Patterns of Endocrine, Behavioural, and Neural Function Underlying Social Deficits after Social Instability Stress in Adolescent Rats

by
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Abstract

Adolescence is a time of social learning as well as a period of heightened vulnerability to stressors and enhanced plasticity, compared with adulthood. Previous research found that repeated social instability stress (SS; daily isolation and return to an unfamiliar peer from postnatal day (PND) 30 - 45) administered in adolescent rats alters social function when tested in adulthood. The main goal of my thesis research was to characterize how SS in adolescent rats affects the development of social brain regions and social behaviour when tested soon after the procedure. In chapter 2, I found that SS potentiated corticosterone release in rats repeatedly paired with an unfamiliar cage-mate after isolation compared with rats that were paired with an unfamiliar cage-mate for the first time after isolation on PND 45. In chapter 3, I found that in social interaction tests (i.e., not in home cage), SS rats had lower social interactions despite having higher social approach with unfamiliar peers relative to control (CTL) rats. Social stimuli carried the same reward value for SS and CTL rats based on tests of conditioned place preference, and SS in adolescence impaired social recognition. Further, SS increased oxytocin receptor density in the nucleus accumbens and dorsal lateral septum in rats compared with CTL rats. In chapter 4, I found that the correlations between time spent in social interaction with an unfamiliar peer and Fos immunoreactivity (a marker of neural activity) in the arcuate nucleus, dorsal lateral septum, and posterior medial amygdala were in the opposite direction in SS rats to those in CTL rats. In chapter 5, I found differences in the expression of proteins relevant for synaptic plasticity and in dendritic arborisation in the lateral septum and medial amygdala. My findings of behavioural and neural differences between SS and CTL rats highlight the heightened vulnerability of the brain to the quality of social experiences

during the adolescent period that may lead to long-lasting deficits in social function in adulthood.

Keywords: adolescence; social instability stress; corticosterone; social behaviour; social brain regions

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List of Abbreviations

ACC	Anterior cingulate cortex
ACTH	Adrenocorticotropic hormone
amBNST	Anteromedial bed nucleus of stria terminalis
aMeA	Anterior medial amygdala
ANOVA	Analysis of variance
ARC	Arcuate nucleus
AVP	Arginine vasopressin
BLA	Basolateral amygdala
CA1	Cornu Ammonis 1 region of the hippocampus
CA2	Cornu Ammonis 2 region of the hippocampus
CA3	Cornu Ammonis 3 region of the hippocampus
CaMKII	Calcium/calmodulin-dependent protein kinase II
CBG	Corticosteroid binding globulin
CeA	Central amygdala
CPP	Conditioned place preference
CRH	Corticotropin releasing hormone
CTL	Control
Ctl/acISO	Controls exposed to acute isolation
DAB	3,3’diaminobenzidine
dlBNST	Dorsolateral bed nucleus of stria terminalis
dLS	Dorsal lateral septum
EPM	Elevated plus maze
GAD67	Glutamic acid decarboxylase isoform 67
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HPC	Hippocampus
HR	High responders

IL	Infralimbic cortex
iLS	Intermediate lateral septum
ir	Immunoreactive
LA	Lateral amygdala
lHb	Lateral habenula
LR	Low responders
LS	Lateral septum
MeA	Medial amygdala
MeAD	Anterodorsal medial amygdala
MeAV	Anteroventral medial amygdala
MePD	Posterodorsal medial amygdala
MePV	Posteroventral medial amygdala
mHb	Medial habenula
mPFC	Medial prefrontal cortex
MPOA	Medial preoptic area
MR	Mineralocorticoid receptor
mRNA	Messanger ribonucleic acid
NAc	Nucleus accumbens
NAcC	Nucleus accumbens core
NAcS	Nucleus accumbens shell
OTR	Oxytocin receptor
PBS	Phosphate buffered saline
PFC	Prefrontal cortex
PL	Prelimbic cortex
plBNST	Posterolateral bed nucleus of stria terminalis
pmBNST	Posteromedial bed nucleus of stria terminalis
pMeA	Posterior medial amygdala
PND	Postnatal day

PSD-95	Post-synaptic density protein 95
PVN	Paraventricular nucleus of the hypothalamus
PVThal	Paraventricular nucleus of the thalamus
rIso+Fam	Repeated isolation and return to a familiar cage partner
rIso+Unf	Repeated isolation and return to an unfamiliar cage partner
S.E.M.	Standard error of the mean
SS	Social instability stress
V1AR	Vasopressin 1a receptor
V1BR	Vasopressin 1b receptor
vLS	Ventral lateral septum
VMH	Ventromedial hypothalamus
VTA	Ventral tegmental area

Preface

Exposure to species-typical social experiences in adolescence is important for the development of social brain regions and for the ability to display correct social behaviours in the future. The main goal of my thesis was to manipulate the quality of social experience (rather than the quantity) by repeatedly pairing adolescent rats with unfamiliar peers (social instability) to investigate effects on hypothalamic-pituitary-adrenal (HPA) function, social behaviour, and changes in the structure and function of social brain regions in days soon after the stress procedure. A general summary of my thesis findings is in the form of a table in chapter 6 (see Table 6-1). In the first part of chapter 1, I define several social behaviour tests that are frequently used in investigations with rodents, and I review four of the main brain regions that develop during adolescence and that are involved in the initiation and maintenance of several social behaviours (lateral septum, medial amygdala, prefrontal cortex, hippocampus). Because social instability is a stessor and delays the return of corticosterone (released in response to stress exposure) concentrations to baseline after exposure to social isolation, I describe the development of the HPA axis in adolescence. In the second section of chapter 1, I review the past findings of adolescent social instability on HPA function, social behaviour, and changes in the brain. I describe the goals of my thesis in the final section of chapter 1.

Chapter 1: General Introduction

This chapter has been adapted from the textbook chapter:

Hodges, T. E. & McCormick, C. M. (In press). Stress and social development in a rodent model. Chapter in O. Schultheiss & P. Mehta (Ed.) *'International handbook of social neuroendocrinology'*. Routledge, Taylor & Francis, UK.

Author contribution: I helped plan how the chapter would be organized, I helped with summarizing the literature, I created all of the illustrations, I wrote half of the chapter (section A), I provided input on the content of the second half of the chapter, and I edited the entire chapter after feedback was provided.

Introduction

Neuroscientists (Cajal, 1967) and psychologists (Beach, 1950) have long recognized the importance of a comparative approach to understanding brain and behaviour relationships. The primary contribution of investigations in non-human animals for research in humans and vice versa is the generation of testable hypotheses and principles of development (Gottlieb and Lickliter, 2004). Nevertheless, research studies with animal models are increasingly conducted predominantly in rodents. Because of the increasing interest in the function of social brain regions in human adolescence and how stressors may influence their development, I review here, first, the typical social behaviour of adolescent laboratory rats, how social behaviour is measured, and the development of social brain regions in rats. Second, I review evidence of how social stress exposures (involving the social instability stress model) in adolescence influences social development. Third, I explain the overarching goal of my thesis and the specific questions to be answered in chapters 2-5. The majority of this research was conducted in males, and results described will be for males unless otherwise noted. McCormick and colleagues have reviewed the benefits and challenges of the use of rats for translational research on the consequences of stressors in adolescence (McCormick, Green, and Simone, 2017b), and a greater discussion of the parallels and differences in adolescent development between the two species can be found in that review. For a review of other models of social stress used with adolescent rats, see Burke and colleagues (Burke, McCormick, Pellis, and Lukkes, 2017).

A. Adolescence, social behaviour, and hypothalamic-pituitary-adrenal function in rats

Defining adolescence in rats

The specific ages in rats that define the adolescent period are variable across studies and across strains of rats. Although a liberal definition starts the adolescent period in rats at weaning from the mother (typically on postnatal day [PND] 21) and ends the adolescent period on PND 59 (with sexual maturity attained at about PND 60), this definition involves a lengthy pre-pubertal period. In contrast, in humans, adolescence is defined as beginning at puberty (reviewed in McCormick et al., 2017b). In rats, the physical markers of puberty appear in males at around PND 40 – 47 (as marked by separation of the prepuce from the glans penis, i.e. balanopreputial separation, and sperm in the epididymis) and in females at around PND 32 – 36 (as marked by vaginal opening and first ovulation) (reviewed in McCormick et al., 2017b). Throughout this chapter, I will define the ages of PND 30 – 45 as mid-adolescence in rats and PND 60 and beyond as adulthood.

The measurement of social behaviour in rats

Across development, engaging in positive social interactions with members of the same species promotes health by enhancing resilience to stressors (social buffering) (reviewed in Hostinar, Sullivan, and Gunnar, 2014). In a laboratory setting, social interaction tests involve observing and scoring two or more rats as they perform several social behaviours with each other (i.e., social investigation, following, grooming of peer, sniffing, social play, lying flat or standing still while maintaining physical contact with partner) compared to non-social behaviours (i.e., exploring, inactivity without touching peer, self-grooming, as in Cirulli, Terranova, and Laviola, 1996) in either a familiar or novel environment. It is also common for rats to spend more time investigating

unfamiliar rats than investigating rats with which they are familiar (Cirulli et al., 1996; Perkins et al., 2016). Tests that involve recording the time a rat spends in proximity to another rat but for which no active interaction is possible (e.g., the stimulus rat is behind wire mesh) have been referred to as tests of social interaction, but are perhaps better termed as tests of social approach (see Figure 1-1 for depictions of social interaction and social approach tests). Social interaction tests also are used as a measure of social anxiety in rats and other mammals; a decrease in social interaction from the norm is interpreted as an increase in anxiety (reviewed in File and Seth, 2003). Nevertheless, the reduction in social interaction may be secondary to a general increase in fear or anxiety rather than a reduction in sociality. For example, bright lighting is anxiogenic for rats, and lighting conditions can thereby increase or decrease "social anxiety" (File and Hyde, 1978). Further, social interaction is dissociable from social reward; for example, previous research has found that rats that have decreased social interaction in tests allowing for social contact will show no decrement in social approach when the peer is behind wire mesh (e.g., Green, Barnes, and McCormick, 2013).

The primary social behaviour in adolescent rats is social play with peers. Social play is found across many species and improves social, emotional, and cognitive skills (reviewed in Pellis and Pellis, 2017; Pellis, Pellis, and Himmler, 2014). This increase in interaction with peers parallels the social restructuring in human adolescents, who increasingly value peer interactions over family interactions (reviewed in Nelson, Leibenluft, McClure, and Pine, 2005). Although there are some differences in the development of social play across strains of rats (Himmler et al., 2013b; Himmler et al., 2014b; Ku et al., 2016; Siviy, Love, DeCicco, Giordana, and Seifert, 2003), same-sex

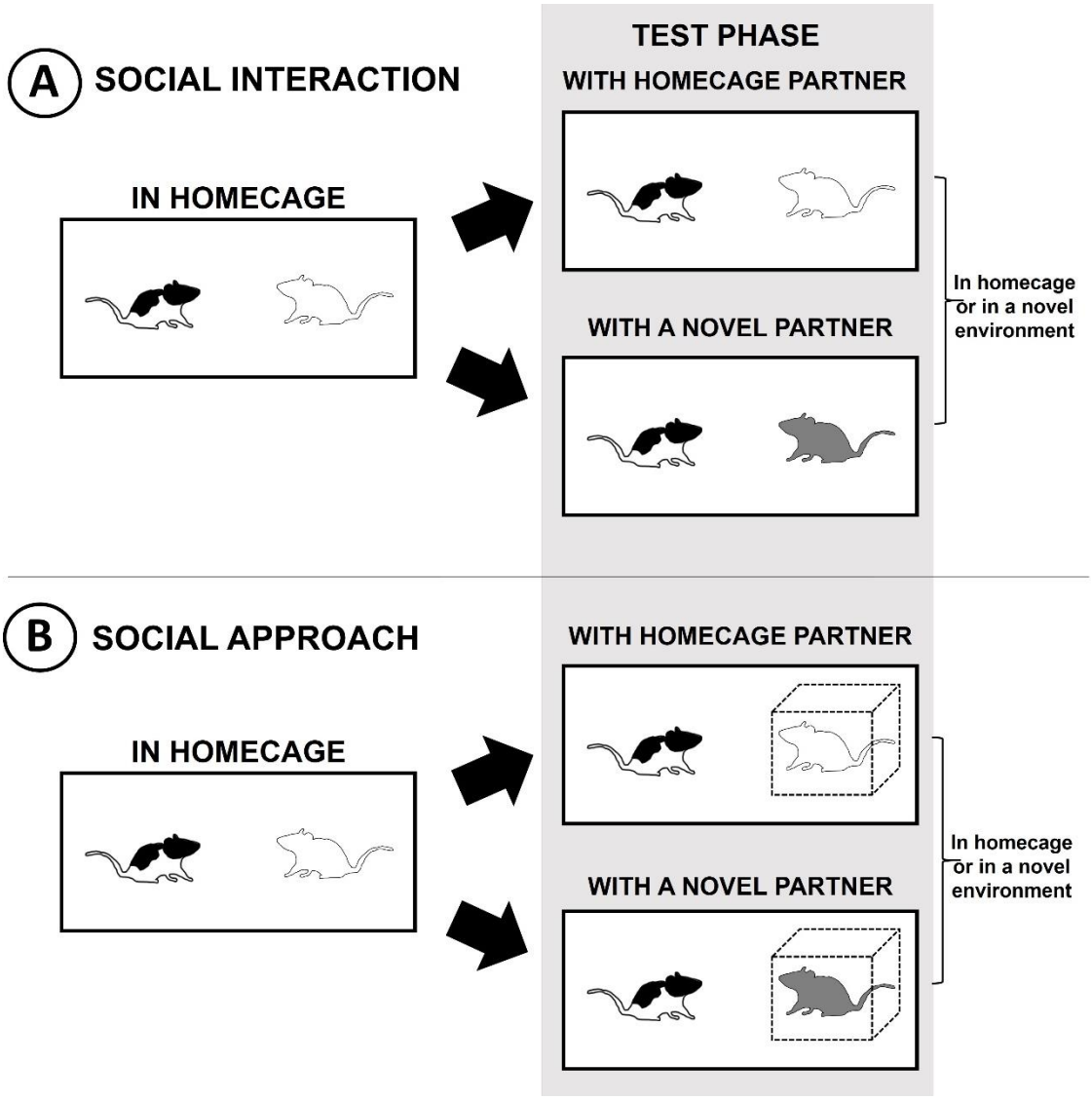

Figure 1-1. Illustrations of (A) the Social Interaction Test and the (B) Social Approach Test procedures in which (A) time spent in physical interaction is measured or (B) time spent near the barrier between the rats is measured in the Test Phase.

play and social investigatory behaviours are consistently higher in adolescent rats than in adult rats (Klein, Padow, and Romeo, 2010; Perkins et al., 2016; Primus and Kellogg, 1989). Further, a short period of isolation increases social play behaviours to a greater extent in adolescent rats (PND 40 – 45) than it does in young adult rats (PND 67 – 72) (Lampe, Burman, Würbel, and Melotti, 2017).

Social play in adolescence is critical for normal adolescent behavioural development into adulthood. For example, rats housed without a cage partner (social isolation) for 13 or more days during adolescence displayed abnormal behaviour in situations of conflict (Einon and Potegal, 1991; Tulogdi et al., 2014; van den Berg et al., 1999a), had impaired object memory (Einon and Morgan, 1976; Einon and Potegal, 1991), had reduced social interaction with a peer (van den Berg et al., 1999a; Lukkes, Mokin, Scholl, and Foster, 2009), increased anxiety-like behaviour (Wright, Upton, and Marsden, 1991), exhibited awkward behaviour with a sexual partner in adulthood (van den Berg et al., 1999a), showed increased conditioned fear (Lukkes, Mokin, Scholl, and Foster, 2009), displayed greater aggressive behaviour with peers (Wall, Fischer, and Bland, 2012), and had impaired social recognition memory (Shahar-Gold, Gur, and Wagner, 2013). Social play behaviours (defined by chasing, wrestling, pinning, pouncing, role reversals) emerge as early as PND 17 (Bolles and Woods, 1964), peak in early- to mid-adolescence (Klein, Padow, and Romeo, 2010; Panksepp, Siviy, and Normansell, 1984; Takahashi and Lore, 1983), and then change in function into precopulatory behaviours (e.g., male-male competition) in adulthood (see review by Pellis and Pellis, 2017). Moreover, the wrestling behaviours that occur during play fighting in adolescence decrease, and more aggressive behaviours (e.g., boxing) increase into adulthood (Meaney

and Stewart, 1981). In addition to the age differences in social interaction and social play, there are sex differences in the development of social behaviours in adolescent rats. Social play behaviours peak earlier in adolescent male rats than in adolescent female rats, and adolescent males engage in more social interaction than do adolescent females (Meaney and Stewart, 1981). In addition, social behaviour becomes more opposite-sex-oriented around the timing of puberty (Meaney and Stewart, 1981). Some of the sex differences in social play involve gonadal hormones; for example, the depletion of testosterone in circulation by gonadectomy in early adolescence reduced the peak in social play behaviour that is typical of mid-adolescent male rats (Cooke and Woolley, 2009).

Play behaviour is also a highly rewarding feature of social interactions with peers in adolescence, and adolescent rats find social play more rewarding than do adult rats (Douglas, Varlinskaya, and Spear, 2004; Yates, Bechmann, Meyer, and Bardo, 2013). In rats, one measure of the reward value of social interactions (and of other stimuli) in the laboratory is the conditioned place preference (CPP) test. In CPP tests that measure social reward, rats are exposed to two or more distinct contexts (i.e., that differ in floor and wall tactile, visual, and sometimes olfactory cues), with one context paired with a socially active peer and a second context paired with either a non-social peer (Calcagnetti and Schechter, 1992), a peer restricted behind a mesh or glass screen (Kummer et al., 2011), an object (Hodges et al., 2017), or no stimuli (Douglas et al., 2004; Peartree et al., 2012; Thiel, Okun, and Neisewander, 2008; Trezza, Damsteegt, and Vanderschuren, 2009; Yates et al., 2013; see Figure 1-2 for depictions of CPP tests). After exposure to each social or non-social context for a number of days (conditioning), rats are free to explore

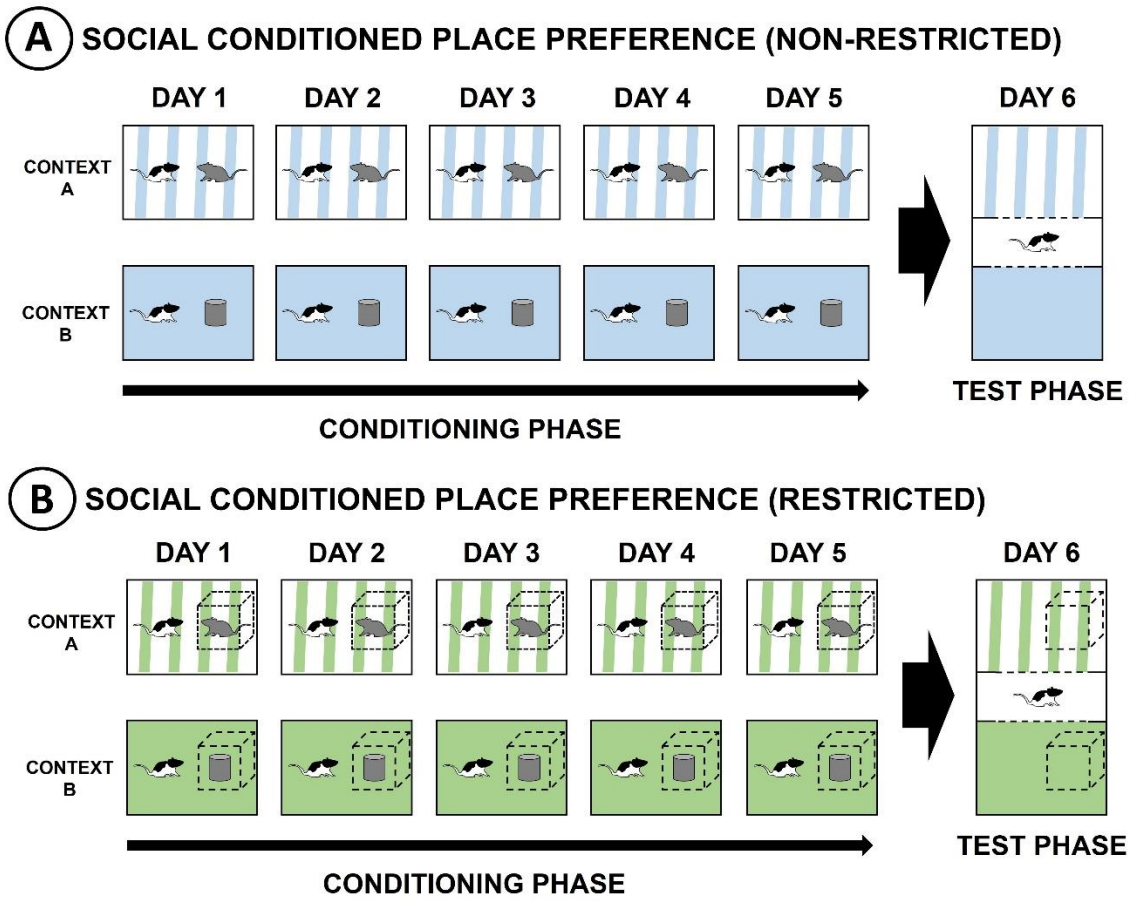

Figure 1-2. Illustrations of conditioned place preference procedures for conditioning between one context and a social stimulus and between another context and a non-social stimulus. One test allows for unrestricted physical contact with the stimuli (A) and one test does not allow for direct physical context (B). In the Test Phase, the time spent in one context versus the other is the index of preference for a social or non-social stimulus.

both contexts in the absence of the social and non-social stimuli (test phase); the difference in time spent in one context versus the other is indicative of the extent to which the social context was rewarding. In general, adolescent rats spend more time in a place that has become associated with social interaction than in control places.

Social cognition, which involves perceiving, recognizing, and evaluating social stimuli before performing a social behaviour, also undergoes change in adolescence in rats, with social recognition being the most used measure (reviewed in McCall and Singer, 2012; van der Kooij and Sandi, 2012). Social recognition is tested with habituation/dishabituation or social discrimination paradigms (see Figure 1-3 for depictions of these paradigms). The social habituation/dishabituation paradigm involves first presenting the test rat with the same stimulus rat multiple times, and then presenting the test rat with a novel stimulus rat (reviewed in Gabor, Phan, Clipperton-Allen, Kavaliers, and Choleris, 2012). During social habituation/dishabituation, typically the test rat will show habituation (a reduction in social investigation) to the first stimulus rat after each presentation, and then a dishabituation response (a return to initial social investigation levels) to the novel stimulus rat. A more direct measure of social recognition is the social discrimination paradigm. The social discrimination paradigm of social recognition consists of presenting the test rodent with one or two stimulus rodents that are either socially restricted (kept behind either transparent plastic or wire mesh, e.g., Gur, Tendler, and Wagner, 2014) or free to socially interact (e.g., Markham and Juraska, 2007; Veenema, Bredewold, and De Vries, 2012). The test phase occurs after this familiarization phase and after a time interval without the stimulus rat(s) present. The test phase involves presenting the test rat with one stimulus rodent from the familiarization

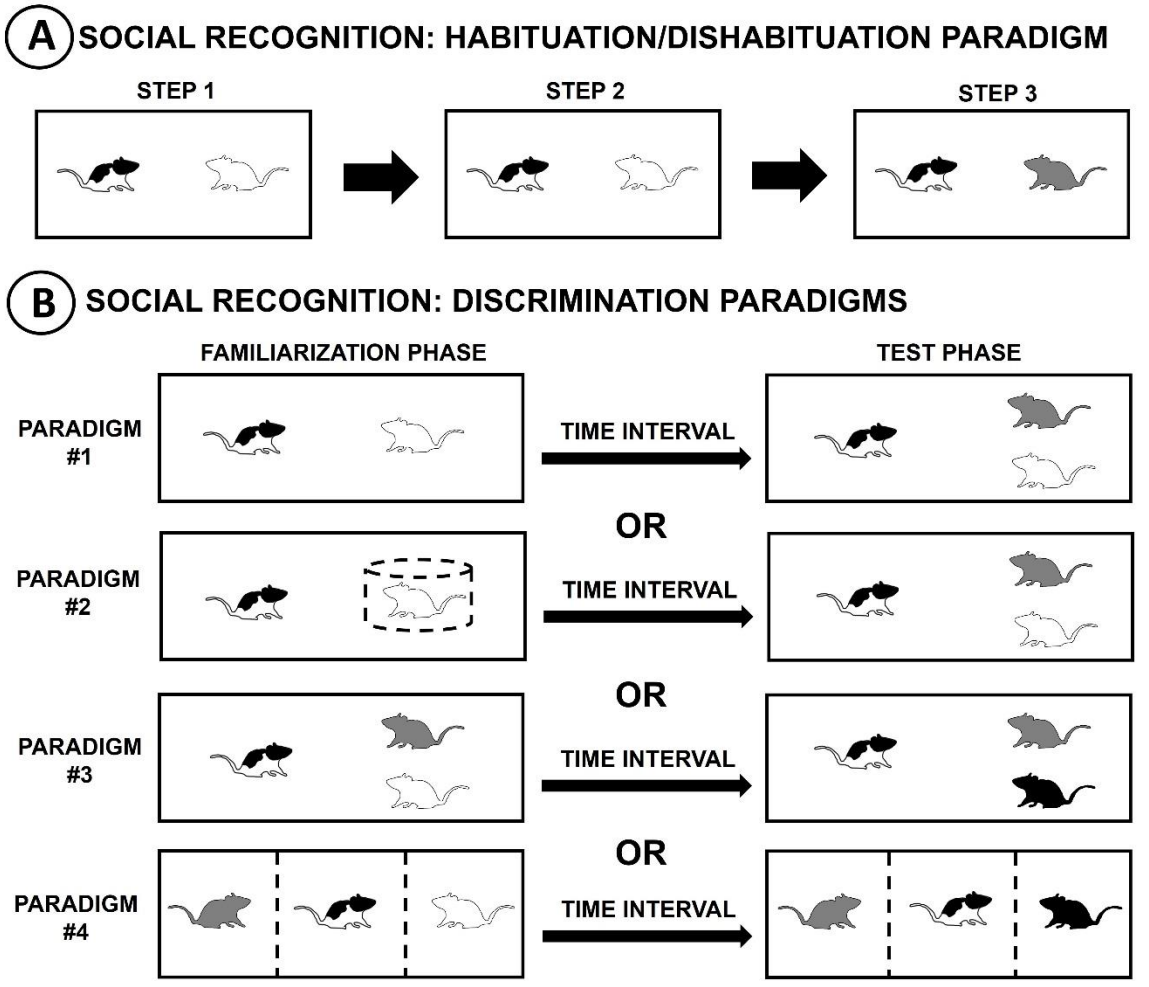

Figure 1-3. Illustrations of Social Recognition Test procedures. In (A), in Step 1, time spent investigating a novel rat is measured. In Step 2, a reduction in time spent investigating (habituation) the rat from Step 1 is expected. In Step 3, a greater time spent investigating (dishabituation) a new novel rat than that spent investigating the rat in Step 2 is the index of social recognition. In (B), Discrimination paradigms of social recognition involve a familiarization phase to either one or two rats with which physical contact is possible (paradigms #1 and #3) or not (paradigms #2 and #4). In the Test Phase, a novel rat is introduced (paradigm #1 and #2) or replaces a rat from the familiarization phase (paradigms #3 and #4), and a preference for investigating the novel vs the familiar rat is the measure of social recognition.

phase and a novel stimulus rodent. The social discrimination paradigm relies on rats' preference for novelty, with rats typically spending more time engaging with novel versus familiar stimuli (reviewed in Gabor et al., 2012). Thus, a rat is said to have exhibited social recognition when they spend more time investigating the novel stimulus rat than the familiar stimulus rat. Social recognition performance declines with age in rats (Markham and Juraska, 2007; Perkins et al., 2016; Prediger, Batista, and Takahashi, 2005). Typically social recognition after a four to five minute familiarization phase is absent after a two-hour interval (Dantzer, Koob, Bluthé, and Le Moal, 1988; Landgraf et al., 1995; Veenema et al., 2012). Social recognition after intervals longer than two hours can be observed when the familiarization phase is longer.

Developmental changes in the nervous system underlie the transitions in social behaviour that occur from the juvenile to adolescent period, and engaging in social behaviour promotes the brain development required to support a complete and appropriate social repertoire in adulthood. The development of social brain regtions in rats is described in the next section.

Social brain regions and their development in adolescence

Social brain regions in mammals consist of the crosstalk between neural structures involved in determining social identity (accessory olfactory bulb), social salience (amygdala, prefrontal cortex), the motivation to perform a social behaviour (ventral tegmental area, nucleus accumbens, ventral pallidum), and the execution of the social behaviour (hypothalamus, medial preoptic area, motor and autonomic pathways) (reviewed in Insel and Fernald, 2004). Through projections from and to these brain regions, the medial amygdala plays a prominent role in social behaviour; for example,

projections from olfactory systems to the medial amygdala and its projections to the lateral septum are key circuits for social recognition in rodents (reviewed in Maroun and Wagner, 2016). To discuss the development and involvement of each of these brain regions in the adolescent period in length is beyond the scope of this chapter, and there are reviews of adolescent rodent brain development elsewhere (e.g., Brenhouse and Andersen, 2011; Crews, He, and Hodge, 2007; Juraska and Willing, 2017; Spear, 2000). I focus on four brain regions that are critical for the initiation and pattern of normative social behaviour in rats: the prefrontal cortex (PFC), the hippocampus, the medial amygdala, and the lateral septum (see Figure 1-4, and see Table 1-1 for each regions' role in social behaviour). Further, although social behaviour involves numerous neurotransmitter systems, and there are notable changes in neurotransmitter systems in adolescence (e.g., Tarazi. Tomasini, and Baldessarini, 1998; Teiche, Andersen, and Hostetter Jr., 1995; Trauth and Slotkin, 2000; Yu, Wang, Fritschy, Witte, and Redecker, 2006), I focus on changes in the neuropeptides oxytocin and vasopressin and their receptors from adolescence to adulthood. Although oxytocin and vasopressin are implicated in anxiety and physiological responses to stressors, they are best known for their role in the regulation of social behaviour, including aggression, maternal behaviour, sexual behaviour, social preference, and social cognition (reviewed in Maroun and Wagner, 2016; Neumann and Landgraf, 2012).

Oxytocin and Vasopressin. Oxytocin and vasopressin are peptides synthesized primarily in the supraoptic and paraventricular nuclei of the hypothalamus. Oxytocin may be synthesized in some other hypothalamic and extra-hypothalamic sites, and vasopressin is also synthesized in the bed nucleus of the stria terminalis, the medial amygdala, the

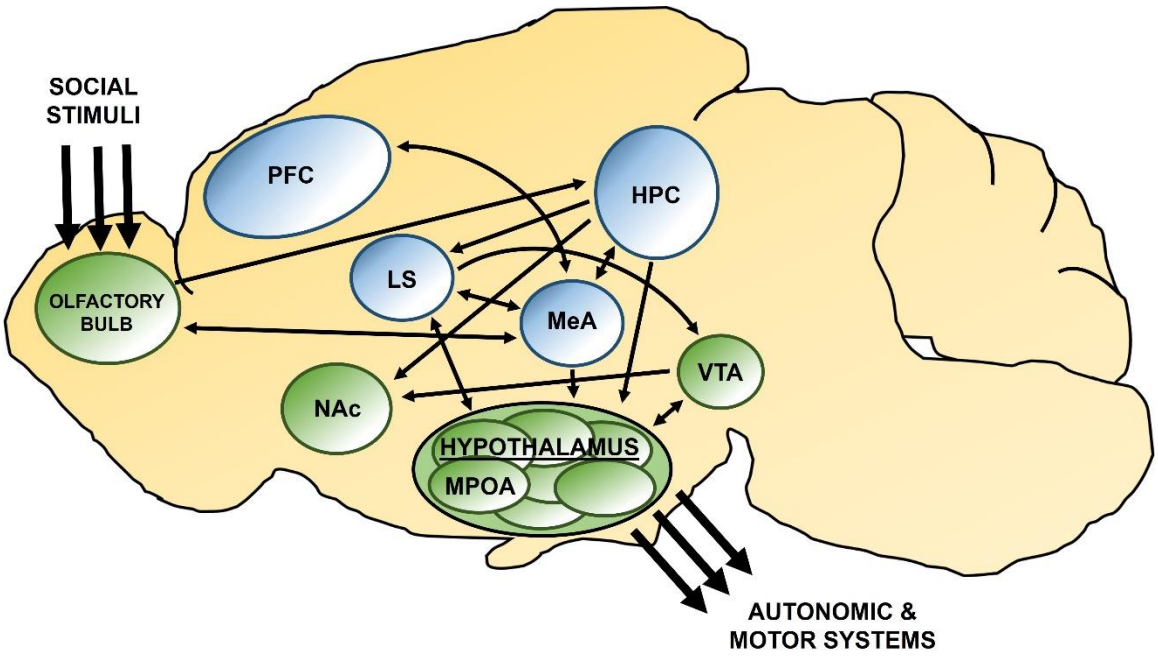

Figure 1-4. Cartoon of a sagittal view of the rat brain illustrating the main brain regions involved in social behaviour. Neural regions coloured in blue are those on which this review focuses. Arrows indicate the main pathways between the regions. PFC = prefrontal cortex; LS = lateral septum; NAc = nucleus accumbens; HPC = hippocampus; MeA = medial amygdala; MPOA = medial preoptic area; VTA = ventral tegmental area.

Table 1-1. Main role of brain regions in social behaviours.

Social roles of the brain regions of interest.	
Prefrontal cortex	coordination of behaviour with a peer[2,6], sexual interest[12], maternal behaviour[15], social interaction[16]
Hippocampus	aggressive behaviour[1,14], behavioural sequencing[10], performing specific social behaviours[9], social recognition (CA2 subregion)[7,17], submissive behaviour[10]
Medial amygdala	aggressive behaviour[8,13], forming a social hierarchy[18], sexual behaviour[8,13], maternal behaviour[8,13], social interaction[11], social recognition[5]
Lateral septum	affiliative behaviour[9], social fear behaviour[20], social interaction[4,19], social recognition[3]

See examples in [1]Becker et al., 1999; [2]Bell et al., 2009; [3]Bielsky et al., 2005; [4]Bredewold et al., 2014; [5]Gur et al., 2014; [6]Himmler et al., 2014a; [7]Hitti and Siegelbaum, 2014; [8]Knapska et al., 2007; [9]Kolb and Nonneman, 1974; [10]Maaswinkel et al., 1997; [11]Murakami et al., 2011; [12]Nakajima et al., 2014; [13]Newman, 1999; [14]Pagani et al., 2015; [15]Sabihi et al., 2014; [16]Sabihi et al., 2017 [17]Smith et al., 2016; [18]Timmer et al., 2011; [19]Veenema et al., 2013; [20]Zoicas et al., 2014.

locus coeruleus, and the olfactory bulb (Veenema, 2012). Oxytocin exerts its effects in the brain primarily through binding at oxytocin receptors (OTR), whereas vasopressin exerts its effects in the brain at 1a and 1b receptor subtypes (V1AR and V1BR, respectively) (actions in the periphery also involve these, as well as additional, receptor subtypes) (Dumais and Veenema, 2016). OTR expression is evident in embryonic rats by about day 15 (Tribollet et al., 1989). Although OTRs and V1ARs are widely distributed in the central nervous system, V1BRs are limited primarily to the Cornu Ammonis 2 (CA2) region of the hippocampus, the paraventricular nucleus, and the olfactory bulb (reviewed in Stevenson and Caldwell, 2012). OTRs increase from adolescence to adulthood in the prefrontal cortex and medial amygdala, and V1ARs in the dentate gyrus of the hippocampus decrease from adolescence to adulthood (Smith, Poehlmann, Li, Ratnaseelan, Bredewold, and Veenema, 2017b). In the lateral septum, OTRs decrease in the ventral part and V1ARs increase in the dorsal part from adolescence to adulthood (Smith et al., 2017b). Early life experiences, particularly experiences in the neonatal period, are known to modify oxytocin and vasopressin systems into adulthood in rats (see reviews by Vaidyanathan and Hammock, 2017; Veenema, 2012), but little is known about effects of adolescent social experiences on the ongoing development of these systems.

Prefrontal cortex. The developmental onset and the display of specific social behaviours are not dependent on the cortex; removal of the cortex or damage to the cortex does not alter the peak of social play in adolescent rats nor reduce the social repertoire of rats (Pellis and Pellis, 2017; Pellis, Pellis, and Bell, 2010). Nevertheless, social contact is reduced in rats with lesions to the PFC (Kolb and Nonneman, 1974) and

the context-dependent coordination of behaviour with a peer requires the PFC across development (Bell, McCaffrey, Forgie, Kolb, and Pellis, 2009; Himmler et al., 2014a). Several social behaviours are modified by oxytocin in the PFC. For example, oxytocin in the medial PFC was a critical factor in the greater reduction of fear in rats when with a peer than when alone (Brill-Maoz and Maroun, 2016), OTR in the medial PFC were required for female mice to show sexual interest in males (Nakajima, Görlich, and Heintz, 2014), and OTR blockade attenuated maternal behaviour in postpartum rats (Sabihi, Dong, Durosko, and Leuner, 2014). Anxiolytic effects of oxytocin administered to the prelimbic region of the medial PFC (Sabihi, Dong, Maurer, Post, and Leuner, 2017) may lead to increased social interaction.

