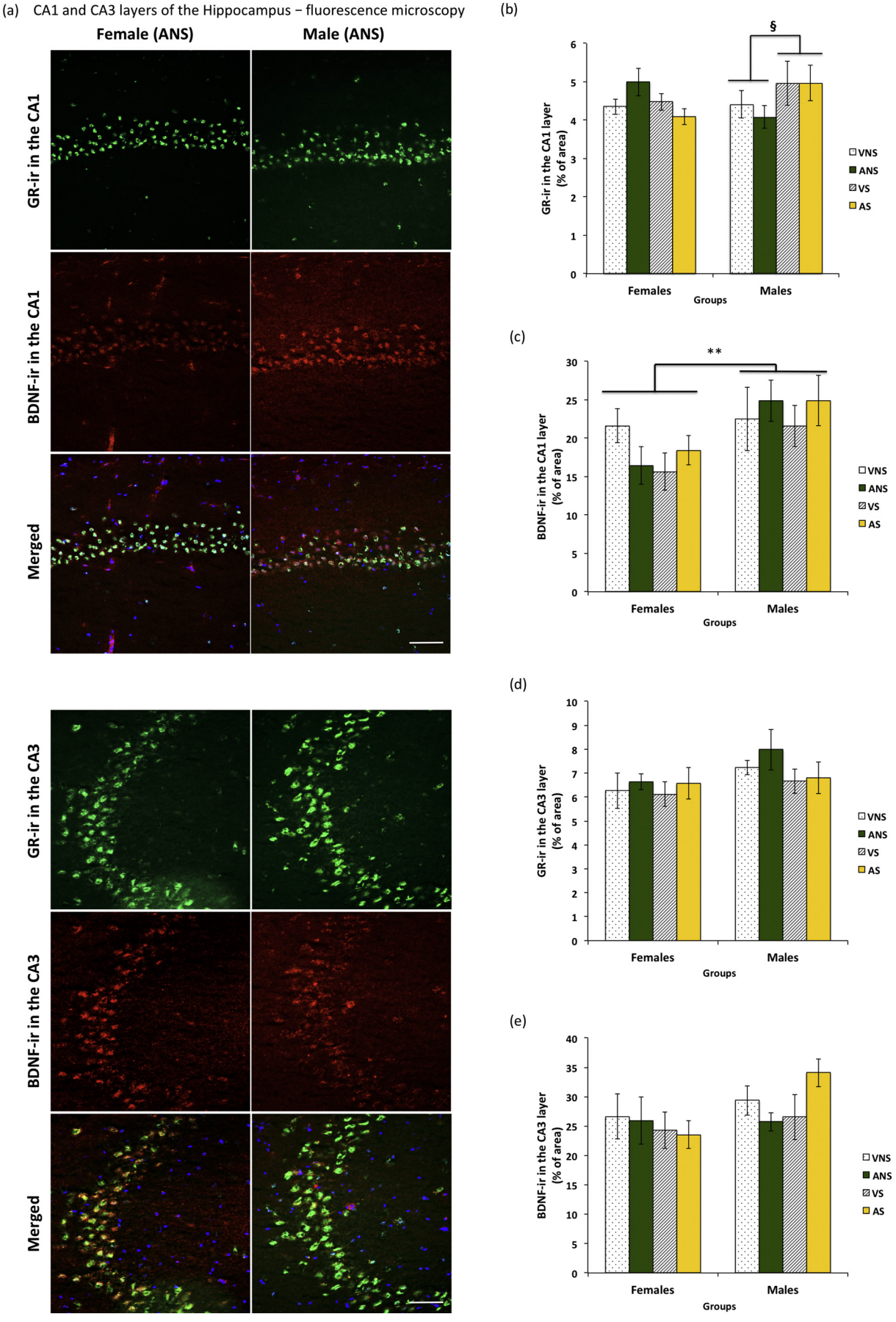


Fig. 6. Females show increased GR and TrkB expression in the central nucleus of the amygdala (CeA).
(6a) Representative confocal photomicrographs (20× oil objective) show glucocorticoid receptor (GR) – ir (green), tyrosine-related kinase B (TrkB) –ir (red) and merged images in the CeA of a female and male rat from the VNS groups. Scale bar 100 μm. (6b–c) Females relative to males showed increased expression of GR and TrkB-ir in the CeA (**, $p < 0.01$). VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: vehicle + stress; AS: ANA-12 + stress; CeA: central nucleus of the amygdala. Data are expressed as means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

VS females. Together, these observed sex differences point to reduced motivation for social contact in females (which may be mediated by boredom) or a higher propensity of males to investigate an unfamiliar conspecific.