Changes in dendritic morphology in the PFC from adolescence to adulthood have been described in rats. For example, dendritic spines in the PFC peak at PND 31 and thereafter decrease into adulthood (Gourley, Olevska, Warren, Taylor, and Koleske, 2012; Koss, Belden, Hristov, and Juraska, 2014). Moreover, white matter increases in the medial PFC (indicative of myelination) from adolescence to adulthood (Markham, Morris, and Juraska, 2007; Willing and Juraska, 2015). There are also sex differences in the adolescent development of the medial PFC, and these involve the sex differences in sex steroids in circulation that increase in adolescence. Neurons in the adolescent female medial PFC decrease from PND 35 to PND 45 and further into adulthood, but no decrease is found in adolescent males (Markham et al., 2007; Willing and Juraska, 2015) or in ovariectomized adolescent females (Koss et al., 2014), suggesting a role of ovarian hormones in apoptosis in the medial PFC.

Depriving adolescent rats of social behaviour can alter the morphological development (Bell, Pellis, and Kolb, 2010; Himmler, Pellis, and Kolb, 2013a) and function (Wall et al., 2012) of the orbitofrontal and prefrontal cortices. For example, dendritic basilar branching (dendritic branching allows for greater neural connectivity) in the orbitofrontal cortex was greater in adolescent rats housed with multiple peers than in those housed alone, and dendritic apical branching in the medial PFC was greater in adolescent rats housed singly than in adolescents that were socially-housed (Bell et al., 2010). Further, rats housed with adult rats during adolescence (thus spent less time in social play) had greater basilar and apical dendritic spines, which are critical for efficient signal transmission, in the medial PFC and orbitofrontal cortex than did rats housed with other adolescents (Bell et al., 2010). Functional differences in these regions were investigated by measuring changes in expression of the immediate early gene protein products such as Fos and Arc; increased expression these proteins is a marker of neural activation. For example, exposure to a novel peer in adolescence increased neural activation in the medial PFC of group-housed adolescent rats but not in singly-housed adolescent rats (Wall et al., 2012).

Hippocampus. There is little neuronal activation in the hippocampus in rats while in social interaction (von Heimendahl, Rao, and Brecht, 2012). Nevertheless, the hippocampus is essential for the performance of several social behaviours. In turn, social behaviours influence the development of the hippocampus. Rats deprived of social interaction had a smaller hippocampal volume compared with rats housed with peers or single-housed rats briefly exposed to a conspecific each day (Kalman and Keay, 2017), and lesions to the hippocampus reduced the time rats spent in social contact with a peer

(Kolb and Nonneman, 1974). The hippocampus, like the PFC, has a role in social coordination, but in rodents it has a greater involvement in the correct sequencing of behaviours during social interactions with a partner than does the PFC. For example, rats with damage to the hippocampus displayed a different set of behaviours in response to an affiliative approach by a novel peer than did control rats; specifically, behaviour became less dependent on that of the peer, and the predictability of aggressive behaviour was diminished (Kolb and Nonneman, 1974). Damage to the hippocampus also increased the amount of aggressive behaviour displayed in social interaction (Becker, Grecksch, Bernstein, Höllt, and Bogerts, 1999; Kolb and Nonneman, 1974; Maaswinkel et al., 1997). The CA2 subfield of the hippocampus is essential for social recognition (Alexander et al., 2016; Hitti and Siegelbaum, 2014; Smith, Avram, Cymerblit-Sabba, Song, and Young, 2016). Further, activation of vasopressinergic neurons in the CA2 region during the familiarization phase (but not the retrieval phase) increased the retention of the social recognition memory from under two hours to seven days (Bluthé, Gheusi, and Dantzer, 1993; Smith et al., 2016). In addition, knocking out V1BRs in the CA2 region reduces social aggression in mice (Pagani et al., 2015; Wersinger, Ginns, O'Carroll, Lolait, and Young, 2002).

In contrast to the peak and pruning of dendritic spines in the PFC that occurs during adolescence, the spine density and length of dendrites in the hippocampus increases from weaning to adulthood in male rats (Gourley et al., 2012; Pokorný and Yamamoto, 1981). Conversely, a reduction in hippocampal dendritic spines between the pre- and post-pubertal period was found in females (Yildirim et al., 2008), consistent with other reports of sex differences in the development of the hippocampus (e.g., Roof and

Havens, 1992). In addition, neurogenesis (formation of new neurons) decreases in the hippocampus from adolescence to adulthood (He and Crews, 2007).

Medial amygdala. The medial amygdala has a key role in several social behaviours (including aggressive behaviour, sexual behaviour, social interaction, social odor processing) (reviewed in Knapska, Radwanska, Werka, and Kaczmarek, 2007; Newman, 1999). OTR and V1AR messanger ribonucleic acid (mRNA) expression in the medial amygdala is positively associated with time spent in social interaction in mice (Murakami, Hunter, Fontaine, Ribeiro, and Pfaff, 2011). Oxytocin in the medial amygdala in males has proven to be essential for the maintenance of memory for familiar females in a social recognition paradigm (Lukas, Toth, Veenema, and Neumann, 2013). Protein synthesis and oxytocin-dependent long-term-depression in the medial amygdala is necessary for the consolidation of long-term (24 hour interval) but not short-term (30 minute interval) social recognition (Gur et al., 2014). In addition, the ability to form a lasting social hierarchy (dominant-subordinate relationship) between rats is dependent on the expression and the activation of OTR in the medial amygdala after their first social encounter (Timmer et al., 2011).

At birth, males and females have the same size of medial amygdala, and its volume becomes larger in males than in females around the beginning of the adolescent period (Hines, Allen, and Gorski, 1992; Mizukami, Nishizuka, and Arai, 1983). The medial amygdala expresses many androgen and estrogen receptors (reviewed in Newman, 1999), and gonadal hormones, the concentrations in circulation of which increase significantly in adolescence, modulate the morphology and functionality of the medial amygdala. For example, the removal of the male gonads in adolescence or

adulthood reduces the volume, number of the dendritic spines, and number of excitatory synapses in the medial amygdala, and results in deficits in male sexual behaviour (Cooke, Breedlove, and Jordan, 2003; Cooke and Woolley, 2009; Zancan, Dall'Oglio, Quagliotto, Rasia-Filho, 2017).

Lateral septum. The lateral septum modulates the motivation for affiliative behaviours with peers and for mating (Luo, Tahsili-Fahadan, Wise, Lupica, and Aston-Jones, 2011; Sartor and Aston-Jones, 2012). Moreover, lesions to the lateral septum increase the non-social and retreating behaviours of rats when with a peer (Kolb and Nonneman, 1974). Oxytocin and vasopressin are involved in the lateral septum's modulation of the motivation for social play in adolescent rats, and in both a sex- and context-specific manner. Oxytocin administered into the lateral septum reduced social play behaviour in adolescent females but not in adolescent males (Bredewold, Smith, Dumais, and Veenema, 2014), and administering a V1AR antagonist to the lateral septum increased social play behaviour in adolescent males and decreased social play behaviour in adolescent females, but only in the home cage and not in a novel environment (Bredewold et al., 2014; Veenema, Bredewold, and De Vries, 2013). As for the medial amygdala, oxytocin in the lateral septum may be essential for social recognition; the administration of an OTR antagonist immediately after the familiarization phase in the lateral septum abolished social recognition (Lukas et al., 2013). Moreover, the overexpression of V1AR in the lateral septum improved social recognition after intervals of 2 – 24 hours, whereas the reduction of V1AR in the lateral septum reduced social recognition (Bielsky, Hu, Ren, Terwilliger, and Young, 2005; Landgraf et al., 1995). In addition, the lateral septum plays a role in both social and non-social anxiety-like

behaviours (Cordero, Just, Poirier, and Sandi, 2016; Liebsch, Montkowski, Holsboer, and Landgraf, 1998), and OTR in the dorsal lateral septum are involved in the acquisition and extinction of social fear behaviour (reduced social investigation after the conditioned pairing of social stimuli with a foot-shock). For example, OTR expression in the dorsal lateral septum increased during social fear conditioning and oxytocin injection into the dorsal lateral septum during extinction abolished social fear behaviour (specifically, increased social investigation) (Zoicas, Slaterry, and Neumann, 2014).

Neuronal density in the lateral septum increases from birth to about PND 14 and thereafter decreases until adulthood (Verney, Gaspar, Alvarez, and Berger, 1987). In the lateral septum, the density of vasopressinergic fibres becomes adult-like in males as early as PND 17, whereas the density of vasopressinergic fibres increase from adolescence to adulthood in females (De Vries, Buds, and Swaab, 1981). In addition, adolescent males have greater vasopressinergic fibers in the lateral septum than do adolescent females, and this sex difference is dependent on the presence of androgens in early life (De Vries, Best, and Sluiter, 1983; De Vries et al., 1981).

Maturation of the hypothalamic-pituitary-adrenal axis in adolescence

Glucocorticoids (primarily corticosterone in rats and cortisol in humans) are released in response to stressors as the endpoint of activation of the HPA axis, which involves the release of corticotropin releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, and the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. The actions of glucocorticoids are mediated to a large extent through actions at their cognate intracellular receptors, the glucocorticoid receptors and mineralocorticoid receptors (GR and MR, respectively). Adolescent rats do not differ

from adult rats for many features of the HPA axis: morphology of the paraventricular nucleus, expression of GR and MR mRNA and protein in the paraventricular nucleus and hippocampus, basal ACTH, basal corticosterone, and concentrations of corticosteroid binding globulin (reviewed in Eiland and Romeo, 2013; Green and McCormick, 2016). Nevertheless, adolescent rats have a greater and/or more prolonged release of corticosterone in response to several (but not all) stressors (reviewed in McCormick et al., 2017b; Romeo, Patel, Pham, and So, 2016). Some features of the HPA axis continue to mature from adolescence to adulthood; prepubertal adolescent male rats had greater restraint-stress induced arginine vasopressin (AVP) heteronuclear RNA expression in the paraventricular nucleus (Viau, Bingham, Davis, Lee, and Wong, 2005); this source of AVP is known to increase HPA stress responses in adults, and thus increased AVP in young rats may be a basis for their higher stress responses than that in adults. The adrenals of prepubertal adolescent male rats had both a greater expression of adrenal melanocortin 2 receptor accessory protein mRNA (to which ACTH binds) and a greater sensitivity to ACTH (Romeo et al., 2014) than did adult male rats, which also is a likely basis for their increased release of corticosterone in response to stressors compared with adults. Further, prepubertal adolescent rats have greater expression of FKBP4 mRNA (which encodes FK506 binding protein 52 to promote the translocation of GR to the nucleus) in the hippocampus than do adult rats (Green, Nottrodt, Simone, and McCormick, 2016), although the functional significance of this increase in prepubertal rats is unknown.

In adult rats, there are clear sex differences in the regulation of the HPA axis; basal estradiol stimulates the release of corticosterone in adult females and basal

testosterone inhibits the release of corticosterone in adult males (reviewed in Handa and Weiser, 2014; McCormick and Mathews, 2007). Nevertheless, gonadal hormone concentrations do not seem to be the basis of the sex differences in the prepubertal period (Romeo, Lee, and McEwen, 2004), and there is some evidence that gonadal hormones may regulate HPA function differently in post-pubertal adolescents than in adulthood (Green and McCormick, 2016). The regulation of adolescent HPA function by gonadal hormones is not well understood.

B. The effects of social instability stress

Social instability stress in adolescence as a means of investigating the malleability of social development

The laboratory of Dr. Cheryl McCormick first began investigating the long-lasting consequences of stress exposures experienced in adolescence in the early 2000s. At that time, there were few investigations of HPA function in response to an acute stressor in adolescence in rats, and even fewer that involved repeated stress exposures (reviewed in McCormick and Mathews, 2007). The choice of a relatively mild social stress procedure was an attempt to obtain effects that might be specific to adolescence; social stressors such as social defeat and isolation housing can result in long-lasting effects in adult rats (Buwalda, Geerdink, Vidal, and Koolhaas, 2011; Einon and Morgan, 1977; Panksepp and Beatty, 1980). Further, although often referred to as a social stressor, isolation housing does not produce the steep rise and prolonged elevation in glucocorticoids that is characteristic of other stress exposures, and instead may involve dysfunction resulting from a lack of stimulation (reviewed in Green and McCormick, 2013b).

The social stress procedure of Dr. McCormick was designed to have two components, an isolation/confinement component and a social instability component, with the procedures administered daily for 16 days covering the range of mid-adolescence. Although the first two publications of her research involved PND 33 – 48 (McCormick, Robarts, Gleason, and Kelsey, 2004; McCormick, Robarts, Kopeikina, and Kelsey, 2005), she subsequently changed the days to PND 30 – 45, which provides more days after the procedure in which to conduct behavioural testing while the animals are still in adolescence. The one hour isolation/confinement component was chosen because it elevates corticosterone concentrations in adolescents for the duration of the session (and there is a rapid recovery to baseline, consistent with milder stress exposures) (McCormick et al., 2001; McCormick, Kehoe, Mallinson, Cecchi, and Frye, 2002) and because of the similarity of the procedure to one of the most commonly used "psychological" stress procedures in adult rats, restraint stress (involves confinement to a tube-like container of dimensions that restrict rat movements). Adolescents and adults do not differ significantly in corticosterone release to one session of one hour of isolation/confinement (Hodges, Green, Simone, and McCormick, 2014). Neonatal rats show a greater release of corticosterone to repeated isolation/confinement compared with age-matched rats undergoing a first isolation/confinement (Knuth and Etgen, 2005; McCormick, Kehoe, and Kovacs, 1998), whereas in adults, there typically is a reduction in corticosterone release to repeated restraint stress relative to that in response to the first restraint session, which has been termed habituation (Grissom and Bhatnagar, 2009). Whether adolescents would show sensitization or habituation to repeated isolation/confinement was unknown.

The social instability component of Dr. McCormick's social stress procedure involves daily changes of cage partners (pair-housed from the time of weaning) for the same 16-day period as the isolation/confinement. Social instability has a long history of use as a stressor in adult rats, with early studies characterizing neuroendocrine changes that occur in groups of males in which the males are interchanged from group to group (e.g., Barnett, 1958; Taylor, Weiss, and Rupich, 1987). For example, increased sympathetic nervous system function was reported after changes in group membership among males, and HPA changes only resulted in the males when cohabitating with females (Mormede et al., 1990). The extent to which social instability would be a stressor for adolescents was not known; given the reward value of social interaction in adolescence, it was possible that new cage partners would be a positive experience. Social relationships, however, were known to be important in the recovery from stressor exposures. For example, PND 35 rats had lower corticosterone concentrations when placed in a novel environment (which typically elevates corticosterone concentrations) with a familiar peer than when with an unfamiliar peer (e.g., Gordon, 2002). Thus, the social instability stress (SS) procedure involves introducing a new cage partners after the isolation/confinement stress exposure. Figure 1-5 illustrates a scheme for organizing partners across the SS procedure.

Previous research has monitored the behaviour of adolescent male rats after daily one hour isolation/confinement and upon return to a new cage partner (new cage partners also are undergoing the SS procedure) and compared their behaviour to that of rats also undergoing daily isolation/confinement but always returned to their familiar cage partner (which is also undergoing daily isolation/confinement). The differences between

ORIGINAL CAGE PARTNERS		DAYS OF THE SOCIAL INSTABILITY STRESS PROCEDURE Numbers indicates the id of the new cage partner of the rat in the first column.																Id of rats with which rats in the first column were never paired (for use in experimental measures requiring a novel rat).				
Rat ID	Cage Mate	PND30	PND31	PND32	PND33	PND34	PND35	PND36	PND37	PND38	PND39	PND40	PND41	PND42	PND43	PND44	PND45					
1	2	13	9	22	21	20	4	5	6	10	8	7	17	11	15	14	2	3	12	16	18	19
2	1	20	12	19	22	21	5	16	13	8	18	17	3	15	9	11	1	4	6	7	10	14
3	4	11	21	12	20	22	19	7	18	9	10	13	2	17	8	5	4	1	6	14	15	16
4	3	15	16	13	10	11	1	8	9	21	17	14	12	7	6	20	3	2	5	18	19	22
5	6	9	10	11	13	18	2	1	22	20	21	16	15	8	17	3	6	4	7	12	14	19
6	5	22	11	18	17	13	7	10	1	14	15	21	9	19	4	12	5	2	3	8	16	20
7	8	18	22	9	11	10	6	3	15	17	14	1	19	4	20	16	8	2	5	12	13	21
8	7	14	18	17	16	19	13	4	12	2	1	11	10	5	3	22	7	6	9	15	20	21
9	10	5	1	7	19	17	20	13	4	3	11	15	6	16	2	18	10	8	12	14	21	22
10	9	19	5	16	4	7	12	6	14	1	3	22	8	20	11	21	9	2	13	15	17	18
11	12	3	6	5	7	4	15	18	17	19	9	8	16	1	10	2	12	13	14	20	21	22
12	11	17	2	3	18	15	10	14	8	13	22	20	4	21	16	6	11	1	5	7	9	19
13	14	1	19	4	5	6	8	9	2	12	20	3	21	18	22	17	14	7	10	11	15	16
14	13	8	17	20	15	16	21	12	10	6	7	4	18	22	19	1	13	2	3	5	9	11
15	16	4	20	21	14	12	11	17	7	18	6	9	5	2	1	19	16	3	8	10	13	22
16	15	21	4	10	8	14	17	2	20	22	19	5	11	9	12	7	15	1	3	6	13	18
17	18	12	14	8	6	9	16	15	11	7	4	2	1	3	5	13	18	10	19	20	21	22
18	17	7	8	6	12	5	22	11	3	15	2	19	14	13	21	9	17	1	4	10	16	20
19	20	10	13	2	9	8	3	22	21	11	16	18	7	6	14	15	20	1	4	5	12	17
20	19	2	15	14	3	1	9	21	16	5	13	12	22	10	7	4	19	6	8	11	17	18
21	22	16	3	15	1	2	14	20	19	4	5	6	13	12	18	10	22	7	8	9	11	17
22	21	6	7	1	2	3	18	19	5	16	12	10	20	14	13	8	21	4	9	11	15	17

Figure 1-5. An illustration of a scheme for pairing of rats for the social instability stress procedure, which involves a daily change of cage partners on postnatal days (PND) 30 – 45. This scheme illustrates pairings for n = 22; new schemes are required for different sample sizes.

adolescent rats that are isolated daily and returned to the same, familiar cage partner and those isolated daily and returned to a new unfamiliar partner are minimal (McCormick, Merrick, Secen, and Helmreich, 2007). However, after the first few daily isolations, the males were less social when being with an unfamiliar partner than with a familiar partner, and this difference diminished in later blocks of days (McCormick et al., 2007). Compared with adolescent males, adolescent females that were returned to an unfamiliar partner after isolation exhibited fewer differences in behaviour from that of control (undisturbed) females and females that were returned to a familiar partner after isolation (McCormick et al., 2007). Age-differences in the effect of SS on homecage behaviour in males or in females had not yet been investigated.

The influence of partner (un)familiarity on hypothalamic-pituitary-adrenal responding to stressors in adolescence versus in adulthood

Previous research investigated HPA function in male and female rats undergoing the SS procedure in either adolescence or in adulthood. Adolescent males showed a reduction in corticosterone release (habituation) in response to the 16th isolation compared with age-matched controls undergoing a first isolation, irrespective of whether returned to a familiar or unfamiliar peer after previous isolations (McCormick et al., 2007). The role of partner familiarity was evident, however, in other measures. Adolescent rats undergoing a 16th isolation and that were housed with an unfamiliar partner after each previous isolation did not differ in expression of CRH mRNA in the central nucleus of the amygdala from rats undergoing their 1st isolation, and both of these groups had higher expression of CRH mRNA compared with rats undergoing their 16th isolation but housed with their familiar partner after each previous isolation (McCormick

et al., 2007). Thus, the habituation of CRH mRNA in the central amygdala (CeA) evident in those returned repeatedly to a familiar partner was not found in those returned to an unfamiliar partner (McCormick et al., 2007).

There is fewer data on neuroendocrine function during the SS procedure for females. It appears, however, that, in contrast to results for males, changes of cage partners impede the habituation to repeated isolation/confinement in adolescent females (McCormick et al., 2007). Nevertheless, both male and female rats that underwent adolescent SS had lower plasma corticosteroid binding globulin at baseline than did rats that underwent 16 days of isolation and return to their cagemate daily (McCormick et al., 2007).

Any changes in HPA function after the SS procedure in adolescence are short-lived. When tested a minimum of two weeks after the SS procedure in adolescence, no differences between SS rats and control rats were found in corticosterone release in response to, and during recovery from, various stressors in either males or females (Mathews, Wilton, Styles, and McCormick, 2008; McCormick et al., 2005; McCormick, Smith, and Mathews, 2008). In contrast, in our study of HPA function 2-3 weeks after the SS procedure administered to adult rats, SS males did not differ from non-stressed controls in corticosterone release before and after 30 min of restraint, whereas SS females showed exaggerated corticosterone release relative to control females (McCormick et al., 2005). Why adult-stressed females show the prolonged change in HPA function that is absent in the other groups has not yet been resolved, but the results do highlight the importance of both sex and developmental stage in the consequences of social stressors.

Further, no effect of adolescent SS on HPA function in adulthood suggests that any long-term alterations in social behaviour are not the result of a dysregulated HPA axis.

Enduring effects of social instability stress in adolescence on social behaviour

Despite minimal differences in social behaviour between newly-paired rats after isolation stress in adolescence in the colony home cages, there is much evidence that the social repertoire is modified by the adolescent SS experience from investigations conducted after the end of the SS procedure and in test arenas rather than in the home cages. When tested weeks after the adolescent SS procedure in adulthood, SS male rats spent less time initiating social interactions with an unfamiliar peer than did control rats (Green et al., 2013). Nevertheless, SS rats did not differ from control rats in proximity to an unfamiliar peer in a social approach test, which indicates that SS rats are not socially avoidant in adulthood (Green et al., 2013). Instead, SS rats may be socially anxious, and this anxiety may be limited to direct physical interactions with conspecifics. SS rats also exhibit more general anxiety-like behaviour in adulthood in tests that involve venturing out onto high, narrow, elevated ledges (Elevated Plus Maze Test) (McCormick et al., 2008), entering the centre of an open field (as opposed to staying close to walls), approaching novel objects (Green et al., 2013), and the latency to drink novel substances (ethanol or 1% sucrose; Marcolin et al., in preparation). General anxiety-like behaviour appears to require an incubation period after adolescent SS; adolescent SS males did not differ from controls in time spent on the elevated plus maze or the open field tests when tested on the last day of the stress procedure (PND 45) (McCormick et al., 2008). Whether social anxiety requires an incubation period before manifesting in adulthood had yet to be investigated.

An alternative, but not mutually exclusive, possibility is that the reduction in social interactions occurs because SS in adolescence thwarts the development of a complete, appropriate, social repertoire. For example, behavioural sequencing (the predictability of the behaviour of one rat by the behaviour of its partner, Maaswinkel et al., 1997) involves the medial PFC and the hippocampus, brain structures that are still developing in adolescence. Thus, a disruption in behavioural sequencing may underlie the effects of SS in adolescence on social interactions.

Male SS rats also exhibit evidence of an altered social repertoire in adulthood when competing against their cage partner for access to sweetened condensed milk that is available daily for 5 minutes for 5 days (Cumming, Thompson, and McCormick, 2014) and when provided with the opportunity to mate with a receptive female (McCormick et al., 2013). Both SS and control rats increased aggressive behaviour from the first day to the last day when competing for sweetened condensed milk, however the increase in aggression was greater for SS rats than for control rats, and SS rats were more aggressive than were control rats on the 5th day (Cumming et al., 2014). The aggression displayed is typically "face whacks", whereby one rat tries to displace the other from the feeder of sweetened condensed milk by striking its snout; thus, the increased aggression in SS rats may involve a higher motivation for the food. Male SS rats show impairments in their sexual performance with females, such as a reduction in the likelihood to ejaculate compared with the likelihood in control males (McCormick et al., 2013). A second study investigated whether females would prefer mating opportunities with control males than with SS males. This experiment revealed that social status may mitigate some of the effects of the SS procedure; when SS rats were the subordinate rat in the dyad, females

showed a marked preference for the control male over the SS male (McCormick et al., 2017a). In contrast, when SS rats were the dominant rat in the dyad, females preferred the SS male over the control male (McCormick et al., 2017a). The dominant-subordinate status of the rats in the dyad was for pairs of SS rats housed together and pairs of control rats housed together. Thus, it remains unknown whether SS rats would be dominant or subordinate when housed with control males. The important finding was that SS in adolescence leads to long-lasting changes in males that were perceptible to females.

Neural mechanisms that may underlie the effects of social instability stress in adolescence on social behaviour

The extent to which differences in the various neural measures underlie the differences in social behaviour between SS and control rats is unknown. That differences are observed in the key neural regions supporting social behaviour, however, is consistent with the possibility that the neural differences underlie the behavioural differences between SS and control males.

Previous research has focused on the hippocampus of SS rats because of findings of reduced performance on spatial tasks in SS rats compared with control rats in males (Green and McCormick, 2013a; McCormick et al., 2012; Morrissey, Mathews, and McCormick, 2011) and in females (McCormick, Nixon, Thomas, Lowie, and Dyck, 2010). As described earlier, the CA2 subregion of the hippocampus is essential for social recognition, and the hippocampus is implicated in anxiety in social interaction tests (e.g., File, Kenny, and Ouagazzal, 1998; Hollis, Wang, Dietz, Gunjan, and Kabbaj, 2010; Marco et al., 2011). In male rats, although the number of proliferating cells in the hippocampus did not differ between SS and control rats, new neurons survived longer in

SS rats than in control rats (McCormick et al., 2012); the functional significance of the increased survival, however, is unknown. Signalling cascades in the hippocampus also differ between SS and control rats, with SS having a higher expression of CaMKIIα (calcium/calmodulin-dependent protein kinase II; important for memory formation) and reduced expression of the autonomously active form of CaMKII compared with expression in control rats tested as adults (McCormick et al., 2012). In adolescence, SS rats had reduced GAD67 (glutamic acid decarboxylase isoform 67, a marker for GABA neurons) in the pyramidal layer of the hippocampus compared with control rats (Hodges et al., unpublished data). Others have found that in adult rats, repeated administration of corticosterone reduced hippocampal GAD67 whereas 21-days of chronic restraint was without effect. Thus, adolescent rats may be more susceptible to GAD67 reduction by stressors than are adults (Lussier et al., 2013). The reductions in GAD67 and CaMKII are consistent with the hippocampal-dependent memory impairments observed in SS rats compared with controls (Morrissey, Mathews, and McCormick, 2011; McCormick et al., 2012; Green and McCormick, 2013a).

In adolescence, there were few differences between SS rats and control rats in the amount of proteins involved in synaptic plasticity in the PFC when tested at PND 46. When tested in adulthood, rats that underwent SS in adolescence had less CaMKIIα and PSD-95 (post-synaptic density protein 95; a scaffolding protein in synapses) in the PFC than did control rats (Marcolin et al., unpublished data), which suggests reduced synaptic transmission in the PFC.

The effects of SS on other brain regions involved in social behaviour, such as the lateral septum and medial amygdala, had yet to be investigated. Further, it was unknown

if social brain regions differed in their response to social behaviour between control and SS rats.

C. The goals of my thesis

Previous research investigated the effect of SS on HPA function, behaviour, and the brain to an extent, but several gaps of knowledge about the effects of adolescent SS remained. The overarching goal of my thesis was to investigate the effects of adolescent SS on HPA function, social behaviour, and the structure and function of social brain regions. Moreover, I wanted to focus on the effects of adolescent SS when tested soon after the stress procedure because very few of the studies were conducted during that period. The studies conducted in chapters 2-5 of my thesis aimed to add new knowledge about the effects of altered social experiences in adolescence on social development.

In chapter 2, I investigated whether adolescents were more vulnerable to the effects of SS compared with adults (an effect of age-group). I also investigated whether the effects were dependent on being repeatedly paired with an unfamiliar cage-partner or with their familiar cage-mate (an effect of partner familiarity). To target effects of age-group, I measured behaviour with cage-partners at the beginning and at the end of the 16-day SS procedure, I measured corticosterone concentrations on the last day of the SS procedure, and I measured neural activity in several brain regions on the last day of the SS procedure in adolescent (SS procedure from PND 30-45) and adult (SS procedure from PND 70-85) rats and compared the results with adolescent and adult rats that underwent the SS procedure for the first time on PND 45 and PND 85 respectively. To target effects of partner familiarity, all measurements were compared with those from

adolescent and adult rats that underwent repeated isolation and return to their cage-mate for 16 days.

In chapter 3, I investigated the extent to which adolescent SS and control rats differed in social behaviour soon after the stress procedure. I tested adolescent SS and controls rats on social interaction, social recognition, and social conditioned place preference. In addition, I investigated whether adolescent SS and control rats differed in OTR and V1AR binding density in social brain regions soon after the stress procedure. I measured OTR and V1AR binding densities in the lateral septum, nucleus accumbens, bed nucleus of stria terminalis, medial preoptic area, central nucleus of the amygdala, basolateral nucleus of the amygdala, and medial amygdala.

In chapter 4, I investigated the functional differences in brain regions between adolescent SS and control rats in response to interacting with a peer. To investigate functional differences, I measured Fos immunoreactivity in several brain regions after SS and control rats interacted with an unfamiliar peer in a novel environment. I measured Fos-immunoreactivity in brain regions involved in HPA function (hypothalamic paraventricular nucleus, arcuate nucleus) to investigate whether adolescent SS rats and controls differed in how stressful they found social interactions, and I measured Fos-immunoreactivity in social brain regions (lateral septum, medial amygdala, CA2 of the hippocampus, nucleus accumbens) to investigate how adolescent SS and control rats differed in their neural activations during social behaviour. In addition, I investigated whether generalized anxiety in early-life could predict a vulnerability to SS in adolescence. I measured anxiety-like behaviour in rats before the SS procedure began and

investigated the association between early-life anxiety and the effects of SS on social interaction after the procedure.

Finally, in chapter 5, I investigated the structural differences in social brain regions between adolescent SS and control rats. Specifically, I targeted measurements of dendritic morphology and markers of pre-synaptic and post-synaptic plasticity in the lateral septum and medial amygdala of adolescent SS and control rats soon after the procedure.

Rationale for Chapter 2

Exposure to glucocorticoids (i.e., corticosterone in rats) is an important means by which life experiences shape brain strcture and function. Previous research found reduced corticosterone concentrations in adolescent rats that underwent social instability immediately after their 16th isolation for 1h compared with rats that were isolated for the first time on PND 45 (the last day of the stress procedure) (McCormick et al., 2007). Further, in my Masters research, I found that adolescent and adults that underwent one exposure to the SS procedure did not differ in corticosterone release after isolation or after being paired with an unfamiliar peer in the homecage (Hodges et al., 2014). The effect of adolescent social instability on corticosterone concentrations after being paired with an unfamiliar peer of the 16th time (the last day of the procedure) and age differences in these effects were unknown. Thus, one aim of chapter 2 was to characterize the effect of adolescent social instability on corticosterone concentrations and neural activation in brain regions involved in stress response and social behaviour in response to being paired with an unfamiliar peer in the homecage on the final day of the stress procedure. The second aim of chapter 2 was to compare the effects of social instability on behaviour in the homecage, endocrine function, and neural activation in several brain regions when the SS procedure was carried out in adolescence versus in adulthood. This comparison allowed me to determine whether the effects of SS were unique to adolescence.

Chapter 2: Adolescent and adult male rats habituate to repeated isolation, but only adolescents sensitize to partner unfamiliarity.

This chapter has been adapted from the published article:

Hodges, T. E., & McCormick, C. M. (2015). Adolescent and adult male rats habituate to repeated isolation, but only adolescents sensitize to partner unfamiliarity. *Hormones and behavior, 69,* 16-30.

Author contribution: For this manuscript Dr. Cheryl McCormick and I designed the experiment and formulated the research questions. I wrote the proposal for ethics approval, ordered the rats required for the experiment, performed the social instability stress procedure on rats (16 day), performed the repeated isolation procedure, took blood samples, measured behaviour in cages, performed perfusions, collected rat brains, sliced rat brains, performed immunohistochemistry on brain slices, performed enzyme-linked immunosorbent assays on blood samples for corticosterone and testosterone, counted cells in coronal brain slices (with help from fellow graduate students Jonathan Simone and Matthew Green), analyzed the data, performed the statistics, I helped to create the figures, wrote the paper, and edited the paper after I received feedback from Dr. Cheryl McCormick.

Introduction

The HPA axis is a means by which an organism coordinates its response to stressors, both psychological and physical. When a stressor is perceived or anticipated, the paraventricular nucleus of the hypothalamus initiates the release of secretagogues that in turn lead to the release of ACTH from the pituitary. ACTH then increases the release of glucocorticoids (primarily corticosterone in rodents) by acting on the adrenal cortex (Herman et al., 2012). Glucocorticoid release helps an organism adapt to, and recover from, the effects of a stressor through influences on the metabolic, cardiovascular, immune, and neurological systems (De Bosscher et al., 2008; McEwen et al., 2012). Nevertheless, prolonged exposure to glucocorticoids in response to repeated (or chronic) stressors may alter endocrine and neural functioning and impair an organism's responses to future stressors (reviewed in Lupien et al., 2009). The ability to recover from the effects of chronic stressors, however, depends on the stage of development of an organism (Eiland and Romeo, 2013; Hollis et al., 2013).

There is growing evidence of a differential susceptibility of adolescents compared with adults to the negative consequences of stressors (reviewed in Green and McCormick, 2013; McCormick and Green, 2013). For example, a reduction in hippocampal volume and deficits in spatial memory was found in adolescent rats three weeks after exposures to chronic stress (Isgor et al., 2004). In contrast, effects of chronic stress exposures in adulthood on spatial memory and dendritic arborisation in the adult hippocampus dissipated within days of termination of the stress (Conrad et al., 1999; Sousa et al., 2000). Further, the pattern of the effects of stressors sometimes differ for the two age groups, as recently found in the basolateral amygdala after exposure to chronic social instability in adolescent and adult rats (Tsai et al., 2014). Developmental

maturation of HPA function and of the central nervous system both have been proposed as bases for the age differences in the consequences of exposure to stressors.

Several studies have reported that adolescent rats have a more prolonged release of corticosterone than do adult rats in response to acute stressors such as intermittent foot shock (Goldman et al., 1973), ether inhalation (Vázquez and Akil, 1993), two hour exposure to a novel environment (Novak et al., 2007), and restraint (Bingham et al., 2011; Cruz et al., 2007; Dziedzic et al., 2014; Foilb et al., 2011; Gomez et al., 2002; Hall and Romeo, 2014; Lui et al., 2012; Romeo et al., 2004a, 2004b, 2006a, 2006b, 2014; Vázquez and Akil, 1993). In contrast, other studies have reported higher or more prolonged release of corticosterone to acute stressors in adult rats than in adolescent rats (e.g. nicotine, Cao et al., 2010; lipopolysaccharide, Goble et al., 2011; confinement to an elevated platform, McCormick et al., 2008; injection of ethanol, Willey et al., 2012), and others have found no differences between adolescent and adult rats in corticosterone release in response to an acute stressor (novelty stress, Goldman et al., 1973; one hour isolation, Hodges et al., 2014; forced swim, Mathews et al., 2008; tetrahydrocannabinol, Schramm-Sapyta et al., 2007). The basis for differential HPA function between adolescents and adults in response to acute stressors remains unknown, although type of stressor is likely an important factor.

Adolescents and adults do not differ in GR or mineralocorticoid receptor binding capacities (Vázquez, 1998; Vázquez and Akil, 1993), in GR mRNA expression (Romeo et al., 2008), or in GR protein immunoreactive cell counts (Dziedzic et al., 2014) in brain regions that regulate the stress response (e.g., the hippocampus, medial prefrontal cortex, paraventricular nucleus). Although earlier studies found no difference between

adolescents and adults in sensitivity of the adrenal gland to ACTH (Goldman et al., 1973; Vázquez and Akil, 1993), a recent study suggests adrenal sensitivity to ACTH may be greater in adolescent than in adult rats after acute restraint, and that the heightened sensitivity of adolescents may involve a greater expression of adrenal melanocortin 2 receptor accessory protein (Mrap) mRNA in adolescent than in adult rats (Romeo et al., 2014). In addition, several studies have reported age differences in the brain regions activated by stressors. For example, pre-pubertal adolescents had greater Fos expression (an index of neuronal activation) in the paraventricular nucleus than did adults after acute 30 minutes of restraint (Lui et al., 2012; Romeo et al., 2006a; Viau et al., 2005). Moreover, rats in early adolescence (postnatal day 28) had greater Fos expression than did adults after two hours in a novel environment in several brain regions (paraventricular nucleus of the hypothalamus, medial amygdala, cingulate gyrus, lateral septum, paraventricular nucleus of the thalamus, and centromedial nucleus of the thalamus) (Novak et al., 2007). Other studies, however, found no difference between adolescent and adult rats in Fos expression in the paraventricular nucleus after either 15 minutes or two hours of restraint (Kellogg et al., 1998). Using a different marker of neural activation, Zif268 (protein product of the immediate-early gene zif268), higher immunoreactive cell counts were found in the paraventricular nucleus of the hypothalamus during recovery from one hour of isolation stress in adolescents compared with adults (Hodges et al., 2014). Thus, a basis for differences between adolescents and adults in HPA function may involve extra-hypothalamic neural regions that continue to mature in adolescence (reviewed in Spear, 2000).

Fewer studies have investigated HPA function in response to repeated stressors in

adolescents compared with adults. Adolescent rats had a higher concentration of corticosterone compared with adult rats in response to a seventh exposure to 30 minutes of restraint, although no difference was found after the first restraint session (Lui et al., 2012; Romeo et al., 2006a). Similar age differences (adolescent > adults after repeated stress) were found in response to five exposures to cat odour (Wright et al., 2011) or to five days of restraint stress (Doremus-Fitzwater et al., 2009). In addition, studies have also reported greater Fos expression in the paraventricular nucleus after the seventh exposure to 30 minutes of restraint in adolescent rats than in adult rats (Lui et al., 2012; Romeo et al., 2006a). These results suggest that the HPA response of adolescents may be differentially affected by repeated stressors compared with that of adults.

Stressors that involve social manipulations may be particularly relevant for age differences in HPA function, considering the developmental stage variation in social behaviour. In both humans and rodents, adolescence is a period of increased novelty-seeking and increased social interactions with peers compared to adulthood (reviewed in Spear, 2000). For example, whereas adolescent rats initiated more play behaviours with conspecifics, adult rats displayed less social play (Klein et al., 2010) and more agonistic behaviours (Meaney and Stewart, 1981) towards conspecifics. Moreover, socially-deprived adolescent rats engaged in more social interactions (Varlinskaya and Spear, 2008) and had a greater preference for the side of a cage that was previously paired with a conspecific (Douglas et al., 2004) than did similarly socially-deprived adults. In addition, our repeated SS procedure (16 days of daily repeated isolation for one hour and return to an unfamiliar cage partner) produced immediate and long-lasting decrements in memory of conditioned fear (Morrissey et al., 2011) and altered behavioural responses to

psychostimulants (McCormick et al., 2005) when experienced in adolescence, but not when experienced in adulthood, which attests to the heightened vulnerability of the adolescent period.

We have investigated some aspects of HPA function in adolescent rats exposed to the SS procedure, but we have not compared adolescent exposure with adult exposure. We found that, irrespective of whether returned repeatedly to unfamiliar or familiar cage partners after daily isolations, there was evidence of habituation to repeated isolation; corticosterone concentrations were lower on postnatal day 45 (mid-adolescence) immediately after the 16th isolation compared with age-matched controls undergoing a first isolation (McCormick et al., 2007). Further, those that had been repeatedly paired with unfamiliar cage partners after isolation showed a significant increase in CRH mRNA in the central nucleus of the amygdala after one hour isolation as did those undergoing a first isolation, whereas those always recovering from repeated isolation with a familiar partner did not (McCormick et al., 2007). The recovery phase was not investigated after the 16th episode of isolation, however, which might uncover additional effects of partner (un)familiarity. When we compared adolescents and adults to an acute isolation, however, the two ages did not differ in corticosterone concentrations after isolation, and both ages had higher corticosterone concentrations when paired with an unfamiliar partner during the recovery period than when with a familiar partner (Hodges et al., 2014). Nevertheless, age differences might emerge under conditions of repeated stress exposures.

Here we directly compare HPA function and neural activations (as measured by Zif268-immunoreactivity) in response to, and in recovery from, repeated (versus acute)

isolation stress, as well as the influence of partner familiarity (social stability vs unfamiliarity/social instability) in both adolescents and adults. In addition to measuring plasma corticosterone concentrations, we also investigated changes in testosterone concentrations, which have been reported to increase in response to various stressors (e.g., reviewed in Chichinadze and Chichinadze, 2008; Foilb et al., 2011) and in response to social interactions (reviewed in Gleason et al., 2009). Lastly, we also monitored behaviour in the home cage during recovery from isolation in both the early and in the later phases of the 16-day procedure to better characterize effects of the SS procedure at both ages. We predicted that partner familiarity would be a greater factor in adolescents than in adults across all measures, and that adults may show greater habituation to the repeated stress exposures than would adolescents.

Methods

Animals

Male Long-Evans rats (N = 144) were obtained from Charles River, St. Constant, Quebec, at 22 days of age. Rats were housed in pairs and maintained under a 12 h light-dark cycle (lights on at 08:00 h) with food and water available *ad libitum*. Use of animals in this experiment was approved by the Brock University Institutional Animal Care Committee and was carried out in adherence to the Canadian Council of Animal Care guidelines.

Stress conditions

Rats were randomly assigned to be tested either as adolescents (PND 30) or as adults (PND 70) and to the three main Groups: a non-stressed control group (Control), some of which would be assigned to an acute one hour isolation group at PND 45

(Control/acute isolation); a one hour isolation and return to a familiar partner repeated daily for 16 days group (repeated isolation+familiar partner); and a one hour isolation and return to an unfamiliar partner repeated daily for 16 days group (repeated isolation+unfamiliar parnter) (PND 30 - 45 for adolescents, PND 70 - 85 for adults) (see Figure 2-1). On day 16, the time points for collection of blood samples and for perfusion for brain collection also were randomly assigned. Two time points were used to assess the stress response of adolescents and adults in the three groups: (1) baseline (immediately after removal from the home cage) and (2) after isolation. A third time point, after one hour back in the colony after isolation, was used to assess the effects of acute versus repeated isolation and partner familiarity (with or without a familiar partner) on recovery from isolation. Isolation containers were 14 cm in diameter and 10 cm in height, whereas adult isolation containers were 20 cm in diameter and 12 cm in height. The cage partners to which rats returned after isolation had also undergone isolation at the same time. Experiments involved three cohorts of rats, with each group represented in each cohort, and experiments were conducted between 09:00 and 13:30 h to minimize the influence of circadian variation.

Behaviour in home cage

Behaviours were monitored in adolescent and adult animals after return to the colony cages after isolation and compared with control rats left undisturbed. A time sampling method was used in which the behaviour of each of two animals in a cage was recorded every 2 min for a total of 20 observations commencing at approximately 10 min after repeatedly isolated rats returned from isolation (approximately 40 minute period of observation) in the initial days of repeated isolation (PND 31-33 for adolescents, PND

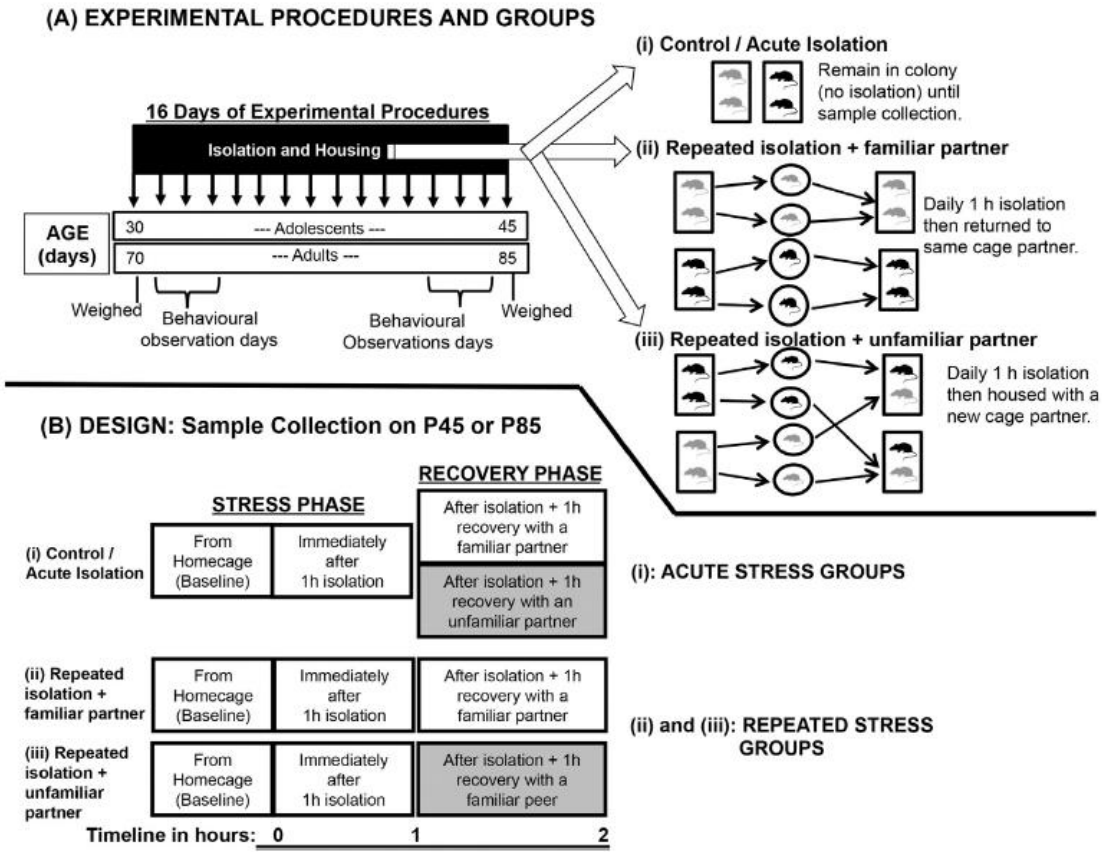

Figure 2-1. (A) A timeline of the 16 days of experimental procedures that resulted in the three main experimental groups (i, ii, and iii) of adolescents and of adults. (B) A timeline of the sample collections for each of the three main experimental groups (i, ii, and iii) on the 16th day of experimental procedures at postnatal day (PND) 45 (for adolescents) or 85 (for adults) during the stress phase (from homecage or after 1 h isolation) and in the recovery phase (after 1 h back in colony with either a familiar or unfamiliar partner). All comparisons are between groups.

71-73 for adults for a total of 60 observations) and in the later days of repeated isolation (PND 42-44 for adolescents, PND 82-84 for adults for a total of 60 observations). Behaviours were observed in control animals at the same time as were rats that had been isolated. Social interactions were defined as any physical contact between cage-mates (social play, allogrooming, huddling, social sniffing, social inactivity, social rest, offensive aggression, defensive aggression; as defined in Cirulli et al., 1996). As in our previous report (McCormick et al., 2007), social interactions were grouped into two categories for statistical analysis: Social Inactivity (social rest and social inactivity) and Social Activity (the remaining social interactions). The total number of interactions per age (adolescent, adult) and per group (control, isolation+familiar partner, isolation+unfamiliar partner) were calculated for the initial days of repeated isolation and for the later days of repeated isolation. 72 pairs of cages were monitored, including 12 pairs of controls, 12 pairs returned to a familiar partner after isolation, and 12 pairs returned to an unfamiliar partner after isolation at each age.

Plasma corticosterone and testosterone assays

A blood sample (approximately 200 µl) was obtained from a tail nick and collected in ice chilled blood collection tubes (Sarstedt) within three minutes. Samples were centrifuged at 1730 x g and 4°C for 10 min, and plasma was collected and stored at -20°C until the assays. Plasma corticosterone and testosterone concentrations were determined using enzyme-linked immunosorbent assay kits (Neogen, Lansing, MI) and a Biotek Synergy plate reader (BioTek Instruments, Inc., USA). Steroids were extracted from 50 µl samples using ethyl ether and the samples were reconstituted in 100 µl of the buffer provided in the kit, then further diluted (20 µl in 980 buffer). Duplicate aliquots

(50 µl) of the final dilution were used for the assay. Intra-assay variance was under 10% and inter-assay variance was under 15%.

Zif268 immunohistochemistry

Immediately after blood sampling, rats were deeply anaesthetized by an overdose of sodium pentobarbital (150 mg / kg), weighed, and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4). Brains were removed from the skulls and post-fixed in a 30% sucrose and 4% paraformaldehyde solution until equilibrated. Coronal sections (37 µm; sections separated by 222 µm) were sliced on a cryostat and collected throughout the medial prefrontal cortex, the hypothalamus, and the hippocampus and were stored in cryoprotectant at -20°C until the time of assay.

Free-floating coronal sections were washed thoroughly in 0.1 M PBS, then in in PBS-X (0.1 M PBS with 3% Triton X-100), and incubated at room temperature in a 0.3% H_2O_2 in 0.1 M PBS-X solution for 30 min. Sections were then washed in PBS-X, blocked at room temperature in 10% goat serum (Sigma) solution for 1 h, and incubated at 4°C overnight in the primary antibody raised against Zif268 (1:10,000; Zif268 rabbit mAb; 15F7; Cell Signaling Technology, Inc.) in PBS-X. The next day, sections were washed in PBS-X and then incubated for 2 h at room temperature in secondary antibody (biotinylated goat anti-rabbit IgG; 1:400; Vector Laboratories, Inc.). After another series of washes in PBS-X, sections were incubated in an avidin-biotin horseradish peroxidase complex (Vector Laboratories, Inc.) for 1.5 h at room temperature. Horseradish peroxidase was visualized with 3,3’diaminobenzidine (DAB) in a 3 M sodium acetate buffer containing 0.05% H_2O_2 (Vector Laboratories, Inc.). After a final series of washes

in PBS-X, sections were mounted on Superfrost Plus slides (Fisher Scientific, Inc.), dried, dehydrated in increasing concentrations of ethanol (70%, 95%, 100%), placed in xylenes, and coverslipped using Permount mounting medium (Fisher Scientific, Inc.). To facilitate identification of subregions of the medial prefrontal cortex and amygdala, those sections were lightly counterstained with neutral red before they were coverslipped. No immunoreactive cells were detected in control sections that were not treated with the primary antibody.

Microscopy and cell counting

Immunostained sections were analyzed using a Nikon Eclipse 80i microscope equipped with a digital camera (Nikon DXM1200F) and Nikon ACT-1 software. Immunoreactive (ir) cell counts were conducted blind to experimental condition and at 400X magnification in a 250 μm^2 area in the paraventricular nucleus of the thalamus (PVThal) and in each hemisphere of the hypothalamic paraventricular nucleus (PVN), the arcuate nucleus (ARC), the medial prefrontal cortex (mPFC; anterior cingulate cortex, infralimbic cortex, prelimbic cortex), the pyramidal layer of the hippocampus (CA1, CA2, and CA3), the granule layer of the dentate gyrus, the lateral amygdala (LA), the basolateral amygdala (BLA), the CeA, the medial posterodorsal amygdala (MePD), the medial posteroventral amygdala (MePV), the medial habenula (mHb), and the lateral habenula (lHb) (brain regions depicted in Figure 2-2). These areas were chosen for examination based on their roles in social behaviour and HPA function (Martinez and Herbert, 2002). Regions were identified according to the atlas of Paxinos and Watson (2005): mPFC sections used for counting were within the coordinates from bregma of 3.00 mm and 2.52 mm; for PVThal, within bregma -3.00 and -3.60; for PVN, within

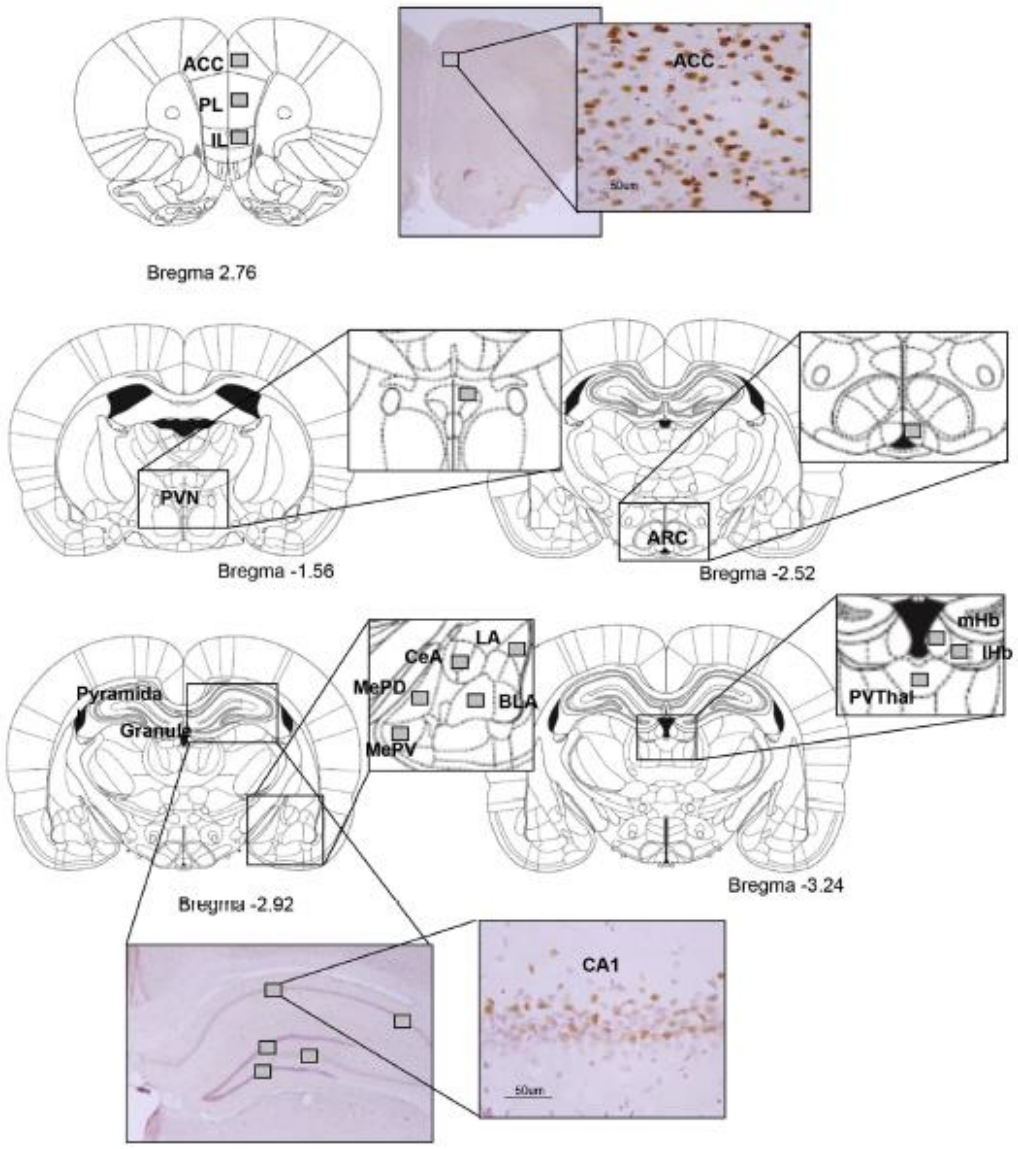

Figure 2-2. Atlas images depicting regions of interest for immunoreactive cell counts (images are used with permission from Paxinos and Watson, The Rat Brain in Sterotaxic Coordinates, 5/e, 2005, Elsevier) and images of Zif268 immunoreactivity in the anterior cingulate cortex and the CA1 of the hippocampus at 400x magnification depicting boxes on images at 100x magnification comparable to the paired atlas images (brain sections were counter-stained with neutral red; in greyscale, the darker stained cells are the Zif268 immunoreactive cells). Boxes depict the areas with regions of interest located under low magnification that were then analyzed at 400x magnification. ACC = anterior cingulate cortex; PL = prelimbic cortex; IL = infralimbic cortex; PVN = paraventricular nucleus of the hypothalamus; ARC = arcuate nucleus; LA = lateral amygdala; CeA = central amygdala; BLA = basolateral amygdala; MePD = posterodorsal medial amygdala; MePV = posteroventral medial amygdala; PVThal = paraventricular neucleus of the thalamus; mHb = medial habenula; lHb = lateral habenula.

bregma -1.44 and -1.72; for ARC, within bregma -2.52 and -2.76; for regions of the hippocampal formation, within bregma -2.64 and -3.24; for CeA, BLA, and LA, within bregma -2.52 and -2.92; for MePD and MePV, within bregma -2.76 and -3.24; for the habenula, within bregma -3.00 and -3.96. The mean number of ir-cells per hemisphere per brain region per rat was used for analysis for all regions of interest except the hippocampal formation. For the hippocampal formation, in each hemisphere of each of three sections, ir-cells were counted in five separate regions, the CA1, CA2, and CA3 (which were averaged and labeled as the pyramidal layer), and in the suprapyramidal and infrapyramidal layers of the dentate gyrus (which were averaged and labeled as the granule layer). Data are not shown nor analyzed for regions that had Zif268 ir-cell counts less than 5 in all of the experimental groups, which was the case for the PVThal, mHb, lHb, infralimbic cortex, prelimbic cortex, CeA, BLA, and LA.

Statistical analyses

Statistical analyses were performed using SPSS version 20 software and consisted of between group analyses of variance (ANOVA) with the factors of Group (Control, Isolation+familiar partner, Isolation+unfamiliar partner) and Age (Adolescent, Adult). For analysis of weight, Day (first day of stress procedures, last day of stress procedures) also was a factor, and for analysis of behaviour in the home cage, Social Interaction Type (Social Activity, Social Inactivity) also was a factor.

The remaining measures (corticosterone, testosterone, Zif268 immunoreactivity) addressed the main aim of the experiment, which was to investigate the effects of acute versus repeated isolation and acute versus repeated partner familiarity/unfamiliarity after isolation. Thus, the design was not balanced, in that the control rats that would undergo

an acute, first isolation could not be included in cells to test the partner familiarity effect until after the first isolation, when they had the first opportunity to be placed with an unfamiliar partner after recovery. Thus, the statistical approach was to analyze baseline samples and samples immediately after isolation in one analysis (Stress analysis: Time [Baseline, Isolation] X Group [Acute Isolation, Repeated Isolation in those previously returned to a familiar partner after isolation, and Repeated Isolation in those previously returned to an unfamiliar partner after isolation) X Age [Adolescents, Adults]). A second analysis involved the samples obtained after one hour of recovery back in the colony after isolation (Recovery Analysis: Stress Exposures [Acute, Repeated] X Partner Familiarity [familiar, unfamiliar] X Age [adolescents, adults]). Nevertheless, the group means are organized in figures to allow visual comparison of the immediately after isolation time point and the one hour of recovery time point within each of the main groups: Controls (which were also the acute isolation group, control/acIso), the repeated isolation group that returned to familiar cage partners (rIso+Fam) and the repeated isolation group that returned to unfamiliar cage partners (rIso+Unf). A third set of analyses (one way ANOVAs comparing the different time points within each Group at each Age) is provided to allow comparison of the Stress phase and Recovery phase. This third analysis, however, is considered supplementary and is limited to the figures and legends. A second set of figures illustrates any main effects or interactions found in the main analyses of Stress and of Recovery measures.

Post hoc analyses consisted of F tests for simple effects, and/or between group t-tests and Fisher least squared differences, where appropriate. Although the experiments initially involved n = 6 for control/acute stressed animals per time point (baseline,

immediately after 1st 1 h isolation, after 1st 1 h return to a familiar peer, after 1st 1 h return to an unfamiliar peer) per age and n = 8 for repeatedly stressed animals per time point (baseline, immediately after 16th 1 h isolation, after 16th 1 h return to a peer) per age, sample sizes were reduced to n = 5-7 in some groups for some measures for several reasons (e.g., unable to obtain blood sample; damaged sections; sections not within a region of interest). An alpha level of $p \leq 0.05$ was used to determine statistical significance.

Partial eta squares are reported as a measure of effect sizes of main effects and interactions in ANOVAs (calculated in SPSS version 20 software), and Cohen's *d* are reported as a measure of effect sizes for post hoc comparisons of two groups (calculated with online calculator at www.cognitiveflexibility.org/effectsize/).

Results

Weight

Daily isolation affected weight gain in adolescents, not in adults, irrespective of partner familiarity. For adolescents, a Group (Control, Isolation+familiar partner, Isolation+unfamiliar partner) X Day (first day of stress procedures, last day of stress procedures) on weight found an interaction of Day and Group ($F_{2, 69} = 7.45$, $p = 0.001$, $n^2p = .18$), with no significant differences among the Groups on the first day of the stress procedure (ps > 0.50 for all groups), and with Control rats weighing more than Isolation+familiar rats ($p = 0.04$, Cohen's $d = 0.58$) and Isolation+unfamiliar rats ($p = 0.007$, Cohen's $d = 0.83$). For adults, the interaction of Day and Group also was significant ($F_{2, 69} = 5.54$, $p = 0.006$, $n^2p = .14$), with no significant differences among the

Groups on the first day of the stress procedure (all ps > 0.17) or on the last Day of the procedure (all ps > 0.10, Cohen's d < 0.48; see Figure 2-3).

Behaviour after isolation

In the initial observation days, adolescents spent more time in social interaction than did the adults, with adolescents increasing and adults decreasing social contact after isolation. The age differences were attenuated in the later observation days. After isolation, rats were more socially active than were control rats, and those with an unfamiliar partner spent more time in social activity than those with a familiar partner after isolation irrespective of age and at both sets of observation days. An Age (adolescent, adult) X Group (Control, Isolation+familiar partner, Isolation+unfamiliar partner) X Social Interaction Type (SI Type: Social Activity, Social Inactivity) ANOVA on times in social interaction during the initial observation days found the three-way interaction significant ($F_{2, 66}$ = 8.43, p = 0.001, n^2p = .20). Analyses then were conducted for the two SI Types separately. For Social Activity, only the main effect of Group was significant ($F_{2, 66}$ = 16.89, p < 0.001, n^2p = .34, other ps > 0.10, n^2p's < .07), with Isolation+unfamiliar rats more socially activity than Isolation+familiar rats (p = 0.002, Cohen's d = 1.03) and Control rats (p < 0.001 Cohen's d = 1.56), and Isolation+familiar rats more socially active than Control rats (p = 0.01, Cohen's d = 0.71). For Social Inactivity, the interaction of Age and Group was significant ($F_{2, 66}$ = 6.97, p = 0.002, n^2p = .17), with the effect of Group not significant in Adolescents (p = 0.06, n^2p = .16), whereas in Adults, Isolation+unfamiliar rats were less socially inactive than Isolation+familiar rats (p < 0.002, Cohen's d = 0.63) and Control rats (p < 0.001, Cohen's d = 1.18), and Isolation+familiar rats less socially inactive than

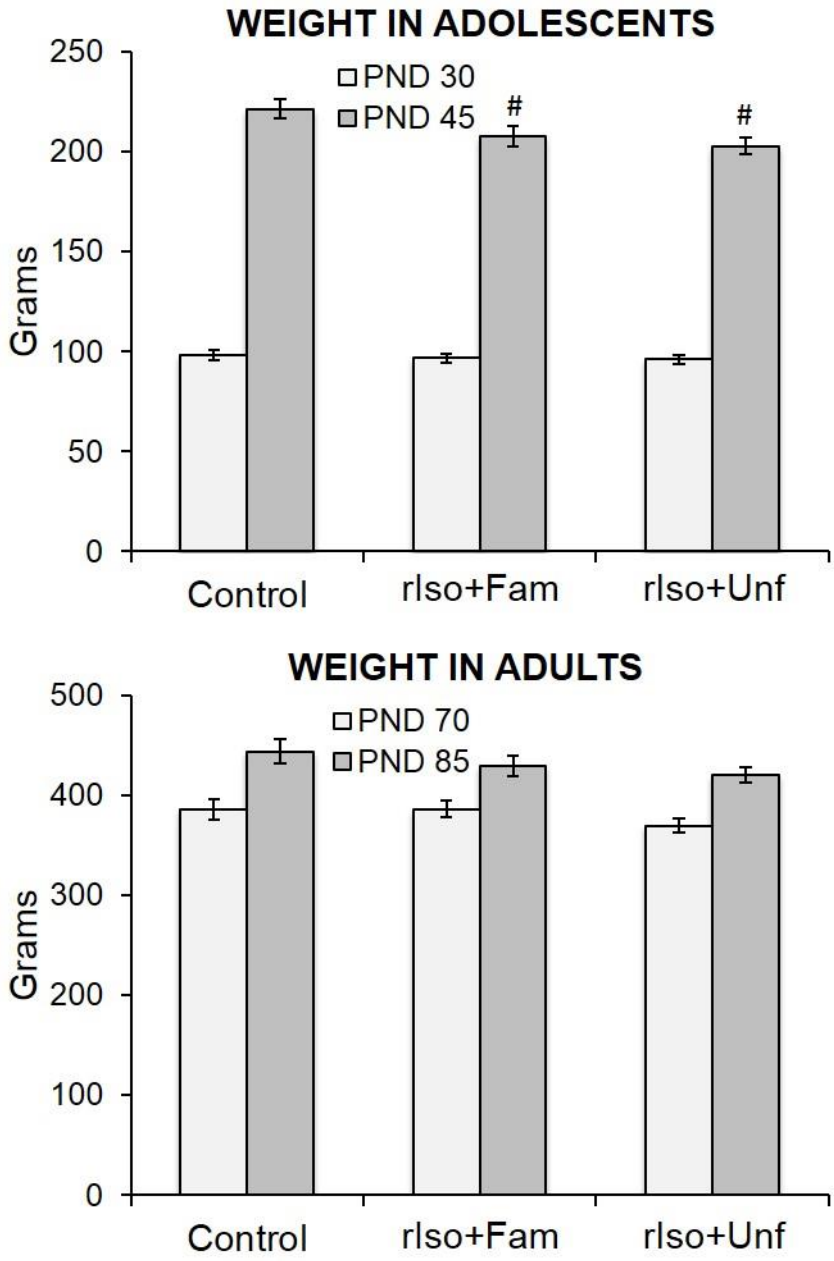

Figure 2-3. Mean (±S.E.M.) weight of adolescent and adult rats before and after 16 days left undisturbed in the home cage (Control), or 16 days of repeated isolation and return to either a familiar (rIso+Fam) or an unfamiliar (rIso+Unf) cage partner. # Lower than Control group ($p < 0.05$).

Control rats (p = 0.001, Cohen's *d* = 0.71). There was no age difference for Control rats (Cohen's *d* = 0.17*)*, whereas there was more social inactivity in adolescents than in adults for both Isolation+familiar and Isolation+unfamiliar rats (ps < 0.001, Cohen's *d* = 1.69 and 2.08 respectively; see Figure 2-4a).

For times in social interaction during the later observation days, the ANOVA found adolescents to have more social interactions than adults ($F_{2, 66} = 3.17$, p = 0.05, n^2p = .09), and a significant interaction of Group and Social Interaction Type ($F_{2, 66} = 32.57$, p < 0.0001, n^2p = .50). Analyses then were conducted for the two SI Types separately. For Social Activity, only the effect of Group was significant ($F_{2, 66} = 20.82$, p < 0.001, n^2p = .39; other ps > 0.06, n^2p's < .07), with Isolation+unfamiliar rats more socially active than Isolation+familiar rats (p = 0.01, Cohen's *d* = 0.65) and Control rats (p < 0.001, Cohen's *d* = 2.32), and Isolation+familiar rats more socially active than Control rats (p < 0.001, Cohen's *d* = 1.11). For Social Inactivity, neither Age, Group, nor their interaction were significant (ps > 0.10, n^2p's < .07; see Figure 2-4b).

Plasma corticosterone

Both adolescents and adults showed evidence of habituation in that there was reduced release of corticosterone to repeated isolation than to a first isolation, irrespective of whether previously housed with familiar or unfamiliar partners. The Stress analysis found a main effect of group ($F_{2, 70} = 4.91$, p = 0.01, n^2p = .12), a main effect of time point ($F_{2, 70} = 79.86$, p = 0.001, n^2p = .53) and an interaction of group and time point ($F_{2, 70} = 4.56$, p = 0.01, n^2p = .12). Post hoc analysis of the interaction indicated that the groups did not differ at baseline (p > 0.95, n^2p = .001), whereas after isolation those undergoing first isolation had higher corticosterone than Repeated

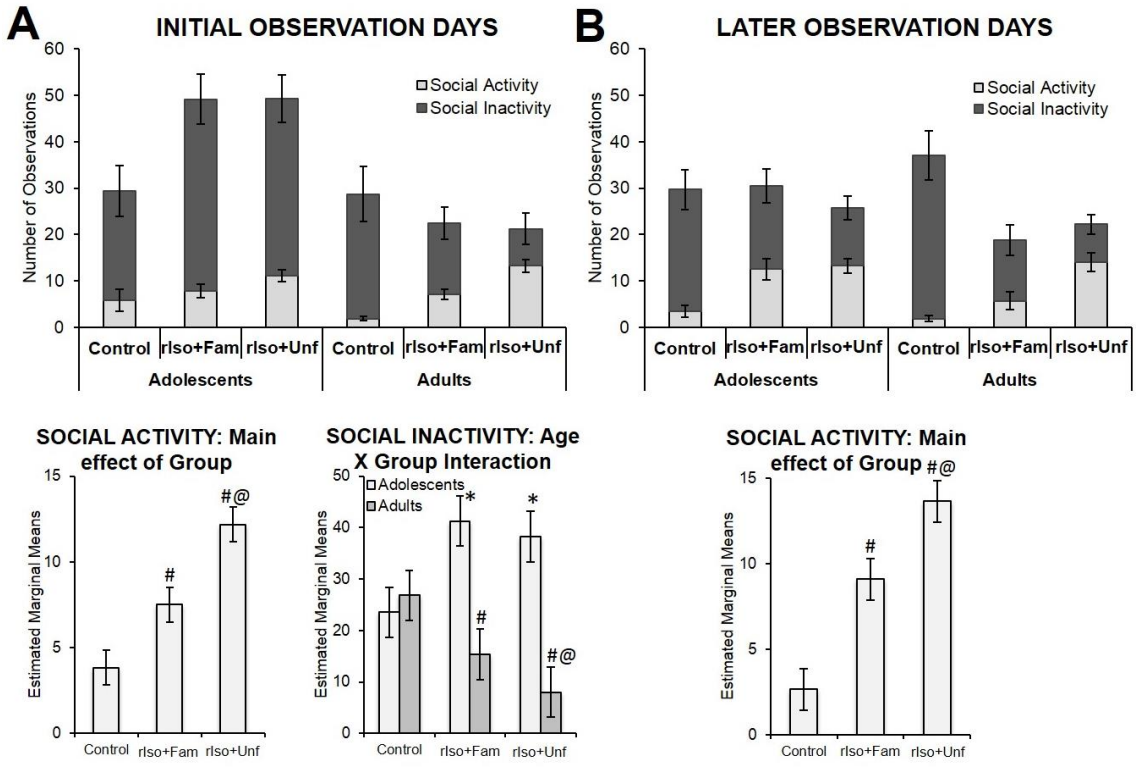

Figure 2-4. Number of socially active and socially inactive behaviours observed in three daily 40 minute periods after isolation (60 observations total) during the ***(A)*** *initial observation days (PND 31-33 for adolescents, PND 71-73 for adults) and* ***(B)*** *later observation days (PND 42-44 for adolescents, PND 82-84 for adults) in adolescent and adult controls (Control) and isolated rats returned to either a familiar (rIso+Fam) or unfamiliar (rIso+Unf) cage partner.* ***(A) Social activity:*** *# higher than Control group ($p < 0.05$); @ higher than rIso+Fam group ($p < 0.05$).* ***Social inactivity:*** ** age difference within group ($p < 0.05$); # lower than Adult Control group ($p < 0.05$); @ lower than Adult rIso+Fam group ($p < 0.05$).* ***(B) Social activity:*** *# higher than Control group ($p < 0.05$); @ higher than rIso+Fam group ($p < 0.05$).*

Isolation+familiar (p = 0.01, Cohen's *d* = 1.11) and Repeated Isolation+unfamiliar (p = 0.01, Cohen's *d* = 0.95) groups, but the Repeated Isolation+familiar and Repeated Isolation+unfamiliar groups did not differ (p = 0.85, Cohen's *d* = 0.08). The main effect of age and all other interactions were not significant (p > 0.10, n^2p's < .05; see Figure 2-5a).