Contrary to anxiolytic properties when acutely administered in the adult male mice (Cazorla et al., 2011), systemic ANA-12 treatment during adolescence had no lasting effect on sociability. This was unexpected and may be due to programming changes occurring between drug administration and behavioral testing, or to ANA-12 bioavailability and amplitude of receptor inhibition in different brain areas. For instance, systemic administration of ANA-12 is reported to be more effective in the striatum compared to the hippocampus and cortex (Cazorla et al., 2011). Interestingly, increases in social interaction have been demonstrated following direct infusion of ANA-12 in discrete brain regions such as the nucleus accumbens (NAc) in adult male rodents (Azogu and Plamondon, 2017b; Walsh et al., 2014; Wook Koo et al., 2016) and the anterior BNST in stressed adult female mice (Greenberg et al., 2014). In our study, ANA-12 also demonstrated effects in the YMCPP and the YMPAT, which are tasks that are shown to specifically activate the prelimbic cortex and the core subregion of the nucleus accumbens (El Rawas et al., 2012). However, social recognition is typically recognized as a prefrontal cortex-dependent task (Bicks



(caption on next page)

Fig. 7. GR and BDNF-ir are increased in the male CA1 pyramidal layers of the dorsal hippocampus
(7a) Representative fluorescence photomicrographs (200× magnification) show glucocorticoid receptor (GR) –ir (green), brain-derived neurotrophic factor (BDNF) –ir (red), and Hoechst (blue, see merged images) in the CA1 and CA3 of the dorsal hippocampus of a female and male rat from the ANS groups. Scale bar 100 μm. (7b) Stressed males expressed higher GR levels in the CA1 compared to non-stress males (§, $p < 0.05$). (7c) BDNF-ir expression is increased in males relative to females (**, $p < 0.01$). (7d–e) Differences were not significant across groups for GR- and BDNF-ir in the CA3. VNS: vehicle + no stress; ANS: ANA-12 + no stress; VS: vehicle + stress; AS: ANA-12 + stress; CA1: cornus ammonis 1; CA3: cornus ammonis 3. Data are expressed as means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2015; Kaminitz et al., 2014). Therefore, ANA-12 effects may be influenced by the route of administration, and could be task-specific. Lastly, pair-housing could have impacted the effects of stress on sociability, as this form of social interaction is reported to buffer the influence of stressful experiences, and use of pair-housed conspecifics in the SIT/SP test may trigger scent familiarity in experimental animals (Beery and Kaufer, 2015; Muroy et al., 2016).

5.1.2. ANA-12 treatment impacted food conditioned place preference independent of stress exposure or sex

Within the YMCPP, our findings show that stress or sex did not affect the hedonic valence of the palatable food. Associative learning observed during the conditioning phase is consistent with reports showing no stress or sex differences in acquisition of conditioned place preference (CPP) using a cocaine-paired environment or food reward (Bobzean et al., 2010; Figlewicz, 2015; Hilderbrand and Lasek, 2014; MacKay et al., 2017; Rubinow et al., 2009). All animals exhibited CPP, using the biased protocol, via increased time spent in the food arm during the post- vs. pre-conditioning sessions. Similar preference level renders it difficult to discern the intensity value of the food reward independent from its hedonic characteristics. This may be influenced by the ability of palatable foods to buffer against the effects of stress exposure on behavior (Ali et al., 2018; MacKay et al., 2017), as decreases in adrenocorticotropic hormone, plasma corticosterone levels and corticotropin-releasing hormone mRNA expression following ingestion of palatable food have been reported at the PVN and are demonstrated to reduce HPA axis stress response (Pecoraro et al., 2004; Ulrich-Lai et al., 2007). Of note, prior ANA-12 treatment led to reduced time in the non-food arm during the post-conditioning session in both sexes, suggesting that blockade of TrkB receptors may have facilitated activation of neuronal circuits involved in regulating sensitivity to hedonic responses. Via actions on the mesolimbic dopamine system, BDNF/TrkB system plays an important role in regulating motivated and reward-seeking behaviors (Cordeira et al., 2010; Xu et al., 2003). Future studies are required to evaluate sex-related differences in BDNF/TrkB signaling to elucidate alterations in appetitive behavior.