Adolescents had higher corticosterone concentrations if with an unfamiliar partner than with a familiar partner during recovery, and more so when in the repeated condition than in the acute condition. No differences were observed among adults. The Recovery analysis found a significant interaction of age and partner familiarity ($F_{1, 46}$ = 6.86, p = 0.01, n^2p = .13) and an interaction of age and stress exposures (p = 0.04, n^2p = .09) on corticosterone concentrations. The interaction of partner familiarity and stress exposures was not significant (p = 0.22, n^2p = .03), and the 3-way interaction approached significance (p = 0.08, n^2p = .07). To investigate the near three way significant interaction further, two-factor ANOVA (stress exposures and partner familiarity) on corticosterone concentrations were conducted separately for the two age groups. For adolescents, the interaction of partner familiarity and stress exposures was significant ($F_{1, 24}$ = 4.10, p = 0.05, n^2p = .15). When adolescents returned to the same cage partner after isolation, the Acute Isolation+familiar rats did not differ from the Repeated Isolation+familiar rats (p = 0.56, Cohen's *d* = 0.32). When returned to a new cage partner, the Repeated Isolation+unfamiliar rats were higher than the Acute Isolation+unfamiliar rats (p = 0.03, Cohen's *d* = 1.51). For the acute groups, higher corticosterone concentrations in those going to an unfamiliar partner (Acute Isolation+unfamiliar) compared to those going to a familiar partner (Acute

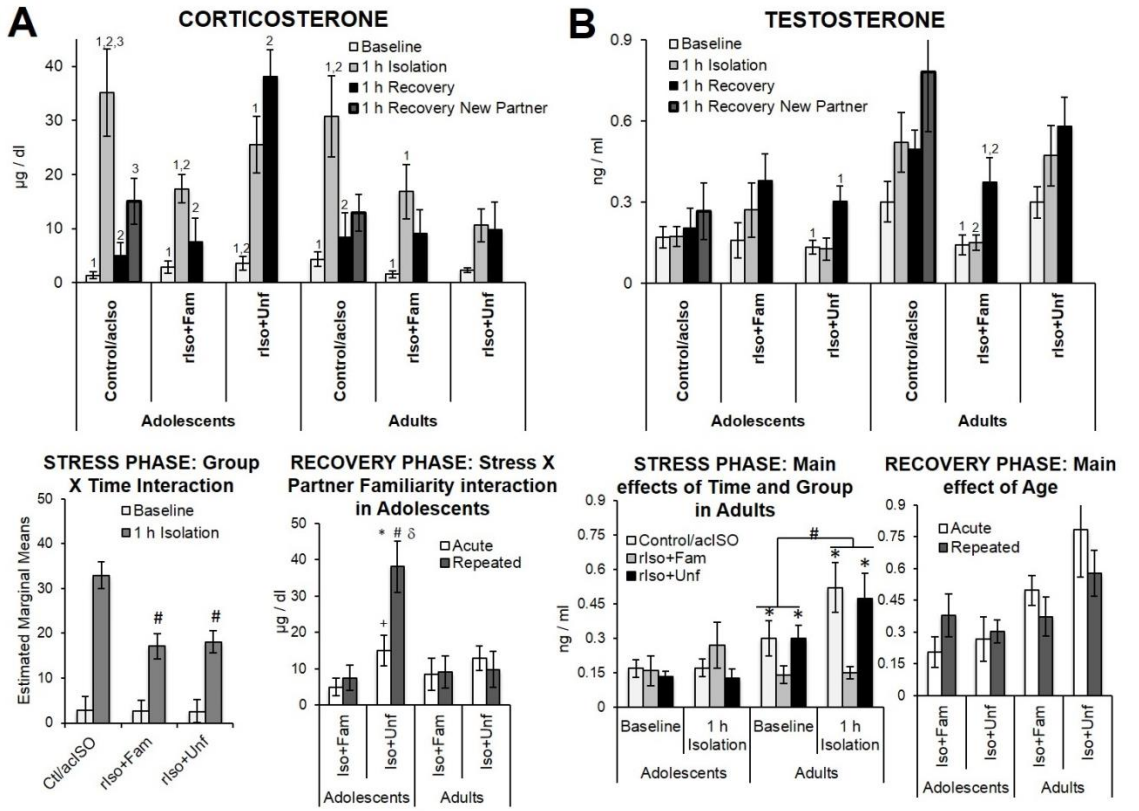

*Figure 2-5. Mean (±S.E.M.) concentrations of **(A)** plasma corticosterone and **(B)** plasma testosterone for acute and repeatedly stressed adolescent and adult rats before and after 1 hour isolation stress and after 1 hour of recovery post-stress with either a familiar or unfamiliar cage partner (n = 5 - 8 per group). Matching numbers above the bars indicate significant comparisons between time points within groups (Control/acIso [acute isolation], rIso+Fam [repeated isolation + familiar partner], rIso+Unf [repeated isolation + unfamiliar partner]) at both ages based on supplementary analyses. Lower panels illustrate significant main effects and interactions from the omnibus analyses. **(A) Stress phase**: # less than Ctl/acISO group ($p < 0.05$). **Recovery phase:** + higher than Acute Iso+Fam group ($p = 0.07$); * higher than acIso+Unf group ($p < 0.05$); # higher than rIso+Fam group ($p < 0.05$); δ higher than adult rIso+Unf group ($p < 0.05$). **(B) Stress phase:** * higher than rIso+Fam group ($p < 0.05$); # higher in 1 h isolation group than baseline group ($p < 0.05$).*

Isolation+familiar) failed to meet statistical significance (p = 0.07, Cohen's *d* = 1.24). For the repeated stress groups, Repeated Isolation+unfamiliar rats had higher corticosterone than the Repeated Isolation+familiar rats (p = 0.002, Cohen's *d* = 2.04; see Figure 2-5a).

For adults neither main effect nor their interaction was significant (p > 0.50, n^2p's < .02). Adolescents and adults were compared in each of the four repeated x partner familiarity groups. Corticosterone concentrations were higher in adolescents than adults only in the Repeated Isolation+unfamiliar groups (p = 0.01, Cohen's *d* = 1.75) (repeated return to a new cage partner), while all others were p > 0.50 (Cohen's *d* < 0.50) (see Figure 2-5a).

Plasma testosterone

There was no effect of isolation on testosterone concentrations in adolescents. In adults, isolation increased testosterone concentrations, and rats in the repeated isolation and familiar cage partner groups had lower testosterone than the other two groups. In the Stress analysis of plasma testosterone concentrations, there was a main effect of Age ($F_{1, 72}$ = 12.72, p = 0.001, n^2p = .15), of Time point ($F_{1, 72}$ = 4.51, p = 0.04, n^2p = .06) and an interaction of Group and Age ($F_{2, 72}$ = 7.25, p = 0.001, n^2p = .17). Thus, analyses were conducted for the age groups separately. In adolescents, neither main effect nor interaction were significant (ps > 0.33, n^2p's < .06). In adults, testosterone was higher after isolation than at baseline ($F_{1, 36}$ = 4.40, p = 0.04, n^2p = .11), and lower in the Repeated Isolation+familiar group compared to both the Control (p = 0.002, Cohen's *d* = 1.61) and the Repeated Isolation+unfamiliar groups (p = 0.003, Cohen's *d* = 0.01), ($F_{2, 36}$ = 7.01, p = 0.003, n^2p = .28; see Figure 2-5b).

After 1 h recovery, adolescents had lower testosterone concentrations than did adults, irrespective of stress history and partner familiarity. The Recovery analysis phase found a significant effect of age only ($F_{1, 48} = 11.99$, $p = 0.001$, $n^2p = .20$; all other ps > 0.08, n^2p's < .06), with higher testosterone concentrations in adults than in adolescents (see Figure 2-5b).

Paraventricular nucleus Zif268

Irrespective of age, only those undergoing an acute isolation increased Zif268-ir cell counts in the PVN after isolation compared to baseline, such that their ir-cell counts were higher at this time point than counts in the repeated isolation groups. There was an interaction of Time point and Group ($F_{1, 59} = 3.86$, $p = 0.03$, $n^2p = .12$): Zif268-ir cell counts were higher after isolation than at baseline only for the Control group ($p < 0.001$, Cohen's $d = 3.39$). The three groups did not differ at baseline, and Zif268-ir cell counts were higher in the Control group compared with the Repeated Isolation+familiar ($p = 0.001$, Cohen's $d = 1.66$) and Repeated Isolation+unfamiliar ($p = 0.02$, Cohen's $d = 0.64$) groups (see Figure 2-6a).

Adults had lower ir-cell counts after 1 h recovery than did adolescents, irrespective of stress history and partner familiarity. Only the factor of Age was significant (Adolescents > Adults, $F_{1, 37} = 17.52$, $p < 0.001$, $n^2p = .32$; all other ps > 0.15, n^2p's < .06; see Figure 2-6a).

Arcuate nucleus Zif268

Similar to the pattern in the PVN, in the ARC, irrespective of age, only those undergoing an acute isolation increased Zif268-ir cell counts after isolation compared to baseline, such that their ir-cell counts were higher at this time

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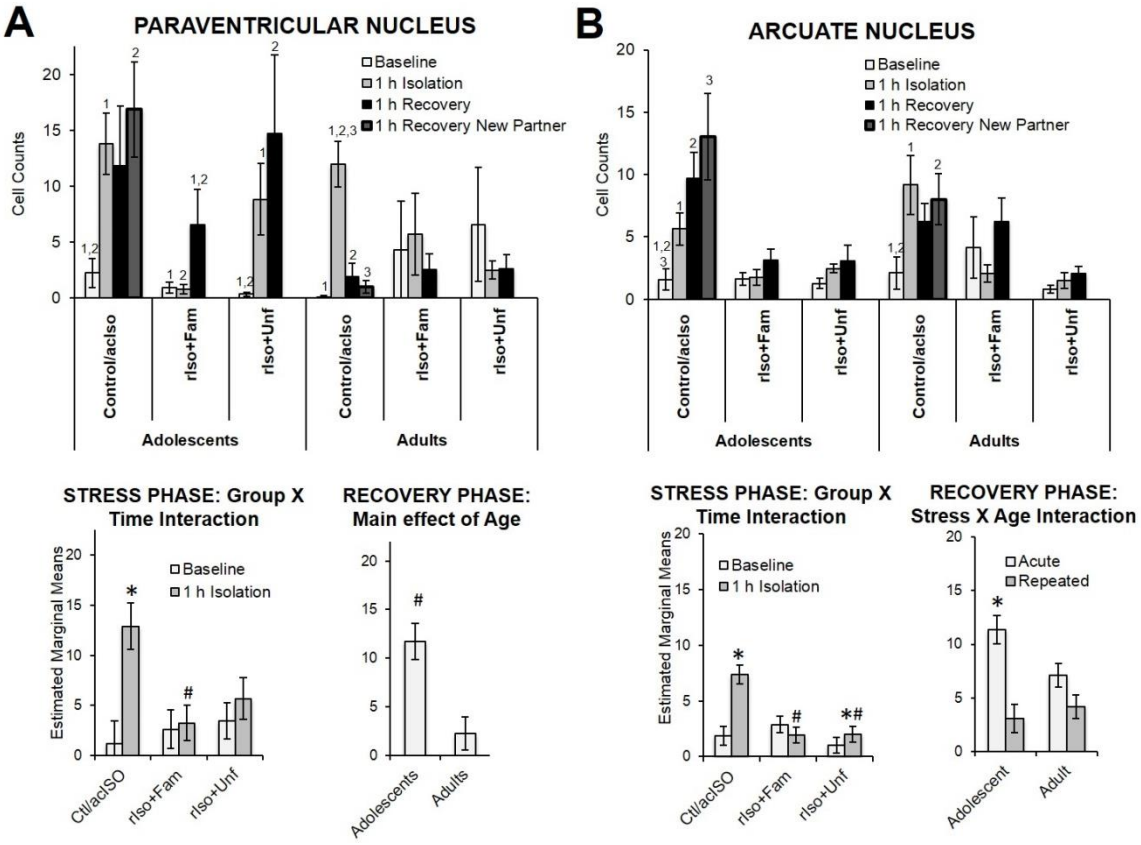

*Figure 2-6. Mean (±S.E.M.) Zif268 immunoreactive cell counts in the **(A)** paraventricular nucleus and **(B)** arcuate nucleus for acute and repeatedly stressed adolescent and adult rats before and after 1 hour isolation stress and after 1 hour of recovery post-stress with either a familiar or unfamiliar cage partner (n = 5 - 8 per group). Matching numbers above the bars indicate significant comparisons between time points within groups (Control/acIso [acute isolation], rIso+Fam [repeated isolation + familiar partner], rIso+Unf [repeated isolation + unfamiliar partner]) at both ages based on supplementary analyses. Lower panels illustrate significant main effects and interactions from the omnibus analyses. **(A) Stress phase: *** higher than baseline ($p < 0.05$); # lower than 1 h isolation Ctl/acISO ($p < 0.05$). **Recovery phase:** # higher than adults ($p < 0.05$). **(B) Stress phase: *** higher than baseline within groups ($p < 0.05$); # lower than Ctl/acISO ($p < 0.05$). **Recovery phase: *** higher than adolescent Repeated stress group ($p < 0.05$); + higher than adult Repeated stress group, $p = 0.09$.*

point than were those in the repeated isolation groups. An Age X Time point (baseline, 1 h isolation) X Group (Control, Repeated Isolation+familiar, Repeated Isolation+unfamiliar) ANOVA on Zif268-ir cell counts in the ARC found an interaction of Time point and Group ($F_{2, 73} = 8.93$, $p < 0.001$, $n^2p = .20$): Zif268-ir cell counts were higher after isolation than at baseline for the Control ($p = 0.002$, Cohen's $d = 1.60$) and Repeated Isolation+unfamiliar ($p = 0.04$, Cohen's $d = 0.78$) groups. The three groups did not differ at baseline, and Zif268-ir cell counts were higher after isolation in the Control group compared with the Repeated Isolation+unfamiliar ($p < 0.001$, Cohen's $d = 1.80$) and Repeated Isolation+familiar ($p < 0.001$, Cohen's $d = 1.71$) groups (see Figure 2-6b).

After one hour of recovery, the acute stress groups continued to have higher ir-counts than did repeated stress groups irrespective of partner familiarity, but the difference was significant only in adolescents. There was an interaction of Age and Stress ($F_{1, 46} = 4.74$, $p = 0.04$, $n^2p = .09$): Zif268-ir cell counts were higher in the acute stress than in the repeated stress groups for adolescents ($p < 0.001$, Cohen's $d = 1.72$) and not for adults ($p = 0.09$, Cohen's $d = 0.69$). The interaction of Partner Familiarity and Stress missed statistical significance ($p = 0.06$, $n^2p = .08$; see Figure 2-6b).

Anterior cingulate cortex Zif268

Adolescents had higher Zif268-ir cell counts than adults in the anterior cingulate cortex (ACC), except in the Repeated Isolation + familiar group, for which ir-cell counts were lower than in the other adolescent groups. The higher ir-cell counts in adolescents were significant after isolation, not at baseline. The interactions of Group and Age ($F_{2, 72} = 4.36$, $p = 0.02$, $n^2p = .11$) and of Age and Time Point ($F_{1, 72} = 4.94$, $p = 0.03$, $n^2p = .06$) were significant. Post hoc t-tests to interpret the interactions

found that adolescents had higher cell counts than adults for the Control (p = 0.03, Cohen's *d* = 0.98) and Repeated Isolation+unfamiliar groups (p = 0.009, Cohen's *d* = 1.10), but not for the Repeated Isolation+familiar group (p = 0.41, Cohen's *d* = 0.30). In adolescents, Repeated Isolation+familiar rats had lower ir-cell counts than did Repeated Isolation+unfamiliar rats (p = 0.003, Cohen's *d* = 1.36), and the lower ir-cell counts of Repeated Isolation+familiar rats compared to Control rats missed statistical significance (p = 0.07, Cohen's *d* = 0.68). Zif268-ir cell counts did not differ for the Age groups at baseline, and adolescents had higher ir- cell counts than did adults 1 h after isolation (p = 0.01, Cohen's *d* = 0.87). The effect of Group was not significant for adults (p = 0.27, n^2p = .07; see Figure 2-7a).

After 1 h of recovery, age and partner familiarity were important factors, and stress history was not. The interaction of Age and Partner familiarity was significant ($F_{1, 39}$ = 6.68, p = 0.01, n^2p = .15): In adolescents, Partner familiarity was not a significant factor (p = 0.28, n^2p = .06), and in adults, those with a familiar cage partner had higher ir-cell counts in the ACC than those with an unfamiliar cage partner (p = 0.02, Cohen's *d* = 1.07). Adolescents and adults with a familiar partner after isolation did not differ in ir-cell counts in the ACC (p = 0.59, Cohen's *d* = 0.25), whereas adolescents had higher ir-cell counts in the ACC than adults among those with an unfamiliar partner after isolation (p = 0.002, Cohen's *d* = 1.47; see Figure 2-7a).

Hippocampal pyramidal layer Zif268

There were no significant group differences on Zif268-ir cell counts in the hippocampus at baseline and after isolation (all ps > 0.10, n^2p's < .07; see Figure 2-7b).

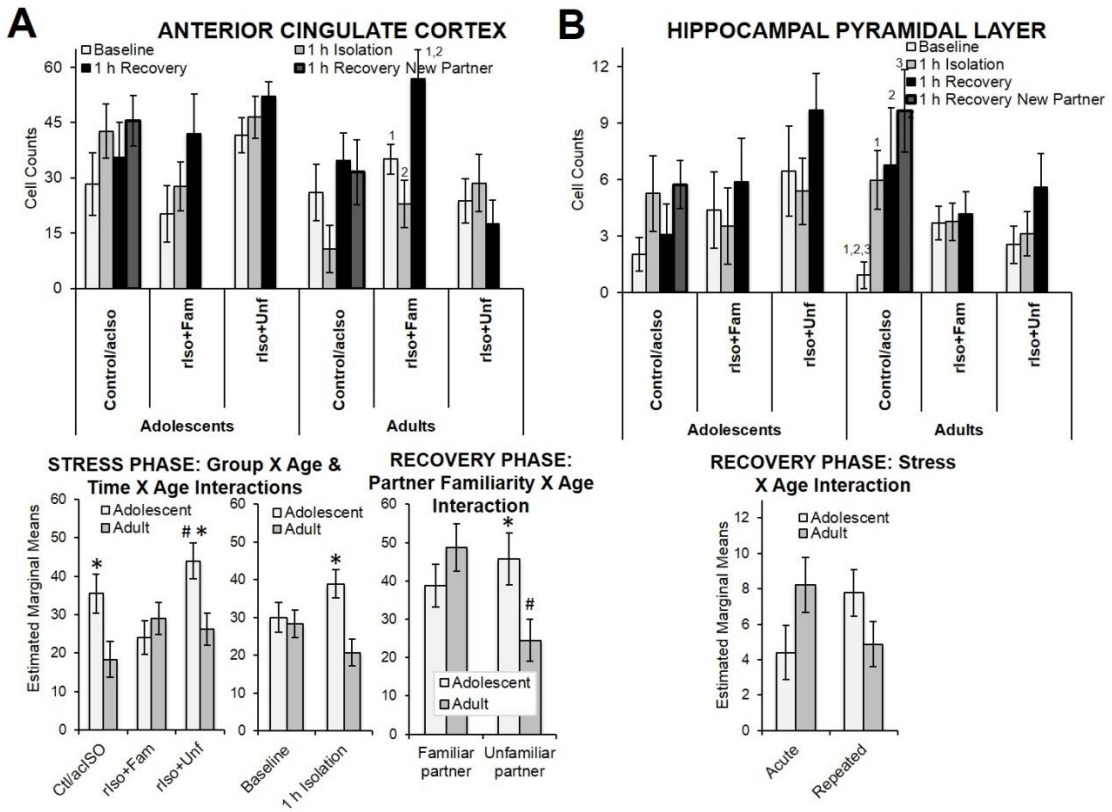

*Figure 2-7. Mean (±S.E.M.) Zif268 immunoreactive cell counts in the **(A)** anterior cingulate cortex and **(B)** hippocampus pyramidal layer for acute and repeatedly stressed adolescent and adult rats before and after 1 hour isolation stress and after 1 hour of recovery post-stress with either a familiar or unfamiliar cage partner (n = 5 - 8 per group). Matching numbers above the bars indicate significant comparisons between time points within groups (Control/acIso [acute isolation], rIso+Fam [repeated isolation + familiar partner], rIso+Unf [repeated isolation + unfamiliar partner]) at both ages based on supplementary analyses. Lower panels illustrate significant main effects and interactions from the omnibus analyses. **(A) Stress phase: *** higher than adults ($p < 0.05$); @ within 1 h isolation, higher than adults ($p < 0.05$); # higher than adolescent rIso+Fam group ($p < 0.05$). **Recovery phase:** * within Unfamiliar, higher than adults ($p < 0.05$); # lower than adult familiar group ($p < 0.05$). **(B)** No significant main effects or interactions.*

In the recovery analysis, only modest effects of age and of stress history were observed. An Age X Partner Familiarity (Familiar or Unfamiliar) X Stress (Acute or Repeated) ANOVA on Zif268-ir cell counts when returned back to the colony for 1 hour after isolation found an interaction of Age and Stress ($F_{1, 46} = 5.55$, $p = 0.02$, $n^2p = .11$), but no post hoc comparison was significant. The main effect of Partner Familiarity missed statistical significance (it was higher when with Unfamiliar Partner than with Familiar Partner, $p = 0.065$, $n^2p = .07$; see Figure 2-7b).

Medial amygdala Zif268

There were no significant group differences on Zif268-ir cell counts in the posterodorsal or posteroventral amygdala in the Stress analysis. For the posterodorsal amygdala, there were no significant differences in Zif268-ir cell counts among the groups at baseline and after isolation (all ps > .20, n^2p's < .04), and for the posteroventral amygdala, only the effect of Age ($p = 0.07$, $n^2p = .05$) and of Condition ($p = 0.07$, $n^2p = .07$) approached significance (see Figure 2-8).

For the posterodorsal amygdala, partner familiarity was not a factor in the Recovery analysis. Ir-cell counts were higher after one hour of recovery in adolescents than adults for the acute stress groups, and in adolescents (not adults), ir-cell counts were higher in those in the acute stress groups compared to the repeated stress groups. The interaction of Age and Stress was significant ($F_{1, 46} = 7.60$, $p = 0.008$, $n^2p = .14$): For adolescents, the acute stress groups had higher cell counts than did the repeated stress groups ($p = 0.015$, Cohen's $d = 1.41$), and there was no difference for adults ($p = 0.62$, Cohen's $d = 0.19$). There was an age difference (adolescent > adult) for the acute stress groups ($p = 0.04$, Cohen's $d = 0.85$), and not for the repeated stress

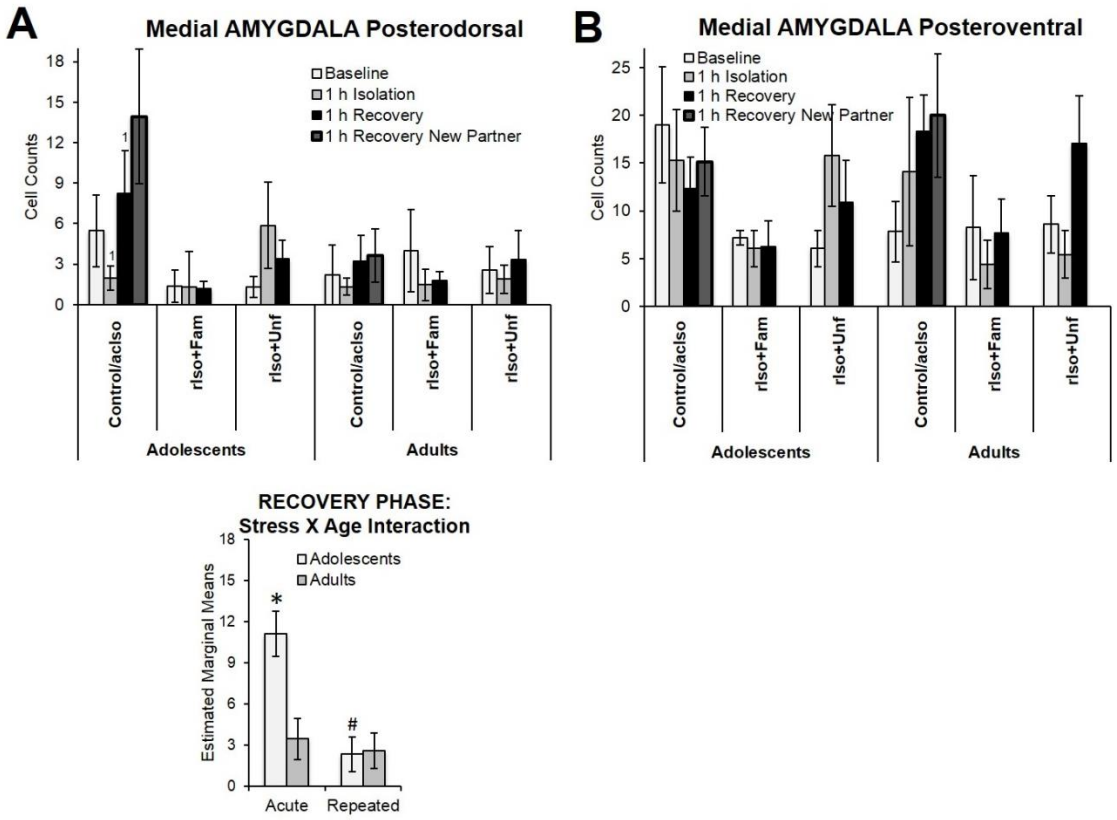

*Figure 2-8. Mean (±S.E.M.) Zif268 immunoreactive cell counts in the **(A)** medial amygdala posterodorsal part and in the **(B)** medial amygdala posteroventral part for acute and repeatedly stressed adolescent and adult rats before and after 1 hour isolation stress and after 1 hour of recovery post-stress with either a familiar or unfamiliar cage partner (n = 5 - 8 per group). Matching numbers above the bars indicate significant comparisons between time points within groups (Control/acIso [acute isolation], rIso+Fam [repeated isolation + familiar partner], rIso+Unf [repeated isolation + unfamiliar partner]) at both ages based on supplementary analyses. Lower panels illustrate significant main effects and interactions from the omnibus analyses. **(A) Recovery phase:** * within Acute, higher than adults ($p < 0.05$); # lower than adolescent Acute group ($p < 0.05$). **(B)** No significant main effects or interactions.*

groups (p = 0.84, Cohen's *d* = 0.54). For the posteroventral amygdala, after one hour back in the colony, only the factor of Stress approached significance (Acute > Repeated, p = 0.06, n^2p = .07; all other ps > 0.10, n^2p's < .05; see Figure 2-8).

Table 2-1 and Table 2-2 provide a summary of the results for all measures of the Stress phase and Recovery phase respectively.

Discussion

Although adolescents and adults differed in behavioural and neural responses to repeated stressors, the only age difference in corticosterone release found was in response to repeated pairings with unfamiliar cage partners, which suggests that adolescents may be more sensitive to social context than are adults.

Behaviour

Consistent with evidence of more social interaction among adolescents than among adults (e.g., Vanderschuren et al., 1997; Varlinskaya et al., 1998; Klein et al., 2010; Varlinskaya and Spear, 2008) and the evidence of higher reward value of social interactions in adolescence than in adulthood (Douglas et al., 2004), adolescents engaged in more social interactions after isolation than did adults in the early days of the repeated, daily isolation. The age difference was no longer found in the later days, which likely reflects developmental maturation of the adolescents from early, prepubertal, adolescence to mid-late, post-pubertal, adolescence rather than an effect of repeated isolation in adolescence; others have reported more social activity after an acute, 30 minute isolation in rats at PND 28 (early adolescence) than in rats at either PND 42 (mid adolescence) or PND 70 (adults) (Varlinskaya and Spear, 2008).

Partner familiarity had an effect on the amount of social interactions that

Table 2-1. Summary of the results for the stress phase.

	Stress phase analysis effects			
	AGE (adolescents compared with adults	TIME (Isolation compared to baseline)	GROUP (Ctl/acISO compared with rIso+Fam and/or rIso+Unf)	FAMILIARITY (rIso+Fam compared with rIso+Unf)
Endocrine				
Corticosterone	n.s.	↑↑	↑↑ (in Ctl/acISO than rIso+Fam and rIso+Unf)	n.s.
Testosterone	↓↓	↑↑ (only in adults)	↑↑ (in Ctl/acISO than rIso+Fam only in adults)	↓↓
Zif268-ir				
Paraventricular nucleus	n.s.	↑↑ (only in Ctl/acISO group)	↑↑ (in Ctl/acISO than rIso+Fam and rIso+Unf)	n.s.
Arcuate nucleus	n.s.	↑↑ (only in Ctl/acISO and rIso+Unf groups)	↑↑ (in Ctl/acISO than rIso+Fam and rIso+Unf)	n.s.
Anterior cingulate cortex	↑↑ (in Ctl/acISO and rIso+Unf groups, and immediately after isolation)	n.s.	↑ (in Ctl/acISO than rIso+Fam only in adolescents)	↓↓ (only in adolescents)
Pyramidal layer of hippocampus	n.s.	n.s.	n.s.	n.s.
Medial amygdala posterodorsal	n.s.	n.s.	n.s.	n.s.
Medial amygdala posteroventral	↑	n.s.	↑ (in Ctl/acISO than rIso+Fam and rIso+Unf)	n.s.

Arrows indicate the direction of an effect: increase, ↑↑ or decrease, ↓↓ = $p \leq 0.05$; ↑ = $p \leq 0.10$ and $p > 0.05$; n.s. (non-significant) = $p > 0.10$. Ctl/acISO = control exposed to acute isolation; rIso+Fam = repeated isolation and return to a familiar cage partner; rIso+Unf = repeated isolation and return to an unfamiliar cage partner.

Table 2-2. Summary of the results of the recovery phase.

	Recovery phase analysis effects		
	AGE (adolescents compared with adults	GROUP (Ctl/acISO compared with rIso)	FAMILIARITY (familiar compared to unfamiliar partner)
Endocrine			
Corticosterone	↑↑ (only in Iso+Unf groups)	↓↓ (in Ctl/acISO+Unf than rIso+Unf only in adolescents)	↓↓ (in rIso+Fam than rIso+Unf only in adolescents ↓ (Ctl/acIso+Fam than Ctl/acIso+Unf only in adolescents)
Testosterone	↓↓	n.s.	n.s.
Zif268-ir			
Paraventricular nucleus	↑↑	n.s.	n.s.
Arcuate nucleus	n.s.	↑↑ (in adolescents) ↑ (in adults)	n.s.
Anterior cingulate cortex	↑↑ (only in Iso+Unf groups)	n.s.	↑↑ (only in adults)
Pyramidal layer of hippocampus	n.s.	n.s.	↓
Medial amygdala posterodorsal	↑↑ (only in Ctl/acISO groups)	↑↑ (only in adolescents)	n.s.
Medial amygdala posteroventral	n.s.	↑	n.s.

Arrows indicate the direction of an effect: increase, ↑↑ or decrease, ↓↓ = $p \leq 0.05$; ↑ or ↓ = $p \leq 0.10$ and $p > 0.05$; n.s. (non-significant) = $p > 0.10$. Ctl/acISO = control exposed to acute isolation; rIso+Fam = repeated isolation and return to a familiar cage partner; rIso+Unf = repeated isolation and return to an unfamiliar cage partner.

adolescents and adults exhibited after isolation, with more social activity when with an unfamiliar than with a familiar partner during both initial and later observation days. These findings are consistent with another report that found more social investigation and social play behaviours but less passive contact behaviours (social rest, allogroom) with an unfamiliar conspecific than a familiar conspecific after 24 hour isolation in adolescent rats (Cirulli et al., 1996), as well as, several studies indicating greater time was spent investigating novel than familiar peers (e.g., Lukas, et al., 2011; Engelmann et al., 1995; Thor and Holloway, 1982). The patterns of behaviour in the home cage, however, do not explain the group differences in plasma corticosterone concentrations observed in response to repeated isolation and pairings with new cage partners.

Corticosterone

In addition to replicating our previous reports that partner familiarity influences recovery from an acute isolation (McCormick et al., 2007; Hodges et al., 2014), we also replicated our finding that adolescent males had reduced corticosterone release to repeated isolation compared to an acute isolation irrespective of whether paired with a familiar or unfamiliar partner (McCormick et al., 2007), and here we show that this finding is the same for adults as for adolescents. A reduction in corticosterone release to repeated, homotypic stressors compared to an acute or to a new (heterotypic) stressor is referred to as habituation, and has been reported using various homotypic stressors in adult rats (reviewed by Grissom and Bhatnagar, 2009; Zhang et al., 2014). In contrast, prepubertal and mid-adolescent males had either a sensitized (Lui et al., 2012; Romeo et al., 2006a; Doremus-Fitzwater et al., 2009; Watt et al., 2009), a habituated (Ariza Translaviña et al., 2014; Melia et al., 1994), or no change in corticosterone release

(Wright et al., 2008) to repeated homotypic stress. The variability across studies may involve the specific ages in adolescence at which the stressors are administered. In addition, the type of stress procedure may be more critical in adolescents than in adults for habituation.

In keeping with the possibility that habituation of corticosterone release is stressor-specific, adolescents had higher corticosterone concentrations and no evidence of recovery from isolation after placement with their 16th new cage partner than after a first placement with a new cage partner. Whereas adolescents showed sensitization of corticosterone release to novel partners, adults did not. One possibility is that adolescents treat each change of cage partner as a heterotypic stressor and thus do not habituate, whereas adults treat the repeated change of cage partners as a homotypic stressor to which they habituate. The heightened corticosterone concentrations of adolescents compared with adults is noteworthy considering our previous report of reduced plasma corticosteroid binding globulin (CBG) concentrations after repeated isolation and pairings with new cage partners (McCormick et al., 2007). CBG is a major transport protein for glucocorticoids in circulation, and a determinant for the percentage of free and bound corticosterone, although the functional consequences of a higher percentage of free corticosterone is unknown (reviewed in Sivukhina and Jirikowski, 2014; McEwen, 2007; Henley and Lightman, 2011). Down-regulated CBG expression and increased free glucocorticoids is found after exposure to various repeated stressors, such as repeated social defeat (Spencer et al., 1996; Stefanski, 2000) or repeated forced ambulation (Neufield et al., 1994). CBG is found in both neurons and glial cells in several brain regions associated with the stress response (e.g. PVN, bed nucleus of stria terminalis,

hippocampus, amygdala; reviewed in Sivukhina and Jirikowski, 2014). Further, glucocorticoid-bound CBG are reported to play a functional role in stress response and to regulate glucocorticoid actions on the brain by binding to CBG receptors (as reviewed in Henley and Lightman, 2011; Petersen et al., 2006; Willnow and Nykjaer, 2010; Nakhla et al., 1988; Strel'chyonok and Avvakumov, 1991). The greater exposure of adolescents than adults to corticosterone and a differential binding of glucocorticoid-bound CBG to receptors in stress-related brain regions may underlie why long term effects (e.g. memory of conditioned fear, Morrissey et al., 2011; behavioural responses to psychostimulants, McCormick et al., 2005) are found in adolescent, but not in adult, rats subjected to repeated social instability stress.

That adolescents habituate to repeated isolation but not to repeated social instability may be related to our previous finding that adolescents that underwent repeated isolation and pairings with an unfamiliar partner increased CRH mRNA expression in the central nucleus of the amygdala after one hour isolation as did rats undergoing a first isolation, whereas rats that were undergoing repeated isolation but always returned to a familiar partner did not increase CRH mRNA to the 16th isolation (McCormick et al., 2007). We proposed that isolation and pairings with an unfamiliar peer remains a highly salient stimulus when repeated in adolescence because of a learned association between isolation and a novel cage partner; thus, isolation is the cue for the new partner to come. One prediction from our current results would be that adult rats would not show increased CRH mRNA in the amygdala to repeated isolation and unfamiliar cage partners, as partner unfamiliarity does not seem to remain as salient a stimulus for adults as for adolescents.

Zif268

Although age differences in corticosterone release were limited to the recovery phase involving repeated pairing with unfamiliar partners, broader age differences were observed in neural activation in HPA-relevant brain regions, particularly during the recovery period. Consistent with our previous report involving acute isolation stress at 30 versus 70 days of age (Hodges et al., 2014), both adolescents and adults increased Zif268-ir cell counts after isolation in the PVN and in the ARC. Increased immediate-early gene expression in both these regions is typically found in response to acute restraint stress (e.g., Cullinan et al., 1995; Kwon et al., 2006; Meyza et al., 2007; Melia et al., 1994). Reciprocal projections between the paraventricular nucleus and the arcuate nucleus are involved in regulating glucocorticoid release; neurons in the arcuate nucleus have the capacity to inhibit HPA activity when stimulated by neurons in the paraventricular nucleus (reviewed in Herman and Cullinan, 1997; Charmandari et al., 2005; Senba and Ueyama, 1997; Baker and Herkenham, 1995). In addition, lesions in the arcuate nucleus increased ACTH and corticosterone concentrations at baseline and enhanced the release of ACTH and corticosterone to stressors (reviewed in Herman and Cullinan, 1997).

In the recovery phase after acute isolation, adolescents had higher Zif268 ir-cell counts in the paraventricular nucleus than did adults. These findings are consistent with our past report of higher Zif268 ir-cell counts in the adolescent (PND 30) PVN than in the adult (PND 70) paraventricular nucleus after isolation stress (Hodges et al., 2014) and to reports of greater Fos protein expression in the paraventricular nucleus of adolescent rats than adult rats in response to acute restraint (Romeo et al., 2006a; Lui et al., 2012;

Viau et al., 2005) and to novel environments (Novak et al., 2007). The adolescent and adult paraventricular nucleus are similar in cell density and volume (Romeo et al., 2007). Thus, the age differences in neural activation in the paraventricular nucleus may involve age differences upstream from the paraventricular nucleus and may be the basis for evidence of prolonged corticosterone release to some stressors in adolescents compared with adults (reviewed in Eiland and Romeo, 2013).

In keeping with the finding of reduced corticosterone release to repeated isolation at both ages, there were higher Zif268 immunoreactive cell counts after a first isolation than after a 16th isolation in the paraventricular nucleus and the arcuate nucleus at both ages, and in adolescents in the medial amygdala. These findings are consistent with several studies in adult rodents that found lower immediate-early gene expression after repeated than acute exposure to a stressor in the paraventricular nucleus (Watanabe et al., 1994; Chen and Herbert, 1995; Girotti et al., 2006; Kwon et al., 2006; Martinez et al., 1998), in the arcuate nucleus (Kwon et al., 2006), and in the medial amygdala (Chen and Herbert, 1995; Martinez et al., 1998). Although the ACC and in the hippocampus pyramidal layer regulate the HPA response to stress and are involved in social behaviour (reviewed in Martinez et al., 2002), only modest effects involving interactions of isolation and partner familiarity and/or age were observed in the present experiments that are difficult to interpret, but suggest a complex pattern of stress and social context differences in neural activation between adolescents and adults.

Testosterone

Consistent with the present findings, we previously found that testosterone increased one hour after return to either a familiar or an unfamiliar peer after an acute

isolation, and testosterone concentrations had a slower return to baseline three hours after isolation if paired with an unfamiliar peer than a familiar peer (Hodges et al., 2014). In the present study, however, the increase was found immediately after an acute isolation and was sustained during the recovery period. Rats that underwent repeated isolation and pairing with a familiar peer, however, had lower testosterone concentrations in the stress period and did not show a rise in testosterone until the recovery period. These findings suggest differential effects on the hypothalamic-pituitary-gonadal axis to repeated isolation when paired with an unfamiliar than with a familiar partner. Nevertheless, several studies have reported an increase in testosterone in response to acute stressors in male rats (e.g., Foilb et al., 2011; Gomez et al., 2002; Gomez et al., 2004). Also, others have reported increases in testosterone concentrations during social interactions (reviewed in Gleason et al., 2009; Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990; Oyegbile and Marler, 2005; Wingfield and Wada, 1989). For example, higher testosterone concentrations were reported in rodents that won agonistic encounters than rodents that did not compete in agonistic encounters (Oyegbile and Marler, 2005). Aggressive behaviours, however, were rarely exhibited in adult rats when returned to either a familiar or unfamiliar peer (data not shown), which suggests that other types of social interactions may play a role. Although figure 2-5 suggests that testosterone concentrations may rise during recovery from isolation in adolescents, no significant differences were observed other than the expected lower testosterone concentrations in adolescents than in adults. Thus, the hypothalamic-pituitary-gonadal axis, which is continuing to mature at 45 days of age (e.g., Varlinskaya et al., 2013a), may be less responsive to social interactions than is the adult axis.

Conclusion

The present results reveal how the functioning of social brain regions may be an important factor in understanding age differences in behavioural and neuroendocrine responses to stressors. Whereas adolescents increased affiliative behaviour after isolation irrespective of partner familiarity, adults did not, although both age groups clearly differentiated between familiar and unfamiliar partners, spending more time in active social investigation when with an unfamiliar partner than when with a familiar partner. Further, adolescents increased corticosterone release to repeated pairings with an unfamiliar cage partner and adults did not. Adolescence is recognized to be an important period of social learning (reviewed in Blakemore and Mills, 2014), and our findings provide further evidence that adolescents perceive social experiences differently than at other stages of development, and suggests that social context may be more relevant for recovery from stressors in adolescence than in adulthood. Social factors may thus be an important basis for the heightened plasticity and vulnerability of the adolescent brain compared relative to adulthood.

Rationale for chapter 3

Previous research has demonstrated deficits in many types of social behaviour in adulthood after adolescent SS. Adolescent social instability reduced time spent in social interaction with unfamiliar peers (Green et al., 2013), increased aggression with a conspecific in food competition (Cumming et al., 2014), and impaired mating behaviour with females (McCormick et al., 2013) when tested in adulthood. My findings from chapter 2 suggest that adolescent rats that underwent the SS procedure are exposed to high concentrations of corticosterone on the final day of the procedure, which may alter the development of social brain regions. Further, adolescent SS reduced the neural activation of the posterodorsal part of the medial amygdala (involved in processing social behaviour with familiar and unfamiliar peers) on PND 45, which could underlie altered social behaviour soon after the procedure. One aim of the research in chapter 3 was to investigate the extent to which adolescent SS lowers social interaction with unfamiliar peers in a novel environment on PND 46 (one day after the stress procedure). This finding would determine whether reduced social interactions with unfamiliar peers is an effect of adolescent SS that is expressed when rats reach adulthood or whether it lasts into adulthood. I investigated whether adolescent SS affected the preference to investigate novel peers over familiar peers using a social recognition test, and the effects of adolescent SS on the rewarding properties of social interactions with unfamiliar peers using a social conditioned place preference test. Additionally, I investigated whether adolescent SS altered oxytocin and vasopressin 1A receptor binding in several social brain regions on PND 46 (oxytocin and vasopressin play major roles in the initiation and maintenance of many social behaviours).

Chapter 3: Social instability stress in adolescent male rats reduces social interaction and social recognition performance and increases oxytocin receptor binding.

This chapter has been adapted from the published article:

Hodges, T. E., Baumbach, J. L., Marcolin, M. L., Bredewold, R., Veenema, A. H., & McCormick, C. M. (2017). Social instability stress in adolescent male rats reduces social interaction and social recognition performance and increases oxytocin receptor binding. *Neuroscience, 359,* 172-182.

Author contribution: For this manuscript, Dr. Cheryl McCormick and I designed the experiment and formulated the research questions. I wrote the proposal for ethics approval, ordered the rats required for the experiment, performed the social instability stress procedure on rats, performed the behavioural testing (with help from fellow students Jennet Baumbach [undergraduate student at the time] and Marina Marcolin [graduate student at the time]), collected rat brains, sliced rat brains, prepared brains for receptor autoradiography, analyzed the data, performed the statistics, created the figures, wrote the paper, and edited the paper after I received feedback from Dr. Cheryl McCormick and our collaborators. Receptor autoradiography was performed by Remco Bredewold and Dr. Alexa Veenema, and pictures of slices were then sent to me to quantify.

Introduction

The ability to display context-appropriate affiliative and aggressive behaviours is necessary for animals to adapt to a changing social environment (reviewed in O'Connell and Hofmann, 2011; 2012). The adolescent brain undergoes several morphological and functional changes in brain regions involved in social behaviour and the salience of social cues (reviewed in Spear, 2000; O'Connell and Hofmann, 2011). Social experiences, in turn, may influence ongoing development and thereby alter future social behaviour, because of the enhanced plasticity of the brain during the adolescent period (reviewed in Buwalda et al., 2011, McCormick et al., 2015). Indeed, social experience in adolescence in rats is crucial to the development of appropriate social behaviour (reviewed in Buwalda et al., 2011; Pellis et al., 2014; Pellis and Pellis, 2017). For example, social deprivation during the adolescent period in rats reduced their approach behaviour to pro-social 50-kHz ultrasonic vocalizations in adulthood compared with non-isolated controls (Seffer et al., 2015). Rats isolated in early-adolescence spend less time in contact with unfamiliar conspecifics both in late-adolescence (van den Berg et al., 1999a) and in adulthood (Hol et al., 1999; Lukkes et al., 2009). After social isolation in adolescence, rats are able to perform socially appropriate behaviours, but these are often misapplied (reviewed in Pellis et al., 2014; Pellis and Pellis, 2017).

Although there is much evidence that the absence of social experience in adolescence impairs social behaviour, the quality of social interactions in adolescence may also influence social development. Rats that underwent repeated SS in adolescence (daily one hour isolation and return to an unfamiliar cage partner from PND 30 – 45) spent less time in social interaction with unfamiliar peers (Green et al., 2013),

demonstrated greater aggression with a conspecific in food competition (Cumming et al., 2014), and had impaired mating behaviour (McCormick et al., 2013) that depended on the individual's social status (McCormick et al., 2017a). Here, we investigated the influence of SS on social recognition and social reward (social conditioned place preference) in addition to social interactions. Whereas our previous studies of social behaviour (social interaction, food competition, sexual behaviour) described in this paragraph were conducted in adulthood (> PND 60) a minimum of two weeks after the social instability stress procedure, here tests were conducted within days of the social instability stress exposures while the rats were mid- to late-adolescents (tests began on PND 46). Although some effects of SS are known to be long-lasting (reviewed in McCormick et al., 2015), the effects of SS on social behaviour may be more evident soon after the procedure in the adolescent period, a time in which social interaction, preference for social novelty, and social reward is typically higher than in adulthood (e.g., Douglas et al., 2004; reviewed in Doremus-Fitzwater et al., 2010). In addition, in the test of social interaction, we compared social interactions with an unfamiliar peer relative to that with a cage mate in the test arena to determine whether any decrease in social interaction is limited to novel peers.

We also investigated whether SS and control rats differed in oxytocin receptor and vasopressin 1a receptor binding in brain regions that are relevant for social behavioural function and social salience. The neuropeptides oxytocin and vasopressin are involved in the regulation of appropriate social responses to social stimuli (reviewed in Veenema and Neumann, 2008). Oxytocin and vasopressin regulate social interaction (Bredewold et al., 2014; Dumais et al., 2016a), social reward (Dölen et al., 2013), and

social recognition (Dantzer et al., 1988; Veenema et al., 2012; Bychowski et al., 2013; Lukas et al., 2013; Dumais et al., 2016b) in rodents by acting on oxytocin and vasopressin receptors (reviewed in Stoop, 2014). Further, gonadal hormones, the release of which increases in adolescence, regulate the expression of oxytocin receptors and both oxytocin and vasopressin in brain regions involved in social behaviours (e.g., De Vries et al., 1984; Champagne et al., 2001; Somponpun and Sladek, 2002; see Albers, 2015 for a review). In addition, there are changes in oxytocin receptor binding density and vasopressin 1a receptor binding density from early-adolescence to adulthood in rats (Tribollet et al., 1991; Lukas et al., 2010; Smith et al., 2017b). Thus, any effects of SS on social behaviour may be accompanied by changes in these receptor densities.

Methods

Animals

The experiments involved male Long-Evans rats (test rats, n = 171; stimulus rats for social recognition and conditioned place preference testing, n = 20) obtained from Charles River, Kingston, New York, on PND 22 and given a week to acclimate to the animal colony. Rats were housed in pairs and maintained under a 12 h light–dark cycle (lights on at 05:00 h) with food and water available ad libitum. Use of animals in these experiments was approved by the Brock University Institutional Animal Care Committee and was carried out in adherence to the Canadian Council on Animal Care guidelines.

Social instability stress procedure

Rats were randomly assigned to the adolescent SS, (n = 83) group or to the non-stressed control (CTL, n = 88) group. The SS procedure was as described previously (reviewed in McCormick, 2010; McCormick et al., 2015). Beginning on PND 30, SS rats

were isolated in a 12 cm x 10 cm ventilated plastic container in a room separate from the colony for 1 h each day until PND 45. Immediately after isolation each day, SS rats were returned to the animal colony and housed in a new cage with a new cage partner that had also undergone the 1 h-isolation. The SS procedure was conducted at various times during the lights on phase of the light–dark cycle to minimize habituation to the procedure. Although this procedure may result in some sleep restriction, which can also have consequences for development (da Silva Rocha-Lopes et al., 2018), we have shown that the effects of daily change of cage partners after daily isolation are greater than those of daily one hour isolation and return to the familiar cage partner (McCormick et al., 2007; McCormick and Ibrahim, 2007; Hodges and McCormick, 2015), which is consistent with our definition of the procedure as a social stressor. On PND 45, after the final isolation, SS rats remained with the same cage partner and were left undisturbed except for cage maintenance or test procedures. CTL rats remained undisturbed in their home cages except for cage maintenance from time of arrival at the colony until the test procedures (see Figure 3-1 for experimental design and timeline of procedures).

Social Interaction Test

The social interaction test was conducted on PND 46 in a plastic arena (61 cm x 30 cm x 53 cm). A video-camera mounted on the ceiling above the test apparatus was used to record the task. The apparatus was cleaned before each session with 70% ethanol. For the social interaction test, CTL (n = 32) and SS (n = 32) rats were either (1) paired with their cage partner (Familiar; CTL with familiar CTL, n = 16; SS with familiar SS, n = 16) or (2) paired with an unfamiliar peer of the same treatment condition (Unfamiliar partner; CTL with unfamiliar CTL, n = 16; SS with unfamiliar SS, n = 16). Total time

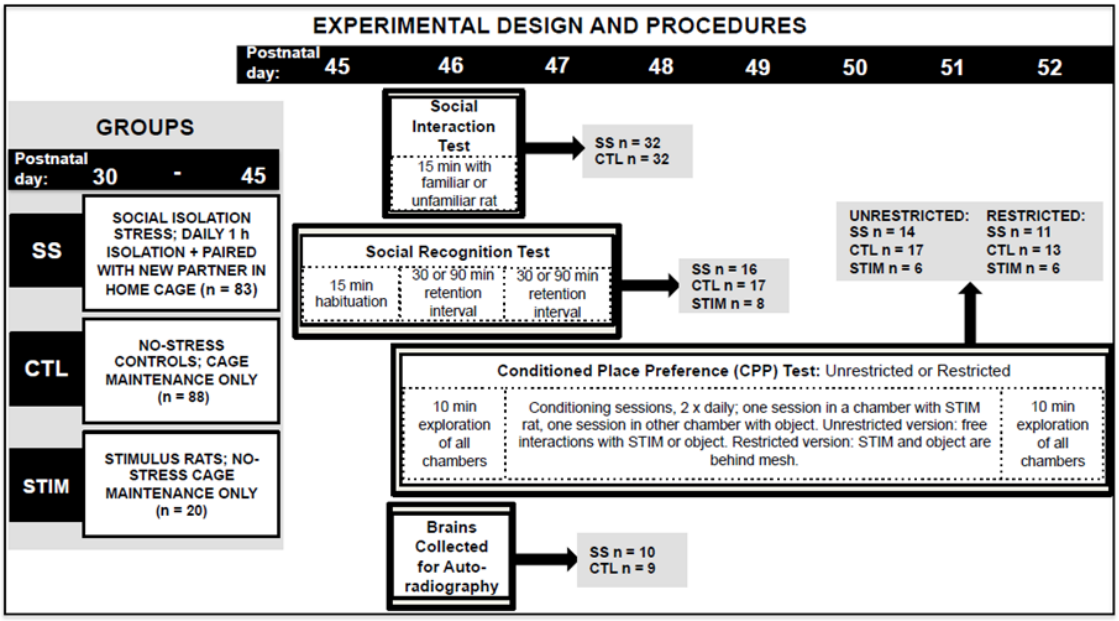

Figure 3-1. Experimental groups and timeline of experimental procedures. Separate cohorts of rats were used for each behavioural test and for the receptor autoradiography. SS = social instability stress; CTL = control; STIM = stimulus rats.

spent in social interaction (including social play, anogenital sniffing, allogroom, following, investigation; as defined in Cirulli et al., 1996 and as in our previous research, Green et al, 2013) during the 15 min test session for each pair of rats was measured by an experimenter blind to both the Stress Group and Familiarity conditions. The pair of rats was treated as a unit of analysis and the total score for the pair was used. Social interaction test sessions were conducted in dim red light between one and five hours after the onset of the dark phase.

Social Recognition Test

The test apparatus was made of plywood coated in vinyl, and was separated into one middle chamber (61 cm x 31 cm x 52 cm) and two side chambers (22 cm x 24 cm x 20 cm) by two metal mesh screens. A video-camera mounted on the ceiling above the test apparatus recorded the test sessions, and the apparatus was cleaned before each session with 70% ethanol.

CTL (n = 17) and SS (n = 16) rats were habituated to the test apparatus individually on PND 45, a minimum of 8 hours after the last 1 hour isolation of the SS procedure. For the habituation procedure, CTL and SS rats were placed in the middle chamber of the test apparatus and left to explore for 15 min before returning to their home cage. The Social Recognition Test was conducted on both PND 46 and PND 47 and involved a Familiarization phase and a Test phase and was adapted from the procedures of Eagle et al. (2013). Stimulus rats (age-matched male rats unfamiliar to the CTL and SS rats; n = 8) were placed into the two side chambers of the test apparatus (one rat per side), and test rats (CTL and SS rats, tested individually) were placed in the middle chamber of the test apparatus to explore for 5 minutes (the Familiarization phase).

Rats were removed from the test apparatus and randomly assigned to either spend a 30 or 90 min retention interval alone in cages containing bedding from the home cage; an impairment in social recognition in SS rats may only be evident with a longer retention interval of 90 min. Stimulus rats were returned to their home cage and cage partner until their next use. After the retention interval, the test rats were placed back into the middle chamber of the test apparatus for 5 min (Test phase) with one stimulus rat from the familiarization phase in one side chamber (Familiar rat) and a Novel stimulus rat in the opposite side chamber; the position of the familiar and novel stimulus rats in the side chambers was counterbalanced across treatments. After the test phase, CTL and SS rats were returned to their home cage and cage partner. On PND 47, CTL and SS rats were tested on the social recognition test again, but they were assigned to the other retention interval (30 or 90 min) from that on PND 46. The time that CTL and SS rats spent investigating each stimulus rat (sniffing the mesh of the side chamber containing a stimulus rat) was measured using a computer program and by an experimenter blind to both the condition of the experimental rat and the novelty of the stimulus rats. All social recognition testing occurred during the dark phase under dim red light.

Conditioned Place Preference Test

The CPP test apparatus consisted of a plastic arena (61 cm x 30 cm x 53 cm) separated into one middle chamber (15 cm x 30 cm x 53 cm) and two side chambers (23 cm x 30 cm x 53 cm) by removable metal dividers (30 cm x 53 cm). The middle chamber had metal mesh flooring and white walls, one side chamber had stripped blue and white walls and a white glossy floor, and the other side chamber had a blue textured floor and glossy white walls. A video-camera mounted on the ceiling above the test apparatus was

used to record on the habituation and test days. The apparatus and the metal dividers were cleaned before each session with 70% ethanol. The CPP tests involved a habituation day, five consecutive conditioning days, and a test day (procedures were based on Douglas et al., 2004 and Peartree et al., 2012).

On PND 46, a separate group of CTL and SS rats (not used for any other behavioural measure) were placed alone into the middle chamber of the test apparatus, the metal dividers were removed, and the rats were allowed to explore all three empty chambers of the test apparatus for 10 min (Habituation day). Entry into each chamber was operationally defined as the experimental rat's two forepaws in contact with the floor of that chamber. Rats did not show a preference for one side of the chamber; no rat spent more than 59% of its time in one chamber and no less than 41% of its time in the other chamber.

Our choice of CPP tests was based on our previous finding that although SS rats spend less time interacting with unfamiliar conspecifics when physical contact is possible, they spend as much time as do CTL rats near unfamiliar conspecifics separated by wire mesh (Green et al., 2013). If social interactions for SS rats are less anxiogenic when the peer is behind mesh, then SS rats may exhibit CPP only when chambers are paired with a stimulus rat that is confined behind mesh). Nevertheless, previous research has shown that although physical contact with a rat is not required for the development of a social CPP, social CPP is stronger when conditioning allows for physical contact (Peartree et al., 2012). Thus, we used two versions of the conditioning days of the CPP test, with different rats assigned to each version. In the Unrestricted CPP test (CTL, n = 17; SS, n = 14), the five conditioning days involved pairing one side chamber with

physical access to a stimulus rat and pairing the other chamber with access to a novel object. In the Restricted CPP test (CTL, *n* = 13; SS, *n* = 11), the five conditioning days involved pairing one side chamber with a stimulus rat confined to a metal mesh container (18 cm x 18 cm x 18 cm) and pairing the other chamber with a similar metal mesh container containing an object. The object was of similar size to the stimulus rats, and thus controlled for the amount of space in the chamber that the test rats could explore. Conditioning sessions of 15 minutes were conducted twice per day (once in the morning and once in the afternoon), with test rats spending time in the chamber with the stimulus rat in one session and in the chamber with the object in the other session (counter-balanced across days and rats). Test rats were placed into one side chamber and were blocked from entering the middle chamber or opposite side chamber by metal dividers. The interval between each morning and afternoon session was at least 3 h. Stimulus rats were age-matched male rats unfamiliar to the CTL and SS rats (Restricted contact, *n* = 6; Unrestricted contact, *n* = 6), and each rat interacted with the same stimulus rat and novel object each day during the conditioning sessions. After each conditioning session, rats were returned to their home cage and their cage partner.

For the Test day on PND 52, CTL and SS rats were placed alone into the middle chamber of the test apparatus, the metal dividers were removed, and they were allowed to explore all three chambers (non-paired middle chamber, Social-paired side chamber, nonSocial-paired side chamber) for 15 minutes. All conditioned place preference testing occurred during the light phase under white light (in keeping with Douglas et al., 2004; Thiel et al., 2008; Trezza et al., 2009; Peartree et al., 2012) to allow the rats to see the visual cues.

Receptor autoradiography

To measure the effect of the SS procedure on OTR and V1AR binding, rats (CTL, *n* = 9; SS, *n* = 10) were decapitated directly upon removal from the home cage on PND 46, 24 h after the last day of the SS procedure. Brains were quickly extracted, frozen in methylbutane on dry ice, and stored at -80˚C. Coronal sections (16 μm) were sliced on a cryostat, collected from approximately bregma 2.52 to approximately bregma -3.72 mm (according to Paxinos and Watson, 2005), and then frozen on slides at -80˚C until receptor autoradiography was performed.

Receptor autoradiography was performed using [^{125}I]-Ornithine Vasotocin Analog (d(CH_2)$_5$[-Tyr(Me)^2,Thr^4,Orn^8,[^{125}I]Tyr^9-NH_2]-OVTA (Perkin Elmer, Boston, MA, USA) as a tracer for OTR and using [^{125}I]-d(CH_2)$_5$(Tyr[Me])-AVP (Perkin Elmer, Boston, MA, USA) as a tracer for V1AR. Receptor autoradiography was conducted in keeping with Smith et al. (2017b). The slides of coronal sections were thawed, dried at room temperature, and then fixed for 2 min in 0.1% paraformaldehyde. Slides were then washed in 50 mM Tris (pH 7.4), incubated in tracer buffer (50 pM tracer, 50 mM Tris, 10 mM MgCl2, 0.01% BSA) for 60 min, and washed in a Tris + 10 mM $MgCl_2$ solution. After washing, slides were dipped in distilled water, dried at room temperature, and then exposed to Biomax MR films (VWR International, Pittsburgh, PA, USA). Coronal sections of CTL and SS rats were processed simultaneously and balanced across both incubation chambers and exposure to films. A 4-day exposure time was used to analyze both OTR and V1AR binding density, and autoradiography films were digitized using a Northern Light Illuminator (InterFocus Imaging, UK).

Image and data analysis

ImageJ (National Institute of Health; http://imagej.nih.gov/ij/) was used to measure optical densities of OTR and V1AR in coronal sections. The mean of bilateral measurements in a fixed number of coronal sections per region of interest per rat had film background subtracted and was used to calculate each OTR and V1AR binding density. A minimum of two coronal sections for each brain region were used to take measurements, and the highest 4-6 measurements of binding density were averaged to obtain a mean for each region of interest. A [^{125}I] standard microscale (American Radiolabeled Chemicals Inc., St. Louis, MO, USA) was used to convert the data to dpm/mg (disintegrations per minute/milligram tissue). Rats with less than 4 measurements of binding density for a specific brain region did not have their mean for that region of interest included in our statistics. Overall, 13 brain regions were analyzed for OTR binding and 10 brain regions were analyzed for V1AR binding. All brain regions chosen are involved in regulating social behaviour (reviewed in O'Connell and Hofmann, 2011; Lim and Young, 2006), and all were identified according to Paxinos and Watson (2005); dorsal lateral septum (dLS; within bregma 1.44 mm and -0.36 mm), ventral lateral septum (vLS; within bregma 1.44 mm and -0.36 mm), intermediate lateral septum (iLS; within bregma 1.44 mm and -0.36 mm), nucleus accumbens shell (NAcS; within bregma 1.44 mm and 0.96 mm), CeA (within bregma -1.92 mm and -3.36 mm), BLA (within bregma -2.16 mm and -3.36 mm), MePD (within bregma -2.76 mm and -3.24 mm), MePV (within bregma -2.64 mm and -3.24 mm), anterodorsal medial amygdala (MeAD; within bregma -1.92 mm and -2.64 mm), anteroventral medial amygdala (MeAV; within bregma -1.92 mm and -2.52 mm), anteromedial bed nucleus of stria terminalis (amBNST; within bregma -0.12 mm and -0.48 mm), posteromedial bed

nucleus of stria terminalis (pmBNST; within bregma -0.60 mm and -0.96 mm), dorsolateral bed nucleus of stria terminalis (dlBNST; within bregma 0.24 mm and -0.24 mm), posterolateral bed nucleus of stria terminalis (plBNST; within bregma -0.12 mm and -0.72 mm), ventromedial hypothalamus (VMH; within bregma -2.64 mm and -3.24 mm), and medial preoptic area (MPOA; within bregma -0.12 mm and -0.84 mm). Measures for the posterodorsal and posteroventral medial amygdala were combined and called posterior medial amygdala (pMeA), and measures for the anterodorsal and anteroventral medial amygdala were combined and called anterior medial amygdala (aMeA) because of functional similarity (reviewed in Newman, 1999). Further, the more anterior part of the lateral septum was measured for OTR binding because of greater density (within bregma 1.44 mm and 0.72 mm), and the more posterior part of the lateral septum was measured for V1AR binding because of greater density (within bregma 0.60 mm and -0.36 mm). OTR binding density was exclusively measured in the basolateral amygdala, posteromedial bed nucleus of stria terminalis, dorsolateral bed nucleus of stria terminalis ventromedial hypothalamus, and medial preoptic area because of low V1AR binding density in these areas. V1AR binding density was exclusively measured in the posterolateral bed nucleus of stria terminalis because of low OTR binding density in this area. See Figure 3-2 for receptor autoradiograms and schematic diagrams indicating all brain regions analyzed.

Statistics

Statistical analyses were performed using SPSS version 20 software and consisted of mixed factor analysis of variances (ANOVAs), independent samples t-tests and paired samples t-tests. Post hoc analysis consisted of t-tests and Fishers LSD, where appropriate.

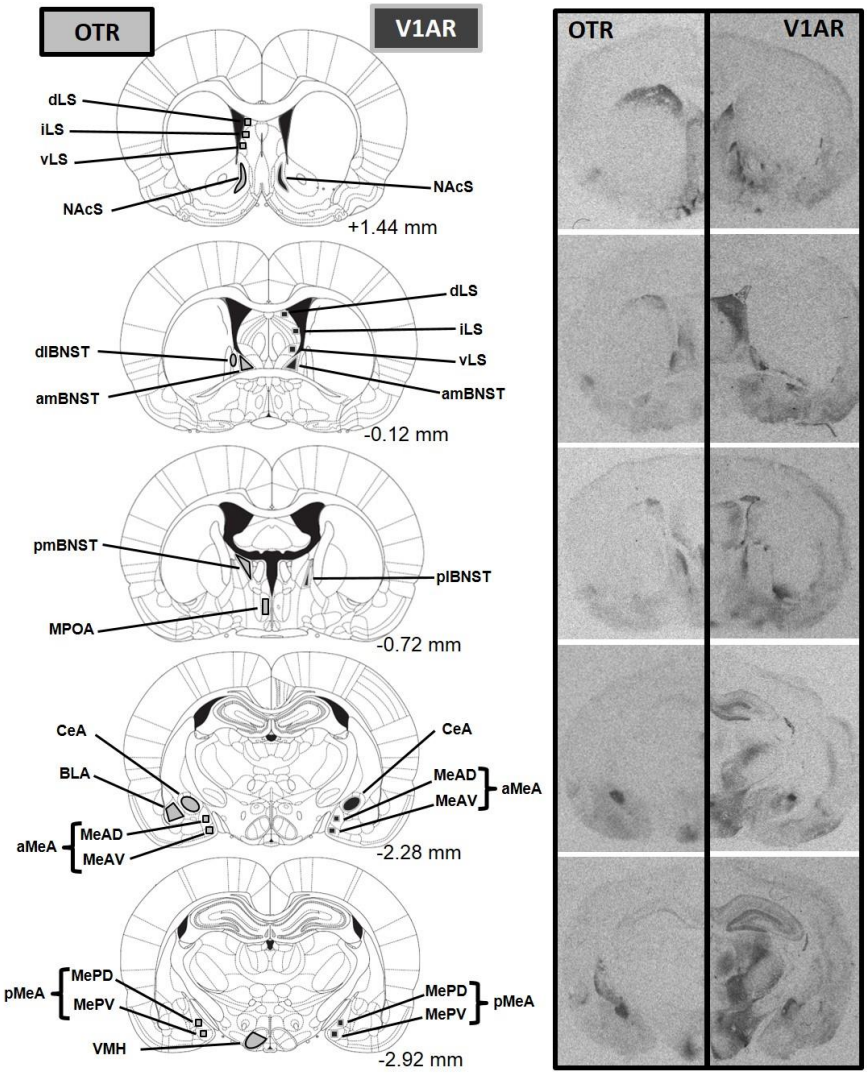

Figure 3-2. Atlas images and their bregma distances depicting regions of interest for oxytocin receptor (OTR) and for vasopressin 1a receptor (V1AR) autoradiography (images are used with permission from Paxinos and Watson, 2005) and corresponding representative autoradiograms of OTR and V1AR in coronal sections. Highlighted in grey on the atlas images are the areas measured for OTR and V1AR binding density. dLS = dorsal lateral septum; vLS = ventral lateral septum; iLS = intermediate lateral septum; NAcS = nucleus accumbens shell; CeA = central amygdala; BLA = basolateral amygdala; MePD = posterodorsal medial amygdala; MePV = posteroventral medial amygdala; MeAD = anterodorsal medial amygdala; MeAV = anteroventral medial amygdala; amBNST = anteromedial bed nucleus of stria terminalis; pmBNST = posteromedial bed nucleus of stria terminalis; dlBNST = dorsolateral bed nucleus of stria terminalis; plBNST = posterolateral bed nucleus of stria terminalis; VMH = ventromedial hypothalamus; MPOA = medial preoptic area; pMeA = posterior medial amygdala; aMeA = anterior medial amygdala.

An alpha level of $p \leq 0.05$, two-tailed, was used to determine statistical significance; nevertheless, given the multifactorial design that might limit power, interactions of $p < 0.10$ were explored using post hoc t-tests. The social interaction test involved the between group factors of Stress Group (CTL, SS) and Partner Familiarity (Familiar, Unfamiliar), and the within group factor of Blocks of Time (First 5 min, Second 5 min, Third 5 min). For the social recognition test, Stress Group was the between group factor, and Retention Interval (30 and 90 min) and Stimulus Familiarity (Familiar, Unfamiliar) were within group factors in the ANOVAs. For the CPP tests, Stress Group was the between group factor and Side of Chamber (Social, non-Social) was the within group factor.

Results

Social Interaction Test

A Stress Group x Partner Familiarity x Block of Time ANOVA found that social interactions increased across the three 5 min blocks of time ($F_{2,56} = 4.51$, $p = 0.015$). The main effects of Stress Group ($p = 0.02$) and Partner Familiarity ($p = 0.001$) were obviated by an interaction between the two factors ($F_{1,28} = 6.40$, $p = 0.017$) (all other interactions, $p > 0.12$). Post hoc t-tests indicated that SS rats spent less time interacting with an unfamiliar partner than did CTL rats ($p = 0.006$), but did not differ from CTL rats when interacting with a familiar partner ($p = 0.95$). SS rats did not differ in time spent in social interaction with a familiar partner than with an unfamiliar partner ($p = 0.26$), whereas CTL spent more time interacting with an unfamiliar partner than with a familiar partner ($p = 0.001$) (see Figure 3-3).

Social Recognition Test

For the Familiarization Phase, a Stress Group x Retention Interval (30 vs 90)

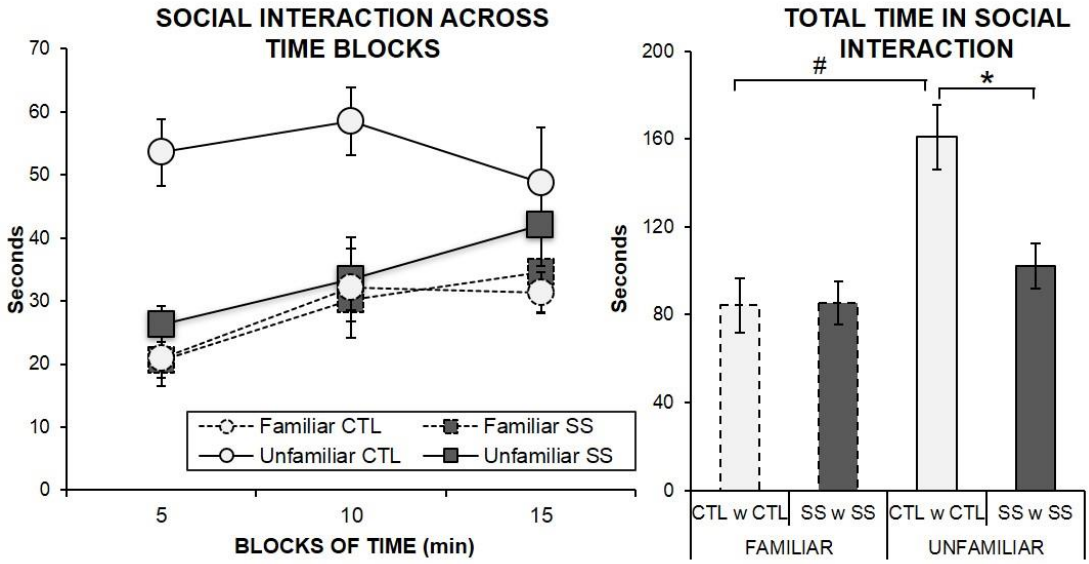

*Figure 3-3. The left panel shows the mean (±S.E.M.) time spent in social interaction across 5 min blocks in a 15 min social interaction session in CTL (control) and SS (social instability stress) rats with a familiar or unfamiliar conspecific. The right panel depicts the significant interaction between Stress Group and Partner Familiarity. # effect of Partner Familiarity within CTL rats ($p < 0.01$); * effect of Stress Group within Unfamiliar condition ($p < 0.01$). w = with.*

ANOVA found that SS rats spent more time investigating the stimulus rats than did CTL rats ($F_{1,31} = 5.145$, $p = 0.03$); no main effect of Retention Interval or interaction was significant (all ps > 0.20).

A Stress Group x Retention Interval (30 vs 90) x Stimulus Familiarity (Familiar vs Novel) ANOVA for the Test Phase found that rats spent more time investigating the novel rat than the familiar rat ($F_{1,31} = 5.83$, $p = 0.022$). Only the interaction between Retention Interval and Stimulus Familiarity approached significance ($p = 0.075$; all other main effects and interactions, ps > 0.27). Post hoc t-tests suggested that the effect of Stimulus Familiarity was driven by the data for the CTL rats (CTL: 30 min, $p = 0.032$ and 90 min, $p = 0.085$; SS: 30 min, $p = 0.126$ and 90 min, $p = 0.930$) (see Figure 3-4).

Conditioned Place Preference Tests

During the Test Phase of the Unrestricted CPP test, rats spent more time on the Social Side than on the nonSocial side ($F_{1,29} = 4.37$, $p = 0.045$; main effect of Stress Group and interaction, ps > 0.75) (see Figure 3-5). During the Test Phase of the Restricted CPP test, CTL rats spent more time in the nonSocial and Social-paired chambers than did SS rats ($F_{1,22} = 5.594$, $p = 0.027$; all other ps > 0.45) (see Figure 3-5).