5.1.3. Sex-specificity is prevalent in passive avoidance and is influenced by prior ANA-12 treatment

Noteworthy, blockade of TrkB receptors improved memory of an aversive event (via increased latency to re-enter the aversive arm) in the YMPAT in adult female rats, particularly in stress females. Sex differences were also present through increased frequencies of risk assessments and aversive arm entries by females, mainly influenced by ANA-12 treatment, supporting enhanced encoding or coping of the aversive experience in females. Consistent with our findings, research supports enhanced acquisition of passive avoidance in females (Kemble and Enger, 1984; Podhorna et al., 2002) and increased escape attempts (an active response) to an aversively conditioned context (Maren et al., 1994; Pryce et al., 1999). These are suggested to reflect a natural tendency to respond defensively to focal sources of aversive stimuli, which may be an adaptive strategy against predation (Kemble and Enger, 1984). Although the precise mechanisms remain to be elucidated, neuroregulatory effects of ovarian hormones on BDNF/TrkB signaling may be of importance. For instance, previous findings from our lab showed influence of ANA-12 to increase both TrkB isoforms in the PFC, NAc and hippocampus of adult female rats, whereas males showed a downregulation in both the NAc and hippocampus (Azogu et al., 2018). We speculate that the influence of plasticity signaling on these sexually dimorphic limbic brain regions that connect to the PVN, and are known to react similarly to BDNF signaling, may modulate sex differences in coping responses. This requires further examination as it could play a role in the sex-specific basis of adult mood disorders, beyond those influenced by stressful experiences.

5.1.4. Influence of estrous cycles on behavioral responding in female rats is state-dependent

Our data support an interplay between stress, TrkB receptor activation and hormonal fluctuations in regulating females' behavioral responses. In agreement with documented estrogenic involvement in social learning in rodents (Choleris et al., 2012; Ervin et al., 2015), findings in the SIT support synergistic effects of estrogenic and BDNF/TrkB signaling pathways to female social recognition memory (Luine and Frankfurt, 2013), although estrous cycle phases were combined into estrus (proestrus + estrus) and non-estrus (metestrus + diestrus) to conduct statistical analyses. In the YMCPP and YMPAT, trends were observed but power calculations did not allow statistical analysis on estrous cycle phases. However, the influence of estrus versus non-estrus stage to diminish place preference in veh-treated females, irrespective of prior stress exposure, is consistent with treatment with estradiol in female rats impairing acquisition of a conditioned place preference task (Galea et al., 2001). Lastly, ANA-12 showed a state-sensitive effect to increased time spent in the aversive arm by ANA-12-treated stress vs. non-stress females in estrus, which may be indicative of retention deficits in the former group (Silva et al., 2004). These findings support the value of examining the impact of each phase of the estrous cycle in further understanding the role of sex hormone cyclicity and prior experience on emotional behavior. Although mechanisms are under study, BDNF has functional interactions with estrogen, GCs, endocannabinoids, and serotonin (De Chiara et al., 2010; Jiang et al., 2016; Numakawa et al., 2010; Pluchino et al., 2013), and may recruit other brain circuits involved in the regulation of stress response. Further investigation and the inclusion of increased sample size per estrous phase are required.

5.2. Biochemical assessments

5.2.1. PVN GR expression is reduced in adult male rats following acute forced swim

Animals were exposed to a 5 min acute forced swim 2 h prior to euthanasia. Within the PVN, sex differences were noted overall in lower GR expression in males compared to females. GR-ir was also reduced in stress males relative to stress females and non-stress males, suggesting the inhibitory influence of testosterone to lower stress reactivity (Viau and Meaney, 1996). In females, increased GR-ir could be due to prolonged GC elevation following acute stress and lower inhibition of the PVN, related to heightened stress sensitivity or effects of ovarian hormones to slow the negative feedback mechanism (Bangasser, 2013; Goel et al., 2014; Iwasaki-Sekino et al., 2009; Seale et al., 2004; Weiser and Handa, 2009). Furthermore, female rats are reported to show enhanced GR and Fos (marker of neuronal activation) expression than males in the hypothalamus following acute and chronic stress (Zavala et al., 2011), indicating sex-specific consequences in neuronal activation that is due to the modulatory effects of the hypothalamic-pituitary-

gonadal (HPG) system on the HPA axis. PVN TrkB-ir remained unaltered in our study. BDNF gene appears to be stress-sensitive, but is PVN TrkB expression similarly influenced? Evidence supports that GCs may directly bind to and activate TrkB, independently of neurotrophin mechanisms (Jeanneteau and Chao, 2013; Numakawa et al., 2009). Further understanding of sex differences in altered plasticity signaling within stress-responsive brain regions is required.

5.2.2. Heightened GR- and TrkB-ir expression in the amygdalar CeA of female rats following 5 min acute forced swim

The CeA possesses a high number of GR-ir neurons relative to other amygdaloid structures (Morimoto et al., 1996), and strongly expresses TrkB mRNA, although being virtually devoid of BDNF mRNA (Krause et al., 2008). Elevated GR and TrkB expressions observed in the CeA of females compared to males, are consistent with higher CRF mRNA expression in the PVN and CeA, and enhanced stress-induced CORT levels typically observed in females vs. males (Azogu et al., 2018; Iwasaki-Sekino et al., 2009). Considering GR-TrkB crosstalk, one may also speculate that elevated GR expression may increase TrkB expression (Jeanneteau et al., 2008), as females are reported to have a differential TrkB activation mechanism (Chan and Ye, 2017).