Oxytocin receptor and vasopressin 1a receptor binding density

SS rats had denser OTR binding than did CTL rats in dorsal lateral septum ($t_{16} = 2.51$, $p = 0.02$) and in the shell of the nucleus accumbens ($t_{12} = 2.87$, $p = 0.01$) (see Figure 3-6). Trends for higher V1AR binding density in the ventral lateral septum ($t_{14} = 2.1$, $p = 0.055$) and lower V1AR binding density in the posterolateral bed nucleus of stria terminalis ($t_{13} = 1.81$, $p = 0.094$) in SS rats compared with CTL rats were found. CTL and SS rats did not differ in OTR or V1AR binding density in any of the other brain regions

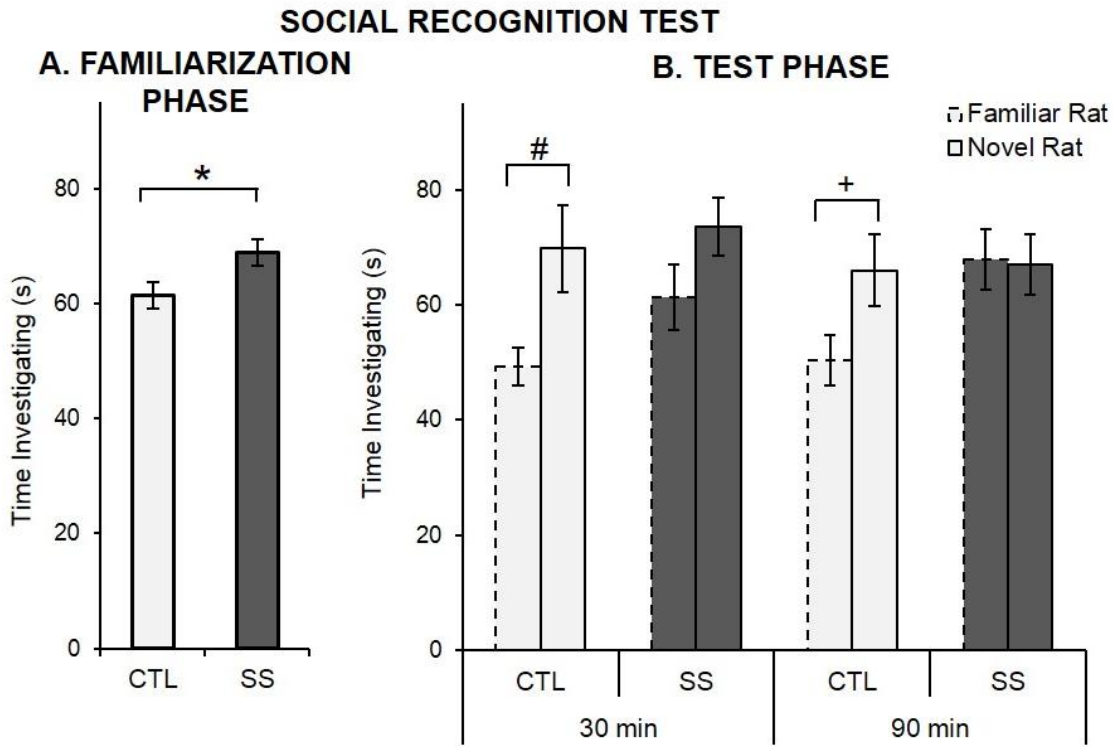

*Figure 3-4. Mean (±S.E.M.) time spent (A.) investigating stimulus rats during the familiarization phase and (B.) investigating the familiar and the novel stimulus rats during the test phase of the social recognition test in CTL (control) and SS (social instability stress) rats. (A.) Familiarization phase: * effect of Stress Group ($p < 0.05$). (B.) Test phase: # preference for novel rat ($p < 0.05$); + preference for novel rat ($p < 0.10$).*

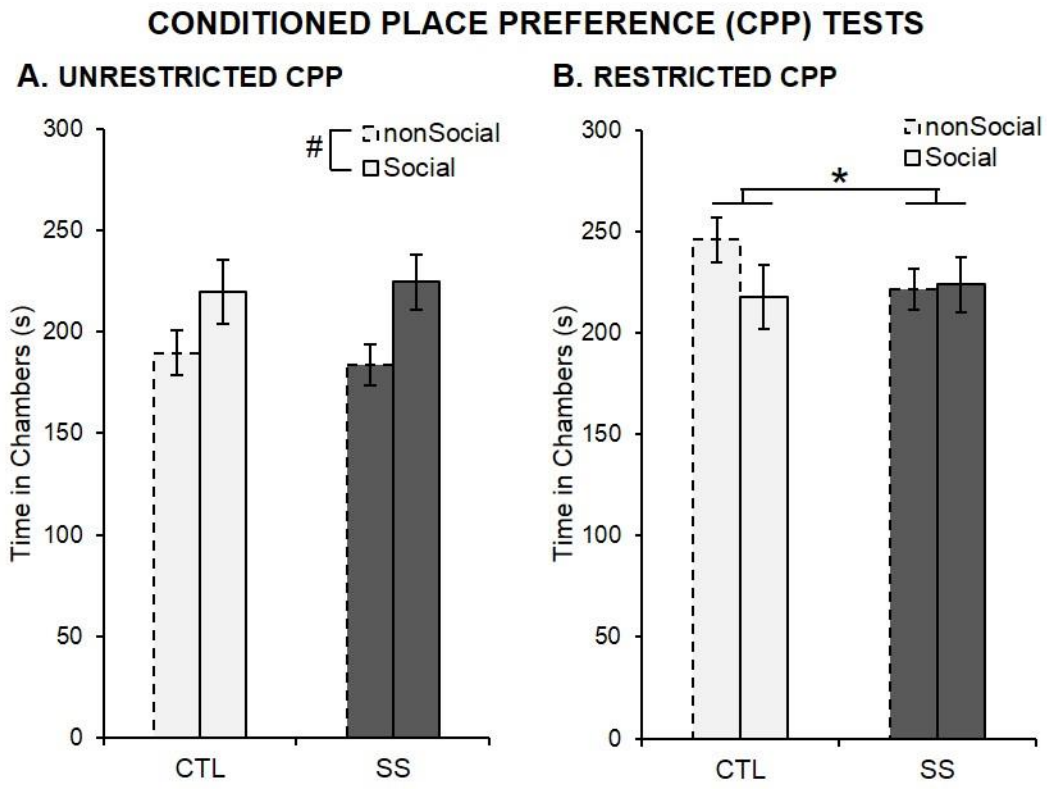

*Figure 3-5. Mean (±S.E.M.) time spent in nonSocial- versus Social-paired chambers during the test phase of the (A.) Unrestricted and (B.) Restricted conditioned place preference (CPP) tests in CTL (control) and SS (social instability stress) rats. (A.) Unrestricted CPP: # preference for Social-paired chamber, main effect ($p < 0.05$). (B.) Restricted CPP: * main effect of Stress Group on time spent in conditioning chambers ($p < 0.05$).*

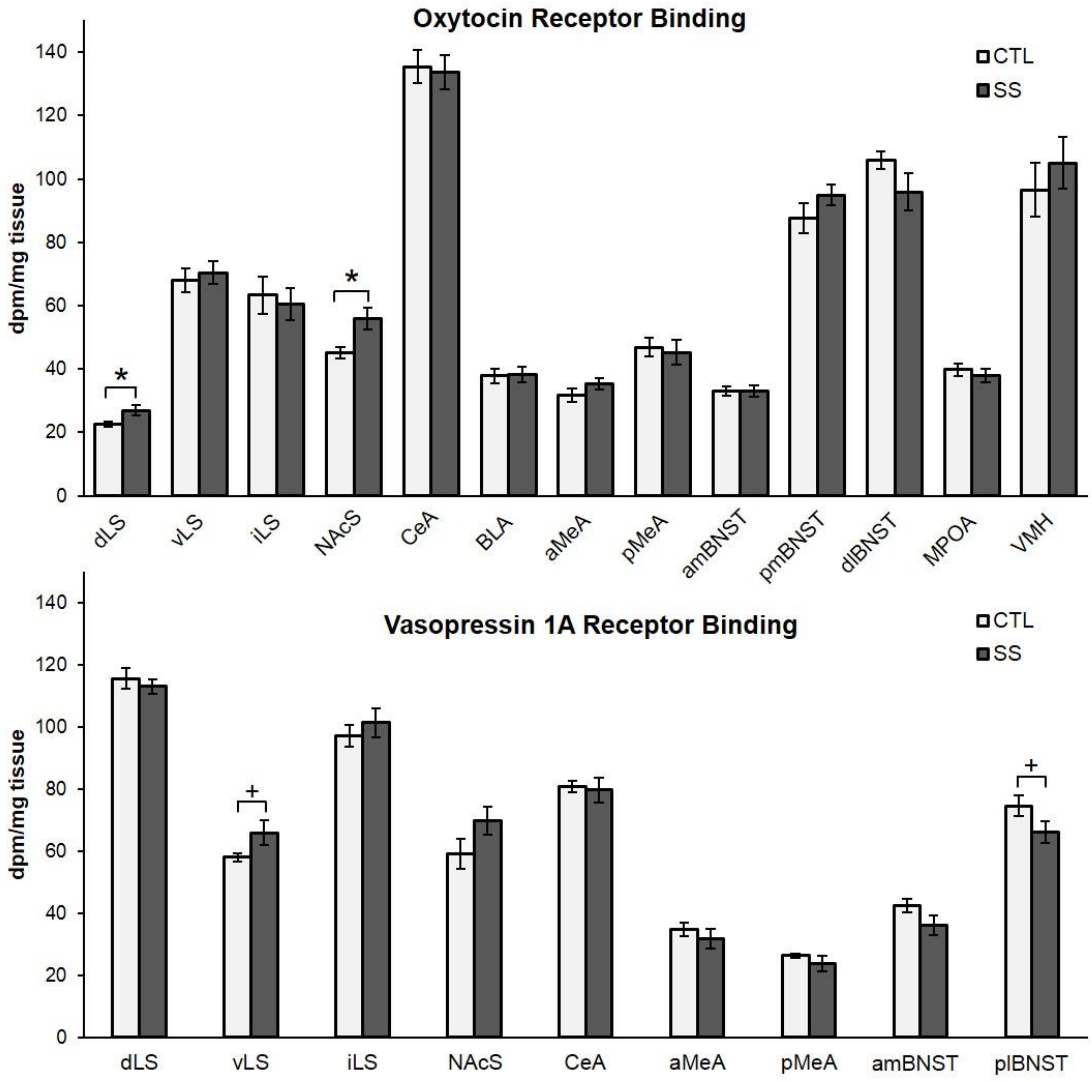

*Figure 3-6. Mean (±S.E.M.) oxytocin receptor (OTR) and vasopressin 1a receptor (V1AR) binding density in brain regions in CTL (control) and SS (social instability stress) rats. * effect of Stress Group ($p < 0.05$); + effect of Stress Group ($p < 0.10$). dLS = dorsal lateral septum; vLS = ventral lateral septum; iLS = intermediate lateral septum; NAcS = nucleus accumbens shell; CeA = central amygdala; BLA = basolateral amygdala; MePD = posterodorsal medial amygdala; MePV = posteroventral medial amygdala; MeAD = anterodorsal medial amygdala; MeAV = anteroventral medial amygdala; amBNST = anteromedial bed nucleus of stria terminalis; pmBNST = posteromedial bed nucleus of stria terminalis; dlBNST = dorsolateral bed nucleus of stria terminalis; plBNST = posterolateral bed nucleus of stria terminalis; VMH = ventromedial hypothalamus; MPOA = medial preoptic area; pMeA = posterior medial amygdala; aMeA = anterior medial amygdala.*

analyzed (all other ps >0.11) (see Figure 3-6).

Discussion

The main findings of the experiments are that rats that underwent SS in adolescence spent less time in social interaction with unfamiliar peers and had reduced social recognition relative to CTL rats. SS and CTL rats did not differ in their performance in social CPP tests. In addition, SS rats had greater oxytocin receptor binding density than did CTL rats in the dorsal lateral septum and in the nucleus accumbens shell.

Social Interaction

We found that SS rats spent less time in social interaction with unfamiliar peers compared with CTL rats when tested within days of the SS procedure while still adolescents. This result is consistent with our previous finding of reduced social interactions with unfamiliar peers in rats that underwent SS in adolescence and were tested several weeks after the procedure in adulthood (Green et al., 2013). These data suggest that the effects of SS on social functioning do not require an incubation period before they manifest and that the effects are long-lasting. The reduction in social approach was observed only in the social interaction test, which allows for physical social contacts. In contrast, adolescent SS rats spent more time in proximity to unfamiliar peers behind wire mesh than did CTL rats in the familiarization phase of the social recognition test. Thus, reduced social interaction does not seem to involve a diminished interest in social investigation. These findings are in accordance with our finding that SS rats tested in adulthood are not socially avoidant (Green et al., 2013).

Whereas CTL rats increased social interaction with an unfamiliar rat compared

with time in social interaction with their cage partner, SS rats did not. The result for CTL rats is consistent with rats' typical preference for novel social stimuli over nonsocial stimuli (Cavigelli et al., 2011). The results for SS rats may reflect a conflict between approach and avoidance, which are independent biological functions. Anxiety (manifested as avoidance) involves a fear of novelty while approach involves the tendency to explore novelty (Denenberg, 1969; Berton et al., 1997). The social interaction test is considered a measure of social anxiety (File and Seth, 2003), and SS rats showed more anxiety-like behaviour than did CTL rats in other tests (elevated plus maze; McCormick et al., 2008; open field; Green et al., 2013). Thus, one possibility is that SS rats may find physical social interactions with unfamiliar peers more anxiety-provoking than they find the investigation of familiar peers and unfamiliar confined peers (in other words, the former test heightens the conflict between avoidance and approach to a greater extent than do the latter two tests); notably, SS rats spent more time investigating rats confined behind mesh than did CTL rats in the familiarization phase of the social recognition test. Another, non-mutually exclusive, possibility is that social stress in adolescence impairs behavioural sequencing (the predictability of behaviour of one rat by the behaviour of its partner; Maaswinkel et al., 1997). Behavioural sequencing involves both the hippocampus and the medial prefrontal cortex (Maaswinkel et al., 1997; Himmler et al., 2014a), structures that continue to develop in adolescence (Pokorný and Yamamoto, 1981; Gambarana et al., 1990; Benes et al., 2000; Gourley et al., 2012), and we have evidence that the SS procedure alters hippocampal development (McCormick et al., 2012). Further, social isolation in adolescence disrupts the coordination of movement between partners (Pellis et al., 1999). Thus, it is possible that the reduction in social

interaction in SS rats involves impaired behavioural sequencing.

Social Recognition

CTL rats spent more time investigating novel stimulus rats than they did investigating familiar stimulus rats whereas SS rats did not. However, SS rats spent more time investigating the stimulus rats during the familiarization phase than did CTL rats. Thus, the reduced discrimination between familiar and novel stimulus rats is not explained by encoding time in SS rats relative to CTL rats. Our finding of impaired social recognition in SS rats is in keeping with past findings of impaired social recognition in adult rats after stressor exposure (Lukas et al., 2011; Eagle et al., 2013; van der Kooij et al., 2014).

We previously reported no difference in either object recognition or object spatial location memory in SS rats and CTL rats when tested in adolescence (McCormick et al., 2012), and that deficits emerged in object spatial location memory in SS rats compared with CTL rats when tested in adulthood (McCormick et al., 2012; Green and McCormick, 2013a). Nevertheless, despite the similarity across testing paradigms, there are differences in the neural mechanisms underlying social memory, object recognition memory, and object spatial location memory (e.g. Everts and Koolhaas, 1997; Tobin et al., 2010; although see Gabor et al., 2015). Thus, we do not know whether the deficits in social recognition would diminish or be exacerbated with time. In addition, whether SS rats have an impairment in social memory, social discrimination, or a reduced preference for novel over familiar peers cannot be determined from the present results.

Conditioned Place Preference

Both CTL and SS adolescent rats formed a social CPP when conditioning involved physical interaction with a peer (Unrestricted condition), but not when conditioning involved a peer behind mesh (Restricted condition). There is a report finding CPP after 8 days of conditioning with peers behind wire mesh in adolescent rats (Peartree et al., 2012), thus we may have found CPP in both tests had more conditioning days been involved. Consistent with our results, stronger CPP is found when it allows for unrestricted physical contact than restricted contact (Calcagnetti and Schechter, 1992; Van den Berg et al., 1999b; Peartree et al., 2012).

Unlike other social CPP paradigms (Calcagnetti and Schechter, 1992; Van den Berg et al., 1999b; Thiel et al., 2008; Peartree et al., 2012) our rats were not isolated at any point of the test procedures other than the 15 min conditioning sessions in chambers paired with objects, which may have reduced social CPP (there is evidence that isolation may enhance social CPP, Douglas et al., 2004; Trezza et al., 2009; Yates et al., 2013). Further, whereas other social CPP paradigms placed the rat alone in the non-social conditioning sessions (Douglas et al., 2004; Thiel et al., 2008; Trezza et al., 2009; Peartree et al., 2012; Yates et al., 2013), social CPP may have been weakened in our procedure by our addition of an object to control for both the presence of any stimulus and the amount of space in each chamber. Nevertheless, the results from the CPP tests provide no evidence that CTL rats and SS rats differ in learning the association between a social stimulus and cues in the chamber or in the reward value of social stimuli.

Oxytocin receptor and vasopressin 1a receptor binding density

OTR binding density was higher in the nucleus accumbens shell and in the dorsal lateral septum of SS rats compared with CTL rats. There were no significant differences

between CTL and SS rats in V1AR binding density, although the reduction in V1AR binding density in the posterolateral bed nucleus of the stria terminalis and the increase in the ventral lateral septum may have been significant with a larger sample of rats. Other repeated stress procedures administered in either early life (Lukas et al., 2010) or in adulthood (Liberzon and Young, 1997) also enhanced OTR binding density in male rats. For example, enhanced OTR binding density was found in the medial preoptic area and ventromedial hypothalamus of male rats that were exposed to repeated maternal separation as pups and tested in adulthood (Lukas et al., 2010). Circulating glucocorticoids (such as corticosterone in rodents) released in response to stressor exposure are known to regulate oxytocin receptor densities; for example, adrenalectomized rats had reduced OTR binding density in both the subiculum and hippocampus that was prevented by corticosterone replacement (Liberzon et al., 1994). Further, a high dose of corticosterone in rats was found to increase OTR binding compared with controls in the ventral hippocampus but not in the amygdala, ventromedial hypothalamus, or dorsal hippocampus (Liberzon and Young, 1997). The adolescent SS procedure is known to result in a greater exposure to corticosterone during the 16 days of the procedure (McCormick et al., 2007; Hodges and McCormick, 2015), which may have influenced OTR binding densities.

The functional consequences of the higher OTR binding densities in SS rats relative to that in CTL rats is unknown. A higher OTR binding density may result from either a higher affinity or a greater expression of OTR. Thus, the higher OTR binding density might represent the potential for higher OTR activation. The enhanced OTR binding density of SS rats in the nucleus accumbens compared with that of CTL rats is

notable considering the role of this region in approach and avoidance behaviours (reviewed in Ikemoto and Panksepp, 1999; O'Connell and Hofmann, 2011). In adolescent male rodents, the injection of an OTR antagonist into the nucleus accumbens was found to reduce social novelty-seeking (Smith et al., 2017a) and reduce the rewarding properties of social interactions (Dölen et al., 2013). Thus, enhanced OTR binding density in the nucleus accumbens may play a role in the increased social investigatory behaviour of peers found in SS rats when the peer is confined behind mesh, but does not explain the reduced time that SS rats spend in unfettered social interactions with a novel peer.

The lateral septum is involved in the exploration of novel stimuli and in a broad range of social behaviours (reviewed in O'Connell and Hofmann, 2011), and there is evidence for a role of vasopressin and oxytocin function. For example, antagonists that target vasopressin receptors administered to the lateral septum increased the percentage of time spent in social play in the home cage (Bredewold et al., 2014) and reduced the percentage of time spent investigating a novel rat in a social recognition test (Veenema et al., 2012) in adolescent male rats. Vasopressin injected in the lateral septum increased the percentage of time spent investigating a novel rat in adult but not in mid-adolescent male rats (Veenema et al., 2012). An OTR antagonist injected into the lateral septum impaired the social recognition of juveniles in adult male rats (Lukas et al., 2013). In contrast, oxytocin administered in the lateral septum was found to reduce social play behaviours in adolescent female rats but had no effect on social play behaviour of adolescent male rats (Bredewold et al., 2014). How oxytocin function is involved in social anxiety may depend on the context. In male mice, oxytocin injection into the dorsal lateral septum abolished the reduction in social investigation found after a social fear conditioning

paradigm (Zoicas et al., 2014), and overexpression of OTR in the lateral septum enhanced freezing in a context paired with social defeat (Guzmán et al., 2013). Despite the growing body of research on the role of these neuropeptides in the lateral septum the available results do not explain how enhanced OTR binding density in the dorsal lateral septum of SS rats is involved in both their greater social investigation and reduced physical social interactions with unfamiliar peers.

It may be that SS rats have an enhanced social salience to social cues. Oxytocin's context-specific regulation of social behaviour is thought to be mediated by shifting social salience (reviewed in Shamay-Tsoory and Abu-Akel, 2016). Animals treated with oxytocin show greater awareness of social cues and increase their aggressive behaviours in contexts involving conflict and increase their affiliative behaviours in contexts involving pro-social behaviour (reviewed in Shamay-Tsoory and Abu-Akel, 2016). In the current study, SS rats may increase their approach behaviours to conspecifics behind wire mesh because of an increase in the salience of positive social cues and may be more sensitive to threat cues of an unfamiliar peer when physical contact is possible. In a previous study, we found that SS rats tested in adulthood are more aggressive in food competition towards a cagemate compared with CTL rats (Cumming et al., 2014), and this finding of increased aggressive behaviour in the context of conflict in SS rats may be regulated by an increase in the salience of threatening social cues. Mating behaviour also differs between SS and CTL male rats in adulthood (McCormick et al., 2013; McCormick et al., 2017a). Although speculative, altered social salience may be the common underlying factor in the social behaviour differences evident in rats after social instability stress in adolescence.

Conclusion

Whereas there is much evidence that the absence of social experiences in adolescence has lasting consequences (Burke et al., 2017), the present results highlight that the quality of the social experiences in adolescence also can shape the social behavioural repertoire.

Rationale for chapter 4

As per results from chapter 3, adolescent rats that underwent SS interacted less with unfamiliar peers in a novel environment than did CTL rats on PND 46. In chapter 2, I found lower Zif268 immunoreactivity in the medial amygdala (involved in social behaviour) in response to being isolated and paired with an unfamiliar peer in the homecage for 1 h in SS compared with CTL rats on PND 45, but it was unknown whether the neural activation in social brain regions differed between SS and CTL rats in response to social interaction with an unfamiliar peer in a novel environment. Moreover, because SS rats had higher plasma corticosterone concentrations in response to being isolated and paired with an unfamiliar peer in the homecage than did CTL rats (described in chapter 2), I predicted that SS rats would have more neural activation in brain regions involved in initiating corticosterone release in response to being paired with an unfamiliar peer. The main aim of this chapter was to investigate neural activation in brain regions involved in the activation of the hypothalamic-pituitary-adrenal axis (paraventricular nucleus, arcuate nucleus) and involved in social behaviour (medial amygdala, lateral septum, CA2 part of the hippocampus, nucleus accumbens) in response to social interaction with an unfamiliar peer in a novel environment using Fos immunoreactivity in adolescent SS rats. In addition, in this chapter we investigated whether anxiety-like behaviour in early-life would be able to predict the amount of time SS rats would spend interacting with unfamiliar peers in a novel environment.

Chapter 4: Predictors of social instability stress effects on social interaction and anxiety in adolescent male rats

This chapter has been adapted from the article in press:

Hodges, T. E., Baumbach, J. L., & McCormick, C. M. (in press). Predictors of social instability stress effects on social interaction and anxiety in adolescent male rats. *Developmental Psychobiology*.

Author contribution: For this manuscript, Dr. Cheryl McCormick and I designed and formulated the research questions for Experiment 1 (Experiment 2 was designed and executed by Jennet Baumbach, who was an undergraduate student at the time, and Dr. Cheryl McCormick). I wrote the proposal for ethics approval, ordered the rats required for the experiment, performed the social instability stress procedure on rats, performed the social interaction test, performed perfusions, collected rat brains, sliced rat brains, performed immunohistochemistry on brain slices, counted cells in coronal brain slices, analyzed the data, performed the statistics, created the figures, wrote most of the paper (Jennet Baumbach wrote the sections on locomotor activity in the open field in the introduction, methods, results, and discussion sections), and edited the paper after I received feedback from Dr. Cheryl McCormick and Jennet Baumbach.

Introduction

Social experience during adolescence, such as social play with same-sex peers, is crucial for proper social, emotional, and cognitive development in rats (reviewed in Burke et al., 2017; Buwalda et al., 2011; Pellis and Pellis, 2017). Disrupting social experience during adolescence can alter behavioural development. For example, rats isolated in the adolescent period spent less time interacting with an unfamiliar peer (van den Berg et al., 1999a; Lukkes, Mokin, Scholl, and Forster, 2009), had impaired social recognition memory (Shahar-Gold, Gur, and Wagner, 2013), had an impaired coordination of movement with partners (Pellis et al., 1999), and had increased anxiety-like behaviour (Wright, Upton, and Marsden, 1991) than did non-isolated control rats. The quality of social interactions rather than the presence versus absence of social interaction in adolescence also influences behavioural development. Rats that underwent SS (daily isolation for one hour and return to an unfamiliar cagemate for 16 days from PND 30 – PND 45) in adolescence had altered social behaviour both soon after the stress exposures; specifically, SS rats spent less time interacting with an unfamiliar peer and had impaired social recognition relative to control rats (Hodges et al., 2017). Several weeks after the stress exposures, SS rats also spent less time in social interaction with an unfamiliar peer (Green et al., 2013), showed greater aggression with a conspecific in food competition (Cumming et al., 2014), and had impaired mating behaviour (McCormick et al., 2013, 2017) compared with control rats.

Brain regions involved in initiating and performing social interactions with unfamiliar peers, such as the lateral septum, medial amygdala, and hippocampus, undergo functional and morphological change in adolescence (Gourley et al., 2012; Verney et al.,

1987; De Vries et al., 1981; Hines et al., 1992; Mizukami et al., 1983). Thus, the quality of social relationships during the adolescent period may alter the developmental trajectory of brain regions involved in social behaviour and thereby alter social behaviour. We previously reported that SS rats had enhanced oxytocin receptor binding density in the lateral septum and nucleus accumbens compared with non-stressed controls (Hodges et al., 2017). Oxytocin is a neuropeptide that acts on central receptors to regulate several aspects of social behaviour, such as social interaction and social recognition (Dumais et al., 2016a, 2016b; reviewed in Dumais and Veenema, 2016). Further, we have evidence of reduced dendritic arborisation and spine numbers in the medial amygdala and lateral septum in SS rats compared with non-stressed controls (Hodges et al., in preparation). The SS procedure leads to elevations in corticosterone release, a glucocorticoid that is the end product of activation of the hypothalamic-pituitary-adrenal axis (McCormick et al., 2007), which may underlie changes in the brain. Changes in dendritic arborisation after chronic stress in early life (Farrell et al., 2016) and in adolescence (Tsai et al., 2014) have been reported, and glucocorticoids are known to be involved in many of the programming effects of early life experiences (reviewed in Albrecht et al., 2017). In addition, SS rats tested in adolescence had a greater release of corticosterone after being paired with an unfamiliar peer for the 16th time than do rats paired with an unfamiliar rat for the first time (Hodges and McCormick, 2015). Thus, SS in adolescence may also alter brain regions involved in initiating and regulating the stress response in response to being exposed to unfamiliar peers, and thereby alter social behaviour.

To better understand the basis of the decreased social interactions evident in SS rats relative to CTL rats, in the present experiment we compared neural activations in several brain regions involved in initiating and regulating either social behaviour (medial amygdala, lateral septum, hippocampus, nucleus accumbens, Bredewold et al., 2014; Gur et al., 2014; Lukas et al., 2013; Murakami et al., 2011; Smith et al., 2016; Smith et al., 2017a) or the release of corticosterone (paraventricular nucleus of the hypothalamus, arcuate nucleus, Leon-Mercado et al., 2017; reviewed in Spencer and Deak, 2017) in response to a 15 min social interaction with a novel rat on PND 46, 24 hours after the SS procedure, in CTL and SS rats. Fos is the protein product of the immediate early gene c-fos, and Fos-immunoreactive cell counts were used as the measure of neural activation.

In addition, to address individual differences in susceptibility to the SS procedure, in a second experiment, we measured the novelty-seeking of rats before administering the SS procedure. Locomotor activity in an open field is a measure of novelty-seeking that has been used to characterize rats as either "low responders" (LR) or "high responders" (HR) (Piazza et al., 1989; Kabbaj, 2006). The designation of HR versus LR predicts behavioural responses to psychostimulants (> in HR, Dellu, Piazza, Mayo, Le Moal, and Simon, 1996; Kabbaj, Devine, Savage, and Akil, 2000), anxiety in an elevated plus maze (EPM) (< in HR, Mällo et al., 2007) and the light-dark box (< in HR, Kabbaj et al., 2000), and depressive behaviours in the forced swim test (< in HR; Mällo et al. 2007) and sucrose preference paradigms (< in HR; Stedenfeld et al. 2011). In previous studies we have found no evidence of greater anxiety in the EPM in SS male rats compared with CTL rats when tested soon after the SS procedure in adolescence (McCormick et al., 2008), but being either low or high in novelty-seeking may influence anxiety that is

specific to social situations in SS rats compared with CTL rats. Thus, we investigated novelty-seeking before the onset of the SS procedure as a predictor of anxiety in the EPM and time spent in social interaction with an unfamiliar peer in SS versus CTL rats. Typically, studies of HR/LR phenotypic differences use outbred strains of rats selected based upon their novelty-seeking behaviour. Rats that demonstrate the highest/lowest 2-3% of responses are outbred in this model (Flagel, 2014), thus, findings are limited to individuals that fall at the ends of the distribution for novelty-seeking. Here, we instead used a normative sample of Long-Evans rats to better understand the relationship between baseline novelty responsiveness and stress susceptibility. We hypothesized that rats high in novelty-seeking may be more resistant to social instability stress than rats low in novelty-seeking.

Methods

Animals

The experiments involved male Long-Evans rats (N = 62) obtained from Charles River, Kingston, New York, on PND 22 and given a week to acclimate to the animal colony. Rats were housed in pairs and maintained under a 12 h light–dark cycle (lights on at 05:00 h) with food and water available ad libitum. Use of animals in these experiments was approved by the Brock University Institutional Animal Care Committee and was carried out in adherence to the Canadian Council on Animal Care guidelines.

Social instability stress procedure

Rats were randomly assigned to the adolescent SS group (n = 34) or to CTL group (n = 28), experiment 1 and 2 combined. The SS procedure was as described previously (reviewed in McCormick, 2010; McCormick et al., 2015). Beginning on PND 30, SS rats

were isolated in a 12 cm x 10 cm ventilated plastic container in a room separate from the colony for 1 h each day until PND 45. The isolation in these containers is akin to restraint stress in Plexiglas restrainers in that rats are confined, although movement is possible. Immediately after isolation each day, SS rats were returned to the animal colony and housed in a new cage with a new cage partner that had also undergone the 1 h isolation. The SS procedure was conducted at various times during the lights on phase of the light–dark cycle to minimize habituation to the procedure. On PND 45, after the final isolation, SS rats remained with the same cage partner and were left undisturbed except for cage maintenance or test procedures. CTL rats remained undisturbed in their home cages except for cage maintenance from time of arrival at the colony until the test procedures. See Figure 4-1 for experimental design and a timeline of all test procedures.

Experiment 1

Social interaction test. The social interaction test was conducted on PND 46 in a plastic arena (61 cm x 30 cm x 53 cm). A video-camera mounted on the ceiling above the test apparatus was used to record the task. The apparatus was cleaned before each session with 70% ethanol. For the social interaction test, CTL (n = 8) and SS (n = 12) rats were paired with an unfamiliar peer of the same experimental condition (CTL with unfamiliar CTL, SS with unfamiliar SS) for 15 min. Total time spent in social interaction during the 15 min test session for each rat was measured by an experimenter blind to the Stress Group conditions. Social interaction included social play, anogenital sniffing, allogrooming, following, investigation, as defined in Cirulli, Terranova, and Laviola (1996) and as in our previous research (Hodges et al., 2017). Social interaction test sessions were conducted in dim red light between one and five hours after the onset of the

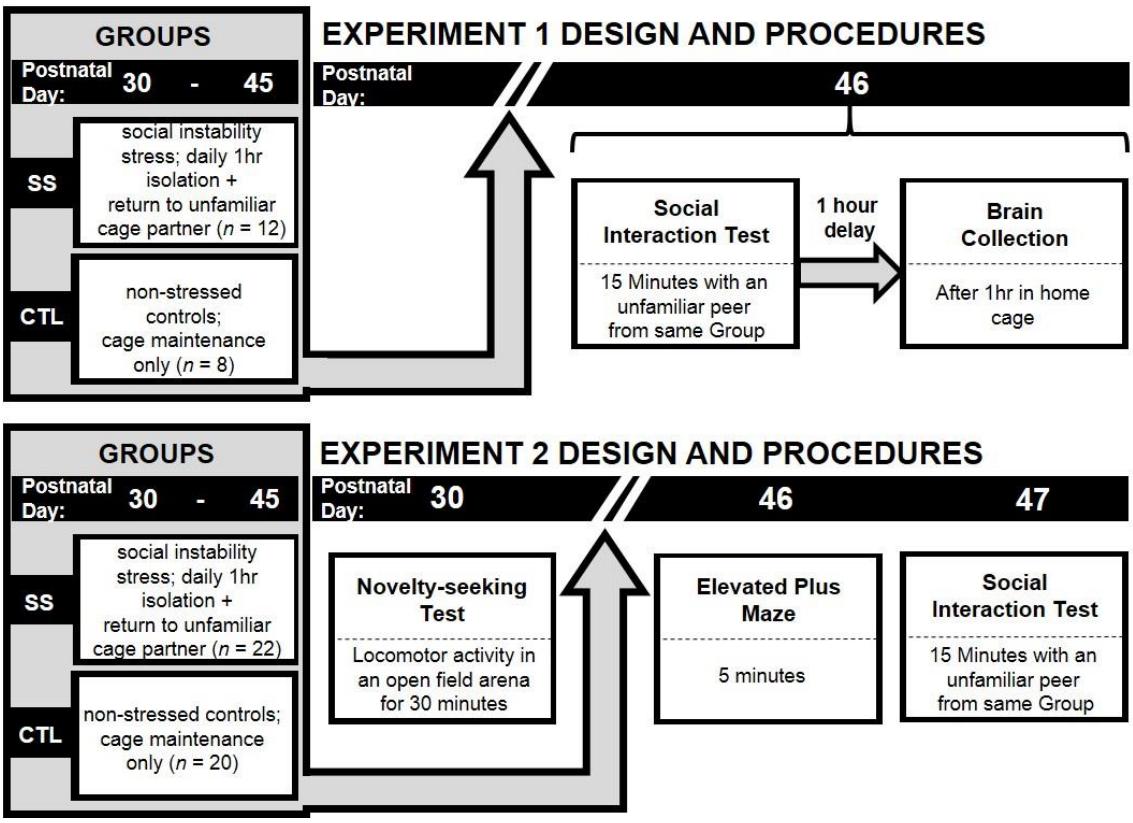

Figure 4-1. Experimental groups and timeline of experimental procedures for Experiment 1 and Experiment 2. SS = social instability stress; CTL = control.

dark phase. After the social interaction test, all rats were returned to their homecages with their homecage partners.

Brain collection and immunohistochemistry assay. One hour after returning to the homecage after the social interaction test, rats were deeply anaesthetized by an overdose of sodium pentobarbital (150 mg / kg), weighed, and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed from the skulls and post-fixed in a 30% sucrose and 4% paraformaldehyde solution until equilibrated. Coronal sections (37 μm; sections separated by 222 μm) were sliced on a cryostat, collected from approximately bregma 2.16 mm to approximately bregma -3.72 mm, and were stored in cryoprotectant at -20°C until the immunohistochemistry assay was performed.

Free-floating coronal sections were washed thoroughly in 0.1 M PBS, then in in PBS-X (0.1 M PBS with 3% Triton X-100), and incubated at room temperature in a 0.3% H_2O_2 in 0.1 M PBS-X solution for 30 min. Sections were then washed in PBS-X, blocked at room temperature in 10% goat serum (Sigma) solution for 1 h, and incubated at 4°C overnight in the primary antibody raised against Fos (1:10,000; Fos rabbit pAb; sc-52;Santa Cruz Biotechnology, Inc.) in PBS-X. The next day, sections were washed in PBS-X and then incubated for 75 min at room temperature in secondary antibody (biotinylated goat anti-rabbit IgG; 1:200; Vector Laboratories, Inc.). After another series of washes in PBS-X, sections were incubated in an avidin-biotin horseradish peroxidase complex (Vector Laboratories, Inc.) for 1.5 h at room temperature. Horseradish peroxidase was visualized with DAB in a 3 M sodium acetate buffer containing 2.5% nickel sulfate and 0.05% H_2O_2 (Vector Laboratories, Inc.). After a final series of washes

in PBS-X, sections were mounted on Superfrost Plus slides (Fisher Scientific, Inc.), dried, dehydrated in increasing concentrations of ethanol (70%, 95%, 100%), placed in xylenes, and coverslipped using Permount mounting medium (Fisher Scientific, Inc.). No immunoreactive cells were detected in control sections that were not treated with the primary antibody.

Microscopy and cell counting. Immunostained sections were analyzed using a Nikon Eclipse 80i microscope equipped with a digital camera (Nikon DXM1200F) and Nikon ACT-1 software. Immunoreactive cell counts were conducted blind to experimental condition and at 400X magnification in a 250 μm x 250 μm area in each hemisphere of our brain regions of interest. Brain regions of interest were chosen based on their involvement in the endocrine stress response (paraventricular nucleus and arcuate nucleus) or their involvement in social recognition and behaviour (medial amygdala, dorsal lateral septum, CA2 subfield of the hippocampus, and nucleus accumbens). All brain regions were identified according to Paxinos and Watson (2005) (see Figure 4-2); the PVN (within bregma -1.44 mm and -1.72 mm), the arcuate nucleus (ARC; within bregma -2.52 mm and -2.76 mm), the MePD (within bregma -2.76 mm and -3.24 mm), the MeAD (within bregma -1.92 mm and -2.64 mm), the MeAV (within bregma -1.92 mm and -2.52 mm), the MePV (within bregma -2.64 mm and -3.24 mm), the dLS (within bregma 1.44 mm and -0.36 mm), the CA2 subfield of the hippocampus (CA2; within bregma -2.64 mm and -3.24 mm), the nucleus accumbens core (NAcC; within bregma 1.92 mm and 0.96 mm), and the NAcS (within bregma 1.92 mm and 0.96 mm). Measures for the MeAD and MeAV were combined and called aMeA and measures for the MePD and MePV were combined and called pMeA because of functional

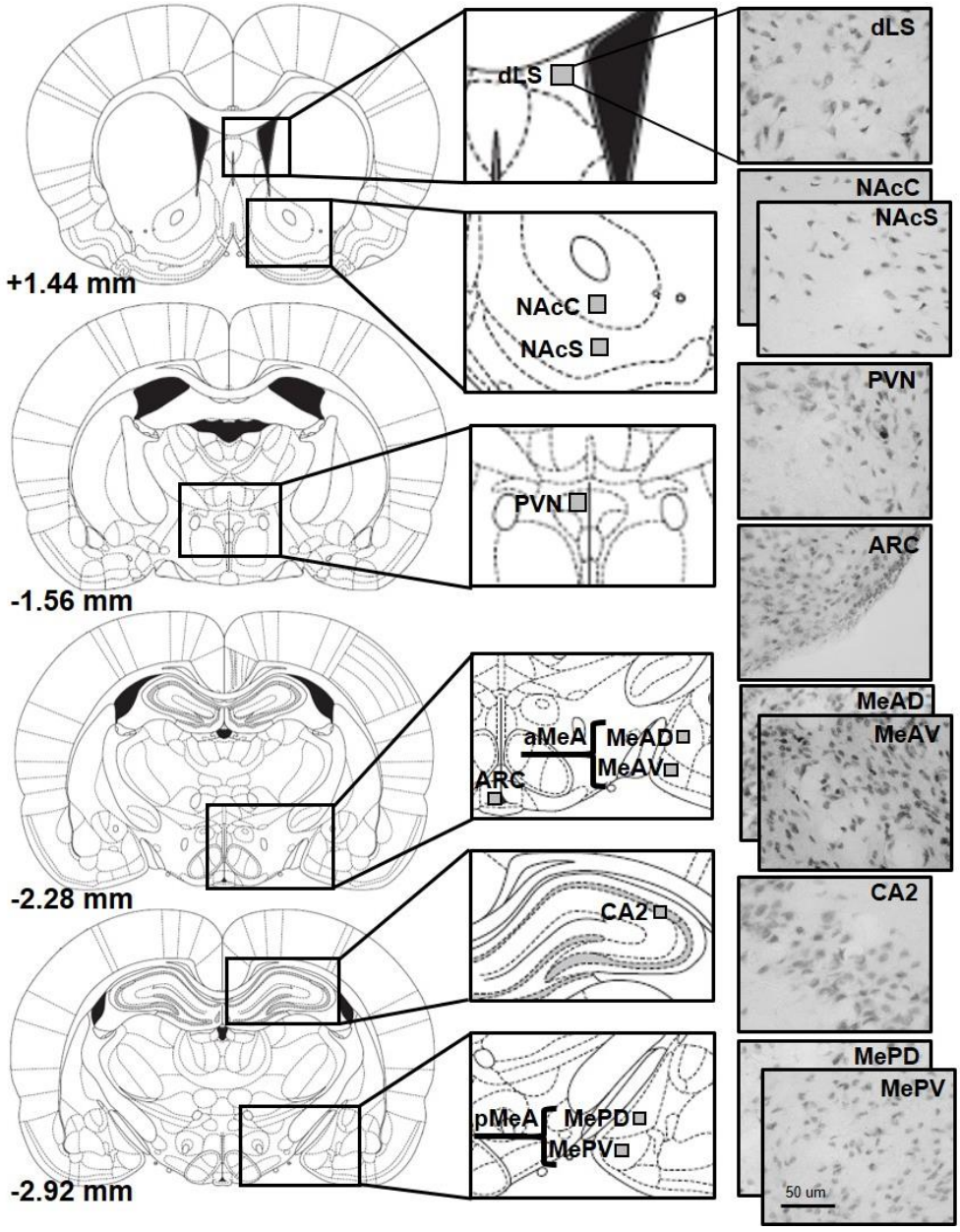

Figure 4-2. Atlas images depicting regions of interest for immunoreactive cell counts (images are used with permission from Paxinos and Watson, 2005) and images of Fos immunoreactivity in each brain regions of interest at 400x magnification. Gray boxes are the areas measured for Fos immunoreactivity for each brain region of interest. Brain regions of interest include the dorsal lateral septum (dLS), the nucleus accumbens core (NAcC), the nucleus accumbens shell (NAcS), the paraventricular nucleus of the hypothalamus (PVN), the arcuate nucleus (ARC), the anterior medial amygdala (aMeA; the anterodorsal medial amygdala [MeAD] and the anteroventral medial amygdala [MeAV] combined), Cornu Ammonis 2 region of the hippocampus (CA2), and the posterior medial amygdala (pMeA; the posterodorsal medial amygdala [MePD] and the posteroventral medial amygdala [MePV] combined).

similarity (reviewed in Newman, 1999) and no difference in cell counts between each pair of regions. Ir-cell counts were averaged across the hemispheres and sections (3 to 4 sections per rat), and the mean number of ir-cells per brain region per rat was used for analysis for all regions of interest.

Experiment 2

Novelty-seeking. On PND 30, before assignment to the SS or CTL condition, rats (n = 42) were placed alone in a 58 cm x 58 cm x 58 cm arena for 30 minutes during the light phase of their cycle. Arenas were cleaned with a 70% ethanol solution between each trial to eliminate exposure to the previous rat's scent. An overhead camera and SMART software were used to track locomotor behaviour by measuring distance travelled in the arena, the measure of novelty-seeking (Dellu et al. 1996). Higher distance travelled in the arena indicates higher novelty-seeking.

Elevated plus maze. On PND 46, CTL (n = 20) and SS (n = 22) rats were placed on a 64 cm x 64 cm plus shaped platform raised 80 cm from the ground for 5 minutes. The EPM consists of four 28 cm arms extending from a square central platform. Walls (30 cm in height) enclose two opposing arms of the maze (closed arms), while the other two are open platforms (open arms). An overhead camera was used to record behaviour during the task, and videos were later dummy coded so scoring could be done blind to experimental condition. Time spent in each area of the maze was measured, as well as the number of entries into each area. An entry was recorded when the head and front two paws simultaneously extended across one of the boundaries. The EPM presents the drive to explore against fear of open spaces (reviewed in Wall and Messier, 2001), thus, more

time spent exploring the open arms of the apparatus is indicative of lower levels of anxiety in this measure (Hogg, 1996).

Social interaction test. On PND 47, rats underwent a 15-min social interaction test with an unfamiliar conspecific of the same experimental condition (CTL with unfamiliar CTL, SS with unfamiliar SS). Testing and behavioural measurements were conducted as described in experiment 1. However, in experiment 2, we measured time spent anogenital sniffing separately from the other social interactions, as social disruption has been shown to selectively increase the prevalence of anogenital sniffing (Neisink and van Ree, 1982).

Statistical analyses

Experiment 1. Statistical analyses were performed using SPSS version 24 software, and consisted of mixed factor ANOVAs and independent t-tests. Post hoc analyses consisted of t-tests and Fisher's LSD, where appropriate. For the Fos immunohistochemistry data, two separate mixed factor ANOVAs were performed; (1) the first included the Fos-ir cell counts for the two brain regions involved in regulating corticosterone release (PVN and ARC) as the within group factor Brain Region and Stress Group (CTL, SS) as the between group factor, and (2) the second included the Fos-ir cell counts of the six brain regions involved in regulating social behaviour, with Brain Region (aMeA, pMeA, dLS, CA2, NAcC, NAcS) as the within group factor and Stress Group (CTL, SS) as the between group factor. Bivariate correlation analyses were used to determine correlations of Fos-ir cell counts in all brain regions of interest and time spent in social interaction for CTL and SS rats separately. Correlations between Fos-ir cell counts and time spent in social interaction were then compared between CTL and SS rats

using the Fisher r-to-z transformation. An alpha level of $p \leq 0.05$, two-tailed, was used to determine significance in all tests. The brain of one SS rat was lost during the Fos immunohistochemistry procedure and could not be used for Fos-ir cell counts in any brain region or in the correlations.

Experiment 2. Statistical analyses were preformed using SPSS version 24 software, and consisted of mixed factor ANOVAs, t-tests and hierarchical regression models. An alpha level of .05 was used to determine statistical significance in all models. Social interaction tests consisted of the within-subjects factor of Social Interaction Type (anogenital vs other physical contact) and the between-subjects factor of Stress Group (SS vs CTL). Between-subjects t-tests were used to evaluate mean group differences on time spent on the open arm, and locomotor behaviour on the EPM, and time spent in other physical contact. For all hierarchical regression models, Stress Group and novelty-seeking (centered) were entered in block 1, and the product of these factors (interaction term) was entered in block 2. CTL subjects were coded as 0, and thus designated as the reference group in our regression analyses.

Results

Experiment 1

Social interaction. Consistent with our previous reports, SS rats spent less time in social interaction than did CTL rats ($t_{18} = 2.230$, $p = 0.039$) (see Figure 4-3).

Fos immunoreactive cell counts: stress-related brain regions. Fos-ir cell counts differed between brain regions ($F_{1,17} = 18.66$, $p < 0.001$) and CTL rats had higher Fos-ir cell counts in the paraventricular and arcuate nuclei than did SS rats ($F_{1,17} = 5.47$, $p = 0.032$), and there was no interaction of the two factors ($p = 0.425$) (see Figure 4-4).

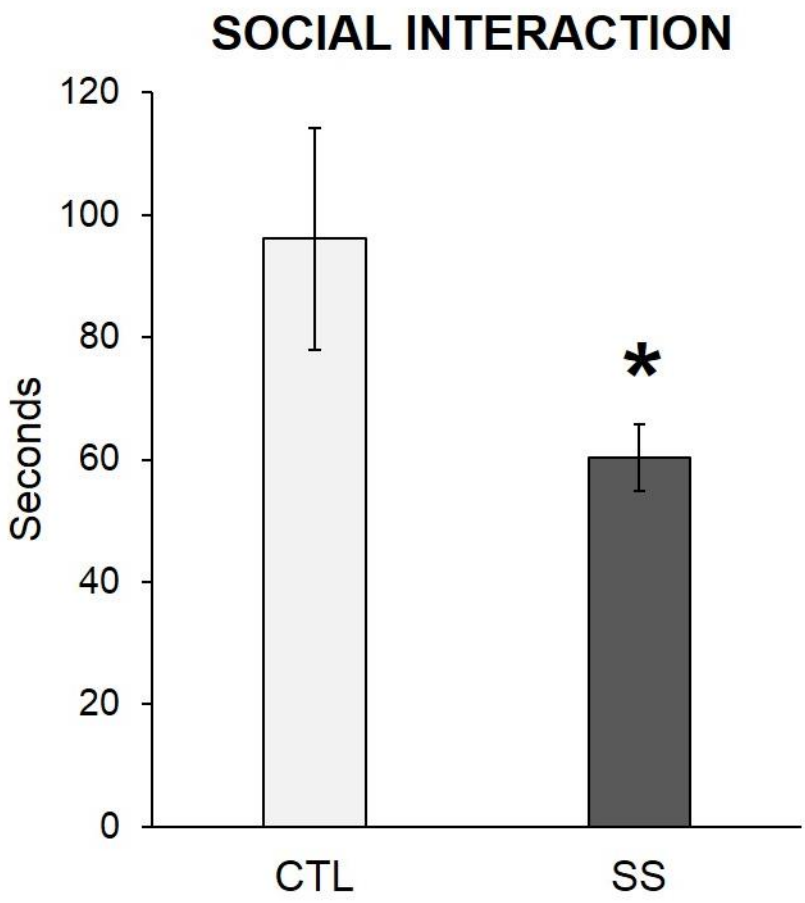

*Figure 4-3. Mean (± S.E.M.) time spent in social interaction with an unfamiliar peer in CTL (control) and SS (social instability stress) rats. * $p < 0.05$.*

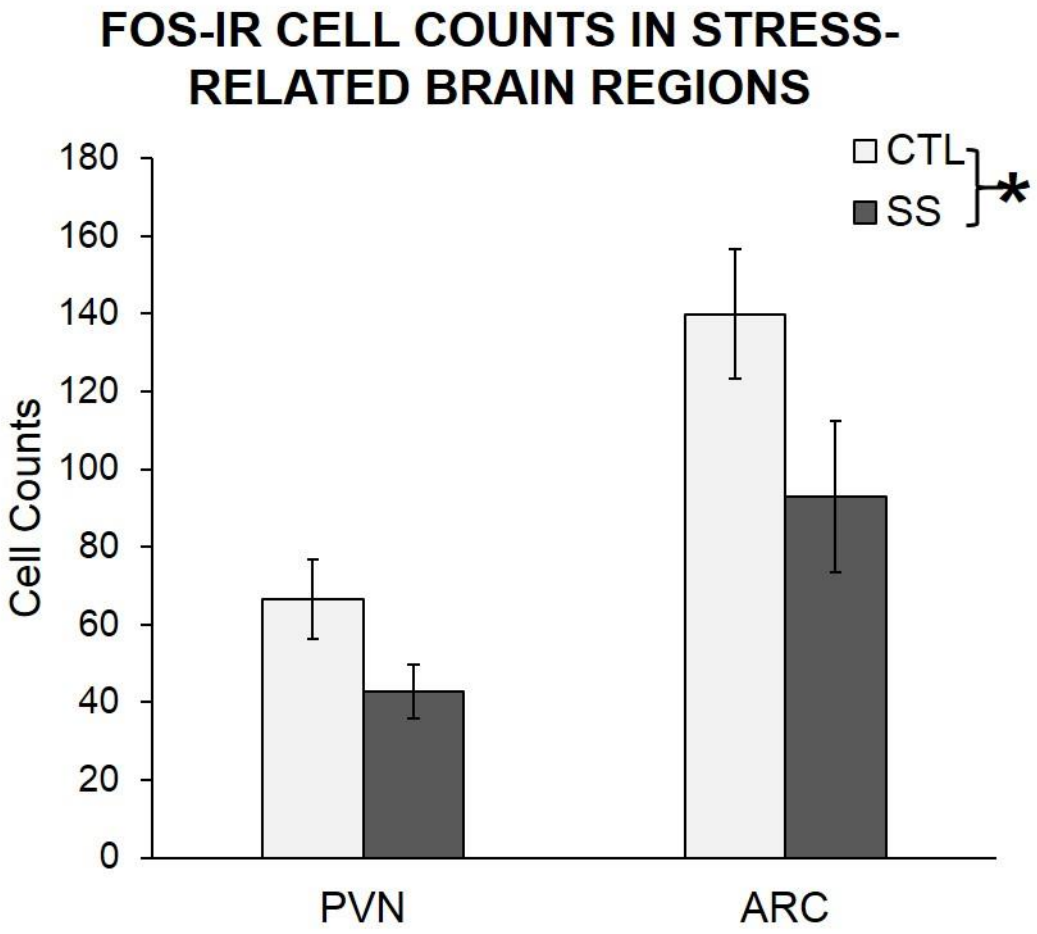

*Figure 4-4. Mean (± S.E.M.) Fos immunoreactive (Fos-ir) cell counts in the paraventricular nucleus (PVN) and the arcuate nucleus (ARC) in response to social interaction in CTL (control) and SS (social instability stress) rats. * Main effect of Stress Group, $p < 0.05$.*

Fos immunoreactive cell counts: brain regions involved in social behaviour. The interaction between Brain Region and Stress Group was significant ($F_{5,85} = 2.61$, $p = 0.03$) (see Figure 4-5). The interaction appeared to be driven by an increase in Fos-ir cell counts in the nucleus accumbens and a decrease in Fos-ir cell counts in the medial amygdala, dorsal lateral septum, and CA2 of SS rats compared with CTL rats. Nevertheless, post-hoc t-tests were not significant for any one brain region.

Correlations among Fos immunoreactive cell counts and time in social interaction. The only significant correlation between social interaction and Fos-ir cell counts was for the arcuate nucleus in SS rats ($r_{11} = .75$, $p = 0.015$), and the strongest association found for CTL rats was for the posterior medial amygdala ($r_8 = .64$, $p = 0.09$). Nevertheless, the associations between social interaction and Fos-ir were significantly different between SS and CTL rats in several brain regions: in the arcuate nucleus ($z = 2.51$, $p = 0.012$), in the lateral septum ($z = 2.07$, $p = 0.039$), in the posterior medial amygdala ($z = 2.39$, $p = 0.017$); the only other difference between SS and CTL rats that was $p < 0.10$ was in the aMeA ($z = 1.68$, $p = 0.093$) (see Figure 4-6). A binomial logistic regression using Fos-ir cell counts in the six brain regions as predictors was able to classify 7 of 8 (87.5%) of CTL rats and 10 of 11 (90.9%) SS rats correctly, indicating that the overall pattern of neural activity differed for the two groups (Nagelkerke R square = 0.51).

Experiment 2

Novelty-seeking behaviour on postnatal day 30. Mean novelty-seeking did not differ between CTL (Mean = 2039, S.E.M. = 48.50) and SS (Mean = 1920, S.E.M. = 67.81) rats in our sample ($t_{40} = 1.40$, $p = 0.17$).

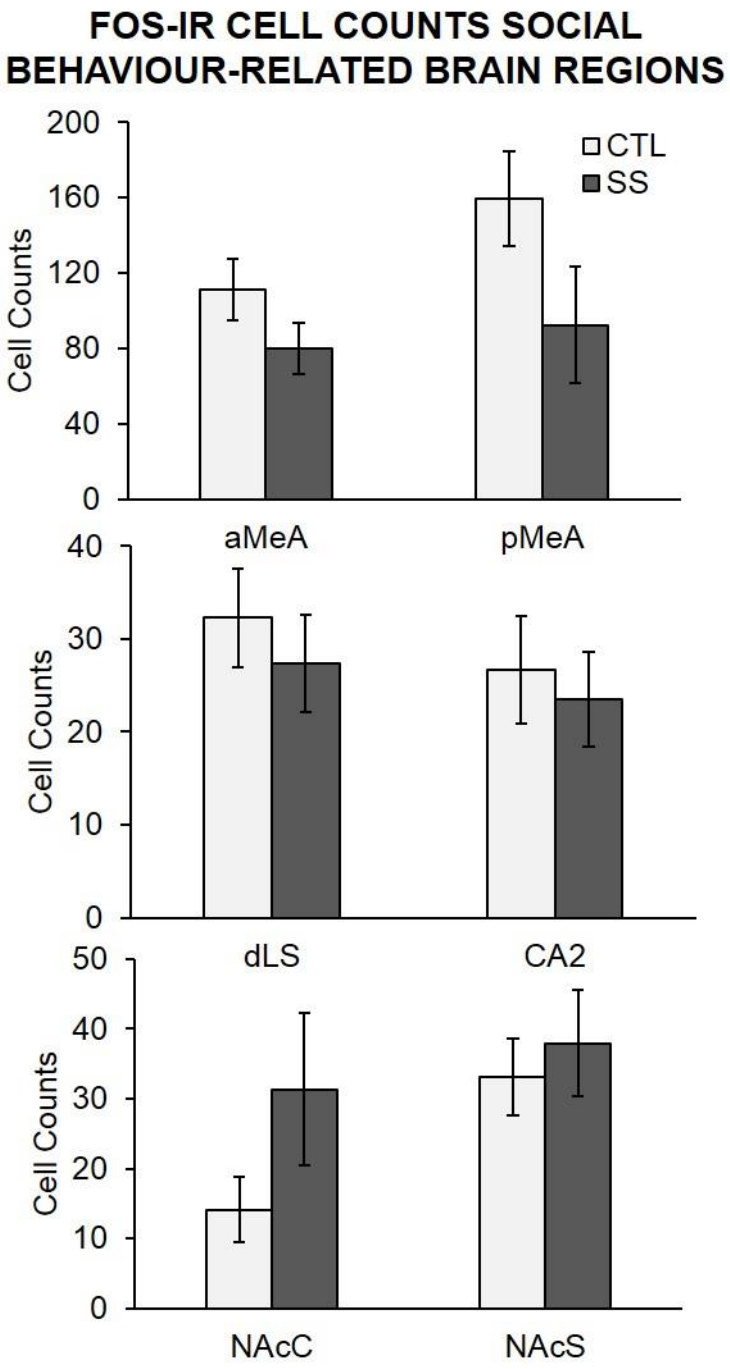

Figure 4-5. Mean (± S.E.M.) Fos immunoreactive cell counts in the anterior medial amygdala (aMeA), the posterior medial amygdala (pMeA), dorsal lateral septum (dLS), Cornu Ammonis 2 region of the hippocampus (CA2), the nucleus accumbens core (NAcC), and the nucleus accumbens shell (NAcS) in response to social interaction in CTL (control) and SS (social instability stress) rats.

A. CORRELATIONS BETWEEN TIME SPENT IN SOCIAL INTERACTION AND FOS-IR CELL COUNTS BY REGION

	PVN	ARC	pMeA	aMeA	dLS	CA2	NAcC	NAcS
CTL	**.371**	-.504	**.636**	**.469**	-.585	**.560**	-.270	.062
SS	.003	**.705**	-.543	-.419	**.469**	.088	-.421	-.299
		*	*		*			

B. SCATTERPLOTS OF TIME IN SOCIAL INTERACTIONS AND REGION SPECIFIC FOS-IR CELL COUNTS

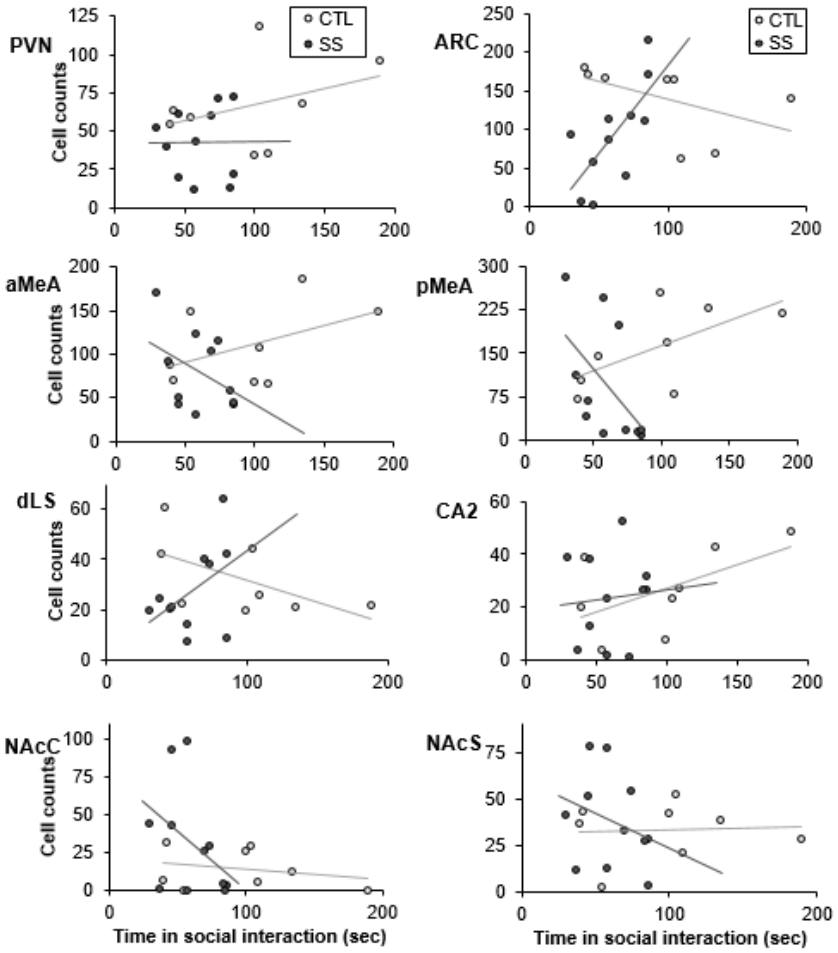

*Figure 4-6. (A) Correlations between time spent in social interaction and Fos immunoreactive (ir) cell counts in brain regions of interest in CTL (control) and SS (social instability stress) rats. Positive correlations are in **bold black** numbers, negative correlations are in **bold gray** numbers, and correlations greater than -0.1 and less than 0.1 are in unbolded black. * indicates differences in the strength of association between CTL and SS for a given neural region, $p < 0.05$. (B) Scatterplots and regression lines demonstrating relationships between time spent in social interaction and Fos-ir cell counts in brain regions of interest in CTL and SS rats. CTL regression lines are in **light gray** and SS regression lines are in **dark gray**. PVN = paraventricular nucleus of the hypothalamus; ARC = arcuate nucleus; aMeA = anterior medial amygdala; pMeA = posterior medial amygdala; dLS = dorsal lateral septum; CA2 = Cornu Ammonis 2 region of the hippocampus; NAcC = nucleus accumbens core; NAcS = nucleus accumbens shell*

Elevated plus maze on postnatal day 46. Consistent with our previous report (McCormick et al., 2008), SS and CTL rats did not differ in time spent on the open arm in the EPM ($t_{40} = 1.59$, $p = 0.128$), nor did they differ in locomotor activity in the EPM (closed arm entries + open arm entries) ($t_{40} = 1.33$, $p = 0.193$). A linear regression using Stress Group and novelty-seeking (first step), and their interaction (second step) as predictors of time in the open arm found the first step model significant ($R^2 = 0.152$, $F_{2,39} = 3.51$, $p = 0.04$), with both Stress Group ($t = 2.03$, $p = 0.049$) and novelty-seeking ($t = 2.10$, $p = 0.043$) emerging as unique significant predictors. The addition of the interaction to the model did not result in a significant F change ($p = 0.13$). When controlling for novelty-seeking at PND 30, SS rats spent more time in the open arm than did CTL rats, and when controlling for Stress Group, those that exhibited higher novelty-seeking at P30 spent more time on the open arm of the EPM at PND 46 (see Figure 4-7).

A linear regression using Stress Group and novelty-seeking (first step), and their interaction (second step) as predictors of locomotor activity on the EPM found neither the first ($R^2 = 0.084$, $F_{2,39} = 1.79$, $p = 0.18$) nor second step ($R^2 = 0.159$, $F_{3,38} = 2.40$, $p = 0.083$) significant. Taken together, the relationship between novelty-seeking on PND 30 and the measure of anxiety on PND 46 (time on open arm in the EPM) was stronger than that between novelty-seeking on PND 30 and locomotor activity in the EPM on P46.

Social interaction test on postnatal day 47. A two factor (Stress Group X Social Interaction Type) ANOVA indicated that rats spent more time in other physical contact than in anogenital sniffing ($F_{1,40} = 88.15$, $p < 0.0001$); the effect of Stress Group ($F_{1,40} = 2.75$, $p = 0.105$) and the interaction ($F_{1,40} = 3.99$, $p = 0.053$) were not significant, although the pattern of means (CTL > SS) was consistent with our previous findings in

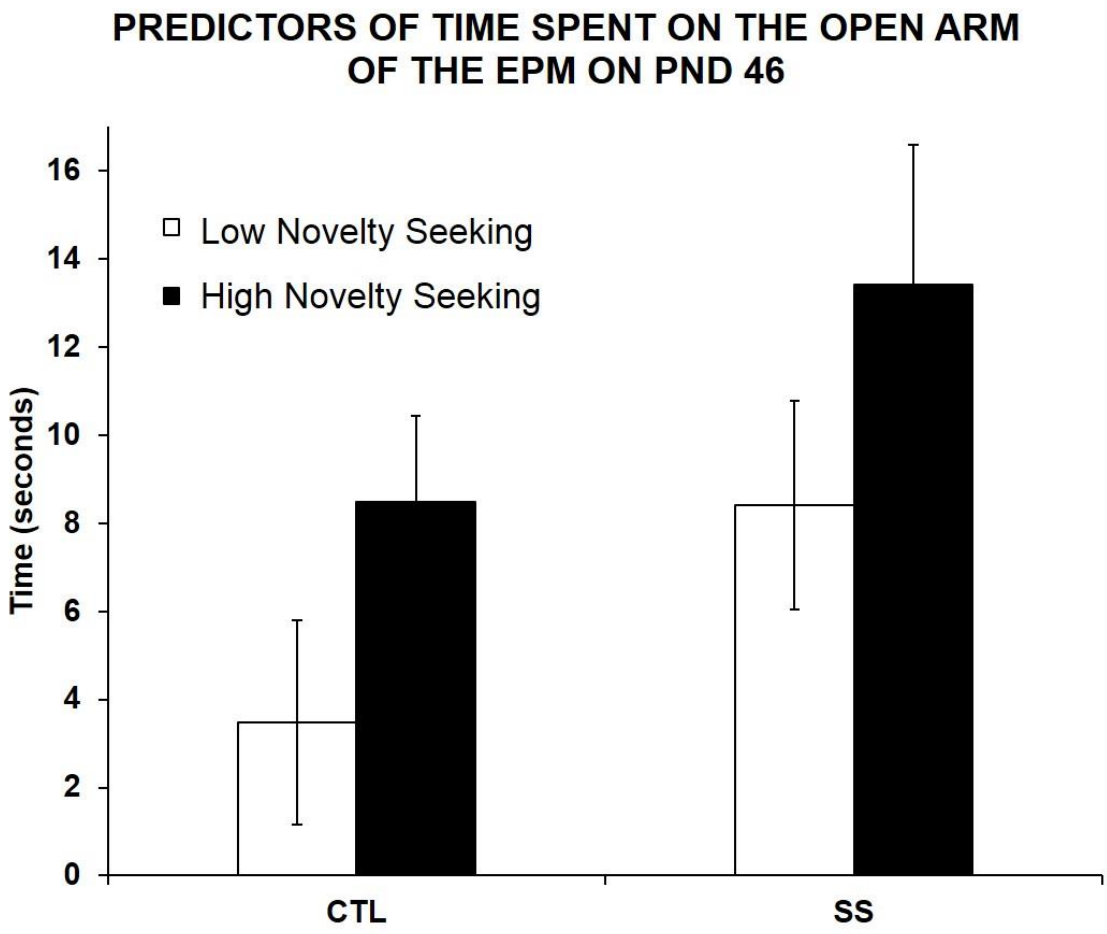

Figure 4-7. Predicted time spent on the open arm of the elevated plus maze (EPM) on postnatal day (PND) 46 in CTL (control) and SS (social instability stress) rats that were characterized as high or low novelty-seeking on PND 30. Error bars represent the standard error of the intercept. High/low novelty-seeking represents +/- 1 standard deviation (SD) from the mean value for novelty-seeking on PND 30 respectively. Our model established a main effect of condition such that SS rats spend more time on the open arm of the EPM than do CTL subjects, over and above the effects of novelty-seeking, and a main effect of novelty-seeking, such that high novelty- seeking rats spent more time on the open arm than did low-novelty seeking rats, over and above the effect of Stress Group.

experiment 1 and in previous reports, and was driven by differences in other physical contact rather than in anogenital sniffing (see Figure 4-8).

A linear regression using Stress Group and novelty-seeking (first step), and their interaction (second step) as predictors of other physical contact found the first step model to explain a modest amount of the variance ($R^2 = 0.112$, $F_{2,39} = 2.45$, $p = 0.10$), with neither predictor significant (Stress Group, $p = 0.09$; novelty-seeking, $p = 0.326$). The addition of the interaction term in Step 2 resulted in a significant model ($R^2 = .22$, $\Delta F = 5.48$, $p = 0.028$), where the interaction term emerged as significant ($t = 2.34$, $p = 0.025$). Novelty-seeking on PND 30 predicted time spent interacting in the Social Interaction task on PND 47 among CTL rats ($t = 2.154$, $p = 0.038$) but not SS rats ($t = -1.39$, $p = 0.17$) (see Figure 4-9). Time spent in social interactions did not differ between SS and CTL rats among those high in novelty-seeking, and CTL rats spent more time in social interactions than did SS rats among those low in novelty-seeking (see Figure 4-9). Figure 4-10 shows the simple correlations between novelty seeking behaviour on PND 30 and other physical contact with an unfamiliar peer on PND 47 between CTL rats and SS rats; the two correlations differed significantly ($z = 2.47$, $p = 0.0135$).

Discussion

The main findings of the two experiments were that rats that underwent social SS in adolescence spent less time in social interaction with an unfamiliar peer than did CTL rats. Moreover, in response to social interaction, the pattern of neural activity in brain regions involved in regulating corticosterone release and in regulating social behaviour differed between SS and CTL rats. Finally, novelty-seeking on PND 30 predicted time spent on the open arm of the EPM and time spent in physical contact other than

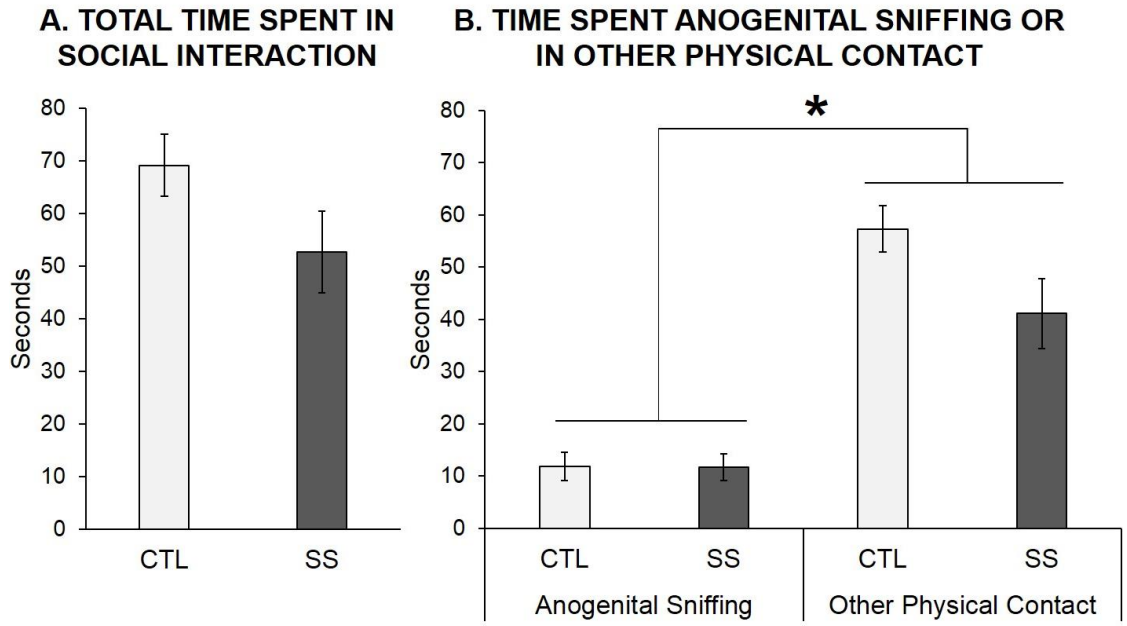

*Figure 4-8. Mean (± S.E.M.) time spent in (A) social interaction with an unfamiliar peer and in (B) social interaction separated into anogenital sniffing and other physical contact measures on postnatal day (PND) 47 in CTL (control) and SS (social instability stress) rats. * Main effect of Social Interaction Type ($p < 0.001$).*

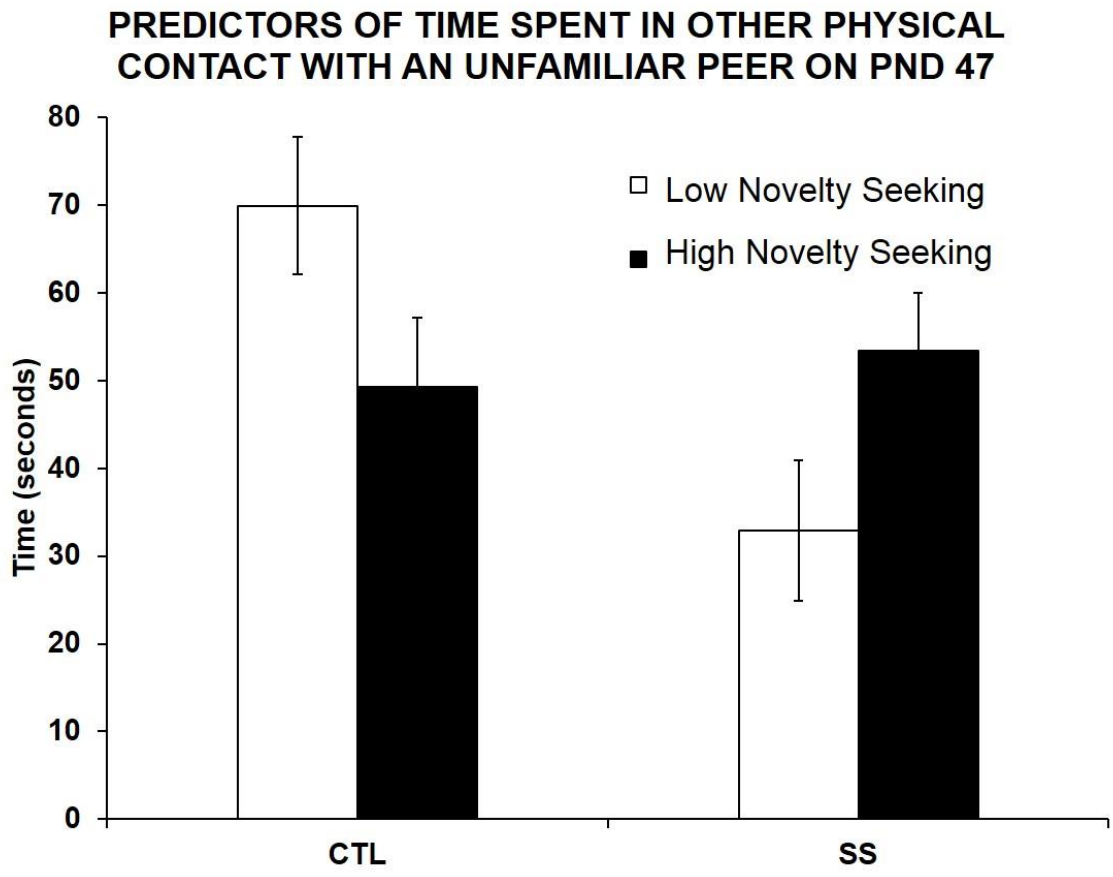

Figure 4-9. Predicted time spent in other physical contact during social interaction with an unfamiliar peer on postnatal day (PND) 47 in CTL (control) and SS (social instability stress) rats that were characterized as high or low responders on PND 30. Error bars represent the standard error of the intercept. High/low novelty seeking represents +/- 1 standard deviation (SD) from the mean value of novelty-seeking on PND 30 respectively. Our model found that Stress Group and novelty-seeking interact to predict time spent in other physical contact. Rats that scored 1 SD below the mean on novelty seeking spent less time in other physical contact than did low novelty seekers in the CTL condition, whereas time spent in other physical contact was unaffected by SS for rats 1 SD above the mean.