5.2.3. Enhanced BDNF and GR expression in the adult male CA1 pyramidal layer is not influenced by TrkB antagonism in adolescence

Our findings showed increased CA1 GR-ir in stressed compared to non-stressed males, which appeared independent of ANA-12 treatment or sex differences. GR upregulation upon acute stressor exposure has been depicted as an adaptive response and a primary mediator in restoring HPA-axis activity (Seckl et al., 1991; Zhu et al., 2014). Comparable GR expression among female groups is intriguing and may be due to protective effects of estrogen in the hippocampus, mediated via estrogen receptors, ERα and ERβ (see reviews (Brann et al., 2007; McEwen et al., 2016)) and/or brain systems involved in sex-specific interpretation of the stimuli's stressful nature and possible coping strategies. BDNF-ir in the CA1 was increased in males versus females, irrespective of treatment or stress. The attenuated BDNF expression observed in our female groups may be detrimental, reducing protection against glucocorticoid-induced cell death (Nitta et al., 1999) and impairing antidepressant effects (Shirayama et al., 2002). Indeed, research has shown that following repeated stress, female rats show elevated levels of BDNF gene methylation compared to males, causing a reduction of BDNF expression and mediating sex-dependent alterations of BDNF signaling in the hippocampus (Niknazar et al., 2016). The ~30-day delay between the end of the 10-day stress paradigm and exposure to acute forced swimming may have buffered the central effects of repeated stress or systemic drug exposure, triggering an intact acute stress response to increase GR-ir and BDNF-ir. Although not significantly different, it is interesting to note that the blockade of TrkB appeared to increase CA1 BDNF-ir in males relative to their veh-treated controls. This is speculated to be due to an influence of ANA-12 administration during adolescence to minimize stress-induced HPA axis activation, and thus foster coping responses. In addition, there is the possibility that short-lived stress may suppress plasticity mechanisms in the CA1 of the hippocampus of veh-treated controls, but these changes in morphology and function are reversible. This remains to be elucidated. Lastly, non-significant observations for BDNF- and GR-ir at the CA3, suggest that this region is less vulnerable to the influence of stressors employed in this study.

6. Conclusion

In summary, sex-specific differences were prevalent in behavioral measures of sociability and social memory, associative learning and passive avoidance memory. Decreased BDNF levels in females' hippocampus and increased GR levels in the PVN and CeA relative to males support the literature on female susceptibility to anxiety and mood disorders. Absence of ANA-12 effects in neurochemical examinations and some of the behavioral tests were not expected. This could be related to ANA-12 bioavailability following systemic administration, and the discrepant amplitude of TrkB inhibition reported between brain areas (Cazorla et al., 2011). Attenuated effects of stress and TrkB blockade may also be due to a selected long assessment interval. In the literature, there is evidence of the impact of stressors on the timing of puberty and internal physiology in rodents and humans (Parent et al., 2015). Together, our data demonstrate inherent sex differences in stress sensitivity that is heightened in females, particularly within limbic brain regions (e.g. hippocampus and hypothalamus) that modulate the HPA axis via a negative feedback loop. In this regard, females are suggested to be more sensitive to limbic dysfunction, while males show dysregulation within the peripheral immune and metabolic system (see (Bourke and Neigh, 2011)). Our main finding of sex-specificity highlights the crosstalk between the HPG and HPA axes.

For future studies, we recommend assessment of pubertal markers in both sexes, and incorporation of the understanding that females have earlier pubertal onset in study design. We also emphasize the importance of conducting research on sex-specific behavioral and neurochemical changes following stress exposure across developmental periods, and the involvement of gonadal hormones in BDNF/TrkB signaling, as these may provide translational insights into predisposing factors that lead to pathophysiology of stress and anxiety or to resilience and improved performance. In this context, it is inappropriate to assume that neuronal circuits and regulatory processes are similar in males and females. Lastly, our findings suggest a need for targeting discrete brain regions in assessing therapeutic drugs that act upon BDNF/TrkB signaling.

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Declaration of conflicting interests

The authors declare no conflicts of interest with respect to the contents of this manuscript.

Author's roles

IA conceived and designed the project, contributed to implementation, data acquisition, analysis and interpretation, wrote the manuscript and approved the final version for appropriate and intellectual content. IC contributed to behavioral data acquisition, analysis and manuscript revision. JN and OI contributed to screening of the immunohistochemistry, analysis and manuscript revision. HP supervised the research project, interpreted the results, and revised the manuscript for intellectual content and approved the final version.

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