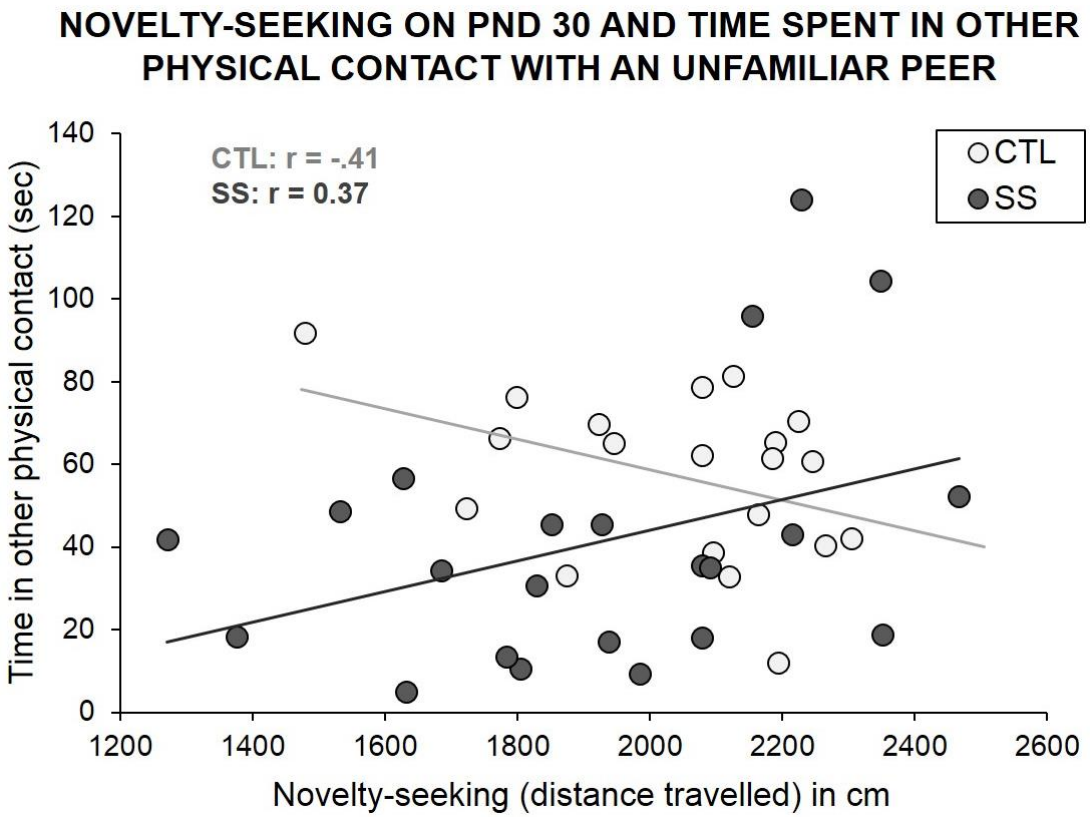

Figure 4-10. Scatterplots and regression lines demonstrating the relationships between novelty-seeking on postnatal day (PND) 30 and time spent in other physical contact during the social interaction test on PND 47 in CTL (control) and SS (social instability stress) rats. In CTL rats r = -.406 as indicated by the ***light gray line*** *(p = .076), and in SS rats r = .37 as indicated by the* ***dark gray line*** *(p = .085). The relationship novelty-seeking and time spent in other physical contact differed significantly between CTL and SS subjects (p < 0.05).*

anogenital sniffing during a social interaction test with an unfamiliar peer. Rats that demonstrated higher levels of novelty-seeking on PND 30 spent more time on the open arm of the EPM than did rats with low levels of novelty-seeking on PND 30 irrespective of Stress Group. Among lower novelty-seeking rats, SS rats spent less time in social interaction with an unfamiliar peer compared with CTL rats, whereas SS and CTL rats did not differ among higher novelty-seeking rats.

Social interaction

Consistent with our previous report (Hodges et al., 2017), in experiment 1, SS rats spent less time in social interaction with unfamiliar peers than did CTL rats when tested on PND 46, 24 h after the last stressor exposure. In experiment 2, we investigated anogenital sniffing separately from other social interaction, and contrary to our prediction, SS and CTL rats spent equal amounts of time in anogenital sniffing. SS rats spent less time in other physical contact during the test than did CTL rats, but the difference was marginal. The difference in day of testing between experiment 1 (PND 46) and experiment 2 (PND 47) is likely not a factor in the different results; we have observed deficits in social interaction several weeks after the last stress exposure when the adolescent SS rats are adult (PND 70) (Green et al., 2013). It is possible that our testing of pairs in the same treatment condition may have affected social interaction. When tested in adulthood, however, this is not the case; the mean time SS rats spent in social interaction with an unfamiliar SS rat did not differ significantly from that when paired with an unfamiliar CTL rat, and CTL rats spent the same time in social interaction when with an unfamiliar CTL as when with an unfamiliar SS rat (Green et al., 2013).

Patterns of neural activity in response to social interaction

The pattern of neural activation as measured by Fos-ir cell counts differed for SS rats and CTL rats in brain regions involved in regulating the stress response and in regulating social behaviour. Moreover, using a binomial logistic regression, the differential patterns of Fos-ir cell counts in the six brain regions involved in regulating social behaviour identified 87.5% of CTL rats and 90.9% of SS rats correctly. Previous studies have reported differential Fos-ir in several brain regions in response to social interaction in rats that were socially deprived in adolescence compared with group-housed controls (Ahern et al., 2016; Wall et al., 2012). In contrast to our findings, however, socially-deprived rats in the aforementioned studies demonstrated increased time spent in social interaction compared with group-housed controls and they show increased aggressive behaviour compared with group-housed controls (Wall et al., 2012). We have not found an increase in aggressive behaviour with unfamiliar peers in SS rats except under conditions of competition against a cage mate for a highly rewarding food, sweetened condensed milk (Cumming et al., 2014).

We have reported that, under homecage conditions, repeated encounters with novel rats leads to heightened corticosterone concentrations in adolescents compared with age-matched rats encountering their first novel rat (Hodges and McCormick, 2015). Thus, one basis for the reduction in social interactions may be a heightened stress response to unfamiliar rats. This interpretation, however, contrasts with the reduction in neural activation of the PVN and ARC of SS rats after interactions with an unfamiliar peer compared with CTL rats. The reduction in Fos-ir cell counts in the ARC in response to social interaction in SS rats compared with CTL rats is in keeping with our past finding of lower Zif268-ir cell counts. Zif268 is another immediate-early gene used as a measure

of neural activity (reviewed in Knapska and Kaczmarek, 2004) in the ARC of SS rats paired with an unfamiliar peer for the 16th time compared with CTL rats paired with an unfamiliar peer for the first time (Hodges and McCormick, 2015). The ARC is involved in the negative feedback of corticosterone release by the hypothalamic-pituitary-adrenal axis; the release of corticosterone in response to a stressor is decreased after corticosterone or glucocorticoid receptor agonists bind to glucocorticoids receptors in the ARC (Leon-Mercado et al., 2017) and lesions to the ARC increase corticosterone secretion at baseline and in response to stressors (Larsen et al., 1994). Greater neural activation of the ARC in response to more time spent in social interaction in SS rats may serve to lessen the release of corticosterone in response to interacting with unfamiliar peers; in SS rats, there was a positive association between Fos-ir cell counts in the ARC and time in social interaction. Further, differences between SS and CTL rats in the associations between Fos-ir cell counts and social interaction are likely because of the greater range in social interaction scores in CTL rats than in SS rats (see Figure 4-6). Thus, the difference in the pattern of neural activations may simply reflect the difference in time spent in social interaction.

A reduction in social interaction may indicate social anxiety and/or an altered behavioural repertoire rather than a reduction in the reward value of peers. In keeping with this possibility, we have found that adolescent SS rats spent more time investigating unfamiliar peers when the peers were confined behind wire mesh (thus, social approach rather than social interaction) despite a reduction in social interactions with unconfined peers (Hodges et al., 2017). Further, in a social conditioned place preference task, SS rats found social interactions with unfamiliar peers as rewarding as did CTL rats (Hodges et

al., 2017). The pattern of greater Fos-ir cell counts in the nucleus accumbens of SS rats compared with CTL rats may reflect a greater reward value of social interactions in SS rats. Previous studies have reported an increase in Fos-ir cell counts in several brain regions, including the lateral septum and the nucleus accumbens core and shell, in response to interaction with an unfamiliar peer in adolescent rats (PND 28-32) (van Kerkhof et al., 2013; Varlinskaya et al. 2013b). The nucleus accumbens is involved in social novelty-seeking (Smith et al., 2017a) and social reward (Dölen et al., 2013), such that the injection of an oxytocin receptor antagonist into the nucleus accumbens reduced these behaviours. Further, we have previously demonstrated increased oxytocin receptor density in the nucleus accumbens of SS rats compared with CTL rats (Hodges et al., 2017). Thus, whereas the pattern of increased neural activity in the nucleus accumbens of SS rats does not appear to play a role in the reduced social interactions with an unfamiliar peer in SS rats, it may play a role in their increased preference of social investigatory behaviour that does not involve direct contact with peers.

The pattern of reduced Fos-ir cell counts in the medial amygdala in SS rats than in CTL rats in response to interacting with an unfamiliar rat, and the opposite pattern of correlations between time spent in social interaction and neural activity in the medial amygdala and lateral septum of SS and CTL rats, may be related to the role of these structures in social recognition (Gur et al., 2014; Bielsky, Hu, Ren, Terwilliger, and Young, 2005; Landgraf et al., 1995; Lukas et al., 2013). We previously reported that SS rats were impaired in social recognition; CTL rats spent more time investigating a novel peer than investigating a familiar peer after intervals of 30 or 90 minutes, whereas SS rats did not discriminate between familiar and unfamiliar peers (Hodges et al., 2017). In

addition, we have found reduced dendritic arborisation and spine density in the medial amygdala and lateral septum (Hodges et al., in preparation). Thus, the SS procedure may reduce social interactions with unfamiliar peers by altering the actions of the medial amygdala and the lateral septum in recognizing familiar and unfamiliar peers.

The hippocampus is essential for the correct performance of several social behaviours (Kolb and Nonneman, 1974; Becker et al., 1999; Maaswinkel et al., 1997), and hippocampal volume changes depending on the amount of social experience the rat obtains; single-housed rats had a smaller hippocampal volume than did rats housed with peers or single-housed rats briefly exposed to social interaction each day (Kalman and Keay, 2017). Further, the CA2 subfield of the hippocampus is also involved in social recognition (Alexander et al., 2016; Bluthé et al., 1993; Hitti and Siegelbaum, 2014; Smith et al., 2016). Nevertheless, there was no evidence of a difference in neural activity in the CA2 subfield of the hippocampus between CTL and SS rats in response to social interaction. We have, however, found reductions in SS rats on other hippocampal dependent tasks such as object spatial location memory (McCormick et al., 2012) and fear conditioned memory for context (Morrisey et al., 2011), as well as altered synaptic plasticity as evidenced by higher expression of CaMKIIα and the survival of new neurons in the hippocampus of SS rats compared with CTL rats; new neurons survived longer in SS rats than in CTL rats (McCormick et al., 2012). Thus, differences between SS and CTL rats specific to the CA2 region may be observed with other measures than Fos-ir.

Novelty-seeking behaviour on postnatal day 30 as a predictor of susceptibility to social instability

The overlap between SS and CTL rats on any of the endpoints we have observed in past studies suggests that there are individual differences in the susceptibility to the effects of adolescent SS. Previous research has identified that novelty-seeking is a predictor of performance on many behavioural tasks (reviewed in the introduction) for which we have observed differences between SS and CTL rats (reviewed in McCormick 2010). Thus, we investigated the extent to which novelty-seeking and the experience of SS predicted anxiety-like behaviour in the EPM. Both experimental condition and novelty-seeking on PND 30 were unique predictors of time spent on the open arm of the EPM, but not of locomotor behaviour in the EPM on PND 46, which suggests that increased novelty-seeking was predicting lessened anxiety rather than simply locomotion. The results are consistent with previous reports of greater anxiety in LR rats than in HR rats (Mällo et al., 2007). Further, social instability stress increased time spent on the open arm independently of novelty-seeking behaviour. A past report from our laboratory (McCormick et al. 2008) did not find an effect of SS on time spent on the open arm at PND 45; the present results suggest that anxiety may depend on controlling for novelty-seeking.

In contrast to the results for anxiety in the EPM, novelty-seeking on PND 30 interacted with stress condition to predict behaviour in the social interaction test. There was a positive relationship between novelty-seeking on PND 30 and time spent in physical contact other than anogenital sniffing on PND 47 in CTL rats, and a negative relationship in SS rats. Among those higher in novelty-seeking, SS and CTL rats did not differ, and among those lower in novelty-seeking, SS rats spent less time in social interaction than did CTL rats. Thus, in accordance with our hypothesis, rats that

demonstrate lower levels of novelty-seeking PND 30 are susceptible to stress-induced social deficits whereas those that demonstrated higher levels of responsiveness to novelty may be resilient to the experience of SS.

These results suggest distinct stress-effects on different behavioural modalities; SS decreased social interactions among lower novelty-seeking rats but increased anxiolytic behaviours independently of novelty-seeking. The effect observed, however may be specific to the SS procedure and to investigations using a continuum of novelty-seeking behaviour; selective breeding models for HR and LR involve rats are limited to the extremes of the distribution (Flagel et al. 2014). In contrast to our results, Rana and colleagues (2016) found that chronic variable adolescent stress increased social behaviours among selectively bred LR rats relative to non-stressed LR rats. HR rats were not included in the study. Our results are more consistent with those of Oztan and colleagues (2011), who reported a decrease in time spent in social interactions after chronic variable adolescent stress, but for both HR and LR rats (classified as top third and bottom third of range of scores in novelty-seeking), rather than among lower novelty-seeking rats only. Study-specific criteria used to classify high- and low- novelty-seeking individuals likely contribute to mixed reports in the literature.

Conclusion

The present results indicate that social instability stress leads to reductions in social behaviour that, in adolescence, do not seem related to generalized anxiety in the EPM. Neural activations in relevant brain regions in response to social interactions, and the relationship between these activations, differ between SS and CTL rats. Further, the results indicate that, depending on the behavioural endpoint, the phenotype of novelty-

seeking has either additive effects with adolescent stress exposures, or interacts with adolescent stress exposure. The results also indicate that the extent to which the effects of adolescent stress exposures produce “detrimental” or “beneficial” changes in behaviour depend on the behavioural endpoint analyzed. Neural and behavioural differences between CTL and SS rats highlight the malleability of ongoing social development in the adolescent period.

Rationale for chapter 5

As per my findings from chapter 2, rats that undergo adolescent social instability are exposed to high concentrations of corticosterone, which can act via binding to glucocorticoid receptors to alter synaptic plasticity. In addition, as per my findings in chapter 4, adolescent social instability changes how the dorsal lateral septum and the posterior medial amygdala are activated in response to interacting with unfamiliar peers. Thus, in chapter 5, I tested the hypothesis that social instability stress alters the morphology of the lateral septum and the medial amygdala. The aim of this chapter was to investigate the effects of adolescent social instability on dendritic morphology and on protein markers of synaptic plasticity in the medial amygdala and lateral septum. All 4 subregions of the medial amygdala (anterodorsal, anteroventral, posterodorsal, posteroventral) were analyzed separately for dendritic morphology because of the different roles they each play in social behaviour, and only the dorsal part of the lateral septum was examined for dendritic morphology in keeping with the region examined in chapter 3 and chapter 4.

Chapter 5: Adolescent social instability stress alters markers of synaptic plasticity and dendritic structure in the medial amygdala and lateral septum in male rats.

This chapter has been adapted from the article recently submitted to the journal *Brain Structure & Function*:

Hodges, T. E., Louth, E. L., Bailey, C. D. C., & McCormick, C. M. (submitted). Adolescent social instability stress alters markers of synaptic plasticity and dendritic structure in the medial amygdala and lateral septum in male rats. *Brain Structure & Function.*

Author contribution: For this manuscript, Dr. Cheryl McCormick and I designed the experiment and formulated the research questions. I wrote the proposal for ethics approval, ordered the rats required for the experiment, performed the social instability stress procedure on rats, performed the social interaction test, collected and prepared brains for western blotting, collected and prepared brains for the Golgi-Cox procedure, performed western blotting, performed the Golgi-Cox procedure, analyzed all data, performed the statistics, created the figures, wrote the paper, and edited the paper after I received feedback from Dr. Cheryl McCormick. Neurons were traced in the laboratory of Dr. Craig Bailey. Emma Louth helped me troubleshoot the Golgi-Cox procedure and taught me how to use the computer programs in the laboratory of Dr. Bailey to trace the neurons, perform sholl analysis on the neurons, and collect images for dendritic spine counting.

Introduction

Social experience during the adolescent period is crucial for the ability to display appropriate social behaviour in the future (reviewed in Pellis et al., 2014; Pellis and Pellis, 2017). For example, rats deprived of social interaction during adolescence displayed reduced social interaction with a peer (van den Berg et al., 1999; Lukkes et al., 2009), awkward behaviour with a sexual partner in adulthood (van den Berg et al., 1999), greater aggressive behaviour with peers (Wall et al., 2012), abnormal behaviour in situations of conflict (Einon and Potegal, 1991; Tulogdi et al., 2014; van den Berg et al., 1999), and impaired social recognition (Shahar-Gold et al., 2013). Brain regions involved in initiating and regulating social behaviour such as the amygdala, lateral septum, prefrontal cortex, and hippocampus, undergo changes in volume, dendritic spine density, white matter, neuronal density, and neurogenesis from adolescence to adulthood (Koss et al., 2014; Markham et al., 2007; Willing and Juraska, 2015; He and Crews, 2007; Mizukami et al., 1983; Verney et al., 1987); thus, social experience may influence ongoing brain development in adolescence. In support of this hypothesis, rats deprived of social interaction had reduced hippocampal volume compared with pair-housed or single-housed rats exposed to a conspecific for 6 minutes a day (Kalman and Keay, 2017), and adolescent single-housed rats had reduced dendritic branching in the orbitofrontal cortex and greater dendritic branching in the medial prefrontal cortex compared with socially-housed adolescents (Bell et al., 2010). Thus, the absence of social behaviour in adolescence alters both the development of social brain regions and social behaviour.

In turn, the quality of social relationships also influences the development of social brain regions and social behaviour. For example, rats that undergo SS in

adolescence show reduced social interactions with unfamiliar peers compared with CTL, and this reduction is evident both after the SS procedure in adolescence (Hodges et al., 2017; 2018) and weeks after the procedure in adulthood (Green et al., 2013). The decrease in social interaction is not accompanied by a reduction in the reward value of social interaction; SS and CTL rats do not differ in time spent in social approach to unfamiliar rats separated by wire mesh (Green et al., 2013) or in conditioned place preference for social interaction (Hodges et al., 2017). The pattern of neural activity during social interaction tests (as measured by Fos protein ir) across a set of brain regions selected for their involvement in social behaviour (medial amygdala, lateral septum, CA2 subregion of the hippocampus, nucleus accumbens) differed between SS and CTL rats (Hodges et al., 2018). We also investigated the oxytocin system because of its importance in social behaviour (reviewed in Veenema and Neumann, 2008), and found increased oxytocin receptor binding density in the dorsal lateral septum and nucleus accumbens shell in SS rats relative to CTL rats (Hodges et al., 2017). SS and CTL rats differ on a variety of social behaviours other than social interaction; compared with CTL rats, SS rats exhibited increased aggressive behaviour when competing against a peer for a food reward in adulthood (Cumming et al., 2014), showed decreased social recognition (Hodges et al., 2017), and had deficits in mating behaviour with a female (McCormick et al., 2013).

To better elucidate the neural mechanisms that might underlie the differences between SS and CTL rats in social behaviour, here we investigate the effect of adolescent SS on dendritic morphology (number of intersections, dendrite diameter, number and distance of terminals, length of matter, branch order), dendritic spine density, and on

markers of synaptic plasticity (spinophilin, synaptophysin, postsynaptic density protein 95 [PSD95], CaMKII in the medial amygdala and lateral septum. The Golgi-Cox method (e.g., Gibb and Kolb, 1998) was used to visualize the dendrites and dendritic spines of neurons in the brain. The medial amygdala and lateral septum were chosen as regions to investigate because of their broad roles in social behaviour. The medial amygdala and lateral septum are involved in social investigation, aggressive behaviour, sexual behaviour, and social recognition (reviewed in Sheehan et al., 2004; Knapska et al., 2007). We also investigated glucocorticoid receptor protein expression in the two groups because of the role of glucocorticoids in the structural and functional remodelling of neurons (reviewed in McEwen et al., 2016) and because of the greater exposure to glucocorticoids experienced by SS rats than CTL rats as part of the SS procedure (McCormick et al., 2007; Hodges and McCormick, 2015).

Methods

Animals

Male Long-Evans rats (N = 40) were obtained from Charles River, Kingston, New York, on PND 22 and given a week to acclimate to the animal colony. Rats were housed in pairs and maintained under a 12 h light–dark cycle (lights on at 05:00 h) with food and water available ad libitum. Use of animals in these experiments was approved by the Brock University Institutional Animal Care Committee and was carried out in adherence to the Canadian Council on Animal Care guidelines.

Social instability stress procedure

Rats were randomly assigned to the adolescent SS (n = 20) group or to the CTL (n = 20) group. The SS procedure was as described previously (reviewed in McCormick,

2010; McCormick et al., 2015). Beginning on PND 30, SS rats were isolated in a 12 cm x 10 cm ventilated plastic container in a room separate from the colony for 1 h each day until PND 45. Immediately after isolation each day, SS rats were returned to the animal colony and housed in a new cage with a new cage partner that had also undergone the 1 h isolation. The SS procedure was conducted at various times during the lights on phase of the light–dark cycle to minimize habituation to the procedure. On PND 45, after the final isolation, SS rats remained with the same cage partner and were left undisturbed except for cage maintenance or test procedures. CTL rats remained undisturbed in their home cages except for cage maintenance from time of arrival at the colony until the test procedures.

Golgi-Cox staining

Brain collection and Golgi-Cox procedure. A subset of CTL (n = 12) and SS (n = 12) rats were decapitated directly upon removal from the home cage on PND 46 and brains were placed in Golgi-Cox impregnation solution for 25 days in the dark. After the 25th day of incubation, brains were placed in sucrose cryoprotectant (30% (w/v) sucrose in phosphate buffered saline, pH 7.4) for 48 h. Brains were then sliced on a vibratome in a 30% sucrose solution into 500 μm-thick sections containing the dorsal lateral septum (from approximately bregma 1.44 mm to bregma -0.24 mm). Sections were also collected to contain the four subregions of the medial amygdala, which differ based on cytoarchitecture, afferent and efferent projections, and functional differences (Canteras et al., 1995; reviewed in Newman, 1999): the anterodorsal and anteroventral subregions of the medial amygdala (from approximately bregma -1.92 mm to -2.64 mm), and the posterodorsal and posteroventral subregions of the medial amygdala (from approximately

bregma -2.76 mm to -3.48 mm) identified according to Paxinos and Watson (2005). Sections were washed twice in distilled water for 5 min, incubated in 2.7% (v/v) ammonium hydroxide for 15 min, and then washed twice in distilled water for 5 min. Sections were then incubated in ascending steps in ethanol (70%, 70%, 95%, 95% for 8 min each; 100%, 100%, 100% for 5 min each) and cleared in two incubations of xylene for 5 min each. After clearing, sections were mounted onto Superfrost Plus slides (Fisher Scientific, Inc.) and coverslipped using Permount mounting medium (Fisher Scientific, Inc.). During the Golgi-Cox procedure one SS rat brain was damaged and unusable. Thus, the final sample for the Golgi-Cox procedure was n = 12 CTL rats and n = 11 SS rats.

Imaging of neurons and analysis of dendrite branches. Imaging and tracing was done blind to experimental condition. Bright field imaging was performed using an upright BX53 microscope (Olympus, Richmond Hill, ON, Canada) fitted with a motorized stage and integrated with Neurolucida software (version 10, MBF Bioscience, Willison, VT). High resolution z axis image stacks were acquired using an Olympus UPlanSApo 30X, 1.05 NA silicone oil-immersion objective (Olympus, Richmond Hill, ON, Canada; total magnification 300x), and each image stack measured 395 μm (x axis) by 296 μm (y axis) with a z axis step size of 1 μm. For an individual Golgi-Cox-stained neuron of interest, its image stacks overlapped in the x and y axes so that when stitched together in three dimensions the entire neuron was contained. Neurons were randomly sampled from the dorsal part of the dorsal lateral septum, anterodorsal medial amygdala, anteroventral medial amygdala, posterodorsal medial amygdala, and posteroventral medial amygdala, and neurons were equally sampled from both hemispheres. Only

stellate neurons were sampled because of their abundance in the medial amygdala (e.g. Dall'Oglio et al., 2008a; 2008b) and lateral septum (e.g. Phelan et al., 1989). A sampled neuron was fully traced and analyzed only if (1) it was fully contained within the section (i.e., no dendrite branches were cut off), (2) it was not occluded by another neuron, and (3) all of its dendrite branches were fully intact. Sholl analyses were used to characterize differences in the dendrite structure of neurons in the medial amygdala and lateral septum between CTL and SS rats. For each rat, three neurons were imaged from each brain region, traced using Neurolucida Software (Version 10, MicroBrightField, Williston, VT, USA), and analyzed using Neurolucida Explorer (MBF Bioscience, Williston, VT, USA). A total of 33-36 dendrites were analysed in each brain region for each experimental group (CTL, n = 11-12 rats; SS, n = 11 rats). For each neuron, the morphological analysis included the following parameters: (1) the number of intersections of dendritic branching and (2) the dendrite diameter within sholl concentric spheres that radiated outward from the soma in multiples of 25 μm, (3) the number of dendrite terminals, (4) the total amount of dendrite matter in length, (5) the highest branch order of dendrites, and (6) the distance to the most distal dendrite terminal. Values for each rat per brain region were averaged, and these rat averages were used as the level of sampling for statistical analysis. Rats were removed from the statistical analyses for a specific brain region if they did not have three intact neurons in that brain region. See Figure 5-1 for examples of the areas from which Golgi-Cox-stained neurons were sampled.

Dendritic spine analysis. The same neurons traced for dendritic branch analysis were chosen for dendritic spine analysis in all brain regions of interest. Dendrites for dendritic spine analysis had to have clearly visible spines. Three neurons per brain region

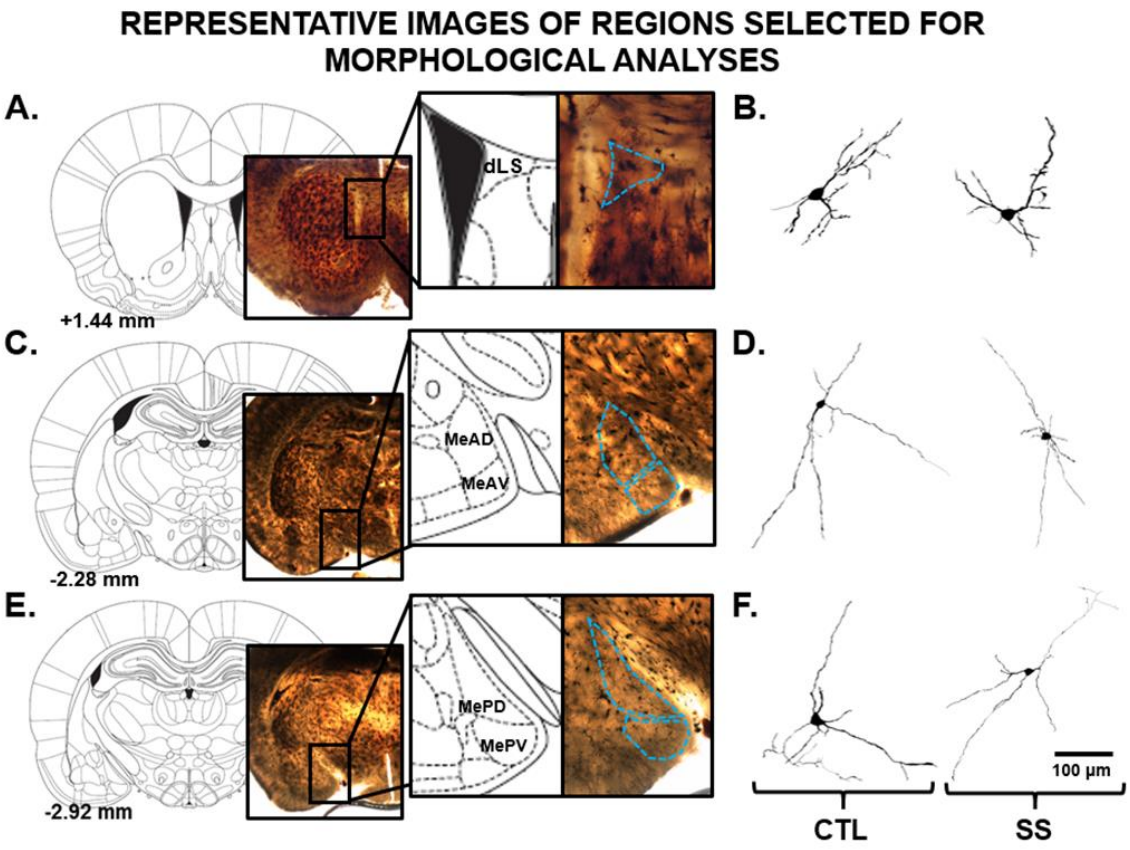

Figure 5-1. Atlas images depicting regions of interest for morphological analyses (images are used with permission from Paxinos and Watson, 2005), corresponding images of Golgi-Cox stained sections, and representative z-projections of traced neurons for the regions of interest. Brain regions of interest include the dorsal lateral septum (dLS) (A), the anterodorsal (MeAD) and anteroventral (MeAV) subregions of the medial amygdala (C), and the posterodorsal (MePD) and posteroventral (MePV) subregions of the medial amygdala (E). Blue dashed lines represent areas that neurons were samples for morphological analyses. Representative z-projections of traced neurons are shown for the dLS (B), MeAD (D), and MePD (F) for rats that underwent adolescent social instability (SS) and non-stressed controls (CTL).

per animal and one dendrite per neuron were chosen and analysed using Image J and blind to experimental condition. Total spines were counted for the length of 80 μm from the cell body for each dendrite. Spines were counted on a total of 27-33 dendrites in each brain region for each experimental group (CTL, *n* = 9-11; SS, *n* = 9-11). Spine density was calculated as the number of spines per 10 μm length of dendrite and averaged per brain region per rat. Rats were removed from the statistical analyses for a specific brain region if they did not have three dendrites with clearly visible spines.

Western blotting

Brain collection, tissue processing, and protein extraction. The remaining CTL (*n* = 8) and SS (*n* = 8) rats were decapitated directly upon removal from the home cage on PND 46, 24 h after the last day of the SS procedure. Brains were extracted, frozen in methylbutane on dry ice, and stored at -80˚C until processing. The lateral septum (from approximately bregma 1.44 mm to approximately bregma -0.24 mm) and the medial amygdala (from approximately bregma -2.16 to approximately bregma -3.36 mm) were identified according to Paxinos and Watson (2005), dissected from each hemisphere of each brain, and placed into separate microcentrifuge tubes on dry ice. Glass beads (0.5 mm diameter) and ice-cold whole-cell lysis buffer (1 M Tris, 150 mM NaCl, 0.1% Triton-X) with protease and phosphatase inhibitors (Roche, USA) were added into each tube containing tissue. All samples were homogenized using a BulletBlender (NextAdvance, USA) at 4˚C for 3 min at a power setting of 6. After homogenization, samples were incubated on ice for 10 min and then centrifuged at 4˚C at 16 000 X g for 10 min. After centrifugation, supernatant was collected as whole-cell soluble protein. Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad, USA) and

a BioTek Synergy plate reader. Samples were then diluted to equal concentrations using whole-cell lysis buffer, and heated 2X Laemmeli buffer was added to each sample at a 1:1 ratio. Samples were then heated for 5 min at 70˚C, centrifuged at 4˚C at 16 000 X g for 10 min, and stored at -20˚C until use in Western blot tests. See Figure 5-2 for an example of the areas from which tissue was sampled for western blotting.

Western blotting procedure. Processed samples were heated for 5 min at 70°C and 5 μg of protein was loaded onto Bio-Rad TGX stain-free fast-cast 12% polyacrylamide gels (Bio-Rad, USA) for SDS-PAGE. Samples from CTL and SS rats were counterbalanced across gels. Once resolved, gels were illuminated for 5 min under UV light for activation of Bio-Rad stain-free total protein determination. After gel activation, proteins were transferred onto PVDF membranes (EMD Millipore, USA), blocked for 1 h in 5% bovine serum albumin in TBS-T (for the primary antibodies anti-spinophilin, anti-synaptophysin, anti- PSD95, anti-CaMKII) or in 5% milk (for the primary antibody anti- GR) at room temperature, and incubated overnight in primary antibody. Primary antibodies used were anti-spinophilin (1:5000, 06-852 Millipore), anti-synaptophysin (1:5000, MAB5258 Millipore), anti-PSD-95 (1:5000, NB300-556 Novus Biologicals), anti-CaMKII (1:2000, 04-1079 Millipore; detects bands for α and β subunits of CaMKII), and anti-GR (1:200, sc-1004 Santa Cruz Biotechnologies). After incubation in primary antibody, membranes were washed (3 x 5 min washes) in TBS-T, and incubated in secondary antibody (AlexaFluor 488 and AlexaFluor 647; Life Technologies, USA) for 45 min in the dark at room temperature with gentle rocking. After incubation in secondary antibody, membranes were washed in TBS-T, and imaged

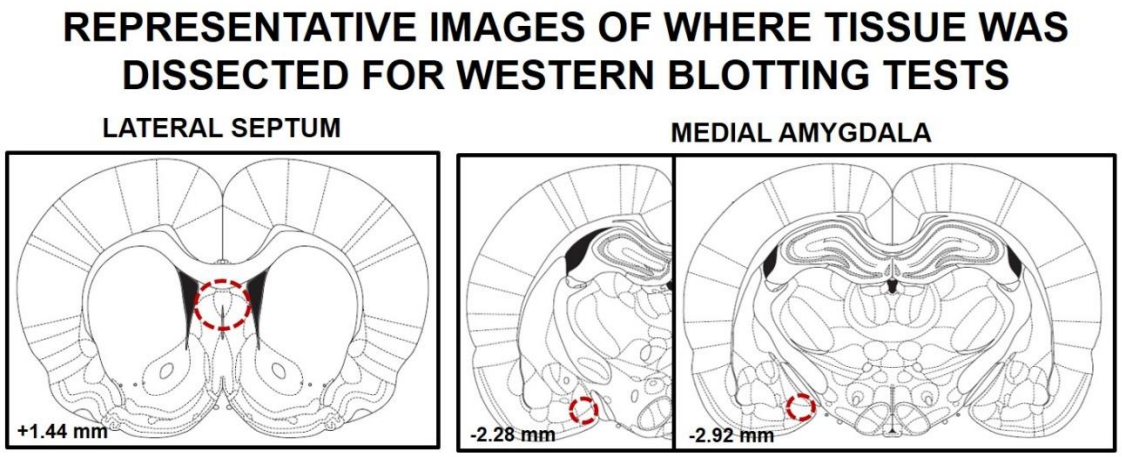

Figure 5-2. Atlas images depicting regions of interest for the western blotting test (images are used with permission from Paxinos and Watson, 2005). Red dashed circles represent where tissue was collected for each brain region (tissue collection for the medial amygdala included the anterior and posterior subregions).

wet using the Bio-Rad VersaDoc MP4000 (Bio-Rad, USA). Staining intensity (intensity X mm) of each band from each lane was normalized to total lane protein using Bio-Rad stain-free total protein determination (Bio-Rad, USA). Results were divided by a constant for each protein so that they would be represented with the same scale across figures; units are thus arbitrary. During the Western blotting test in the medial amygdala for GR and synaptophysin, parts of the membranes containing one CTL rat and one SS rat were damaged, reducing the sample size to 7 per group for these measures.

Statistics

Statistical analyses were performed using SPSS version 24 software, and consisted of mixed factor ANOVAs and independent t-tests. Post hoc analyses consisted of t-tests and Fisher's LSD where appropriate. An alpha level of $p < 0.05$ was used to determine statistical significance in all models. Mixed factor ANOVAs for the three-dimensional Sholl analyses measuring either the average number of intersections or average dendrite diameter consisted of Stress Group (CTL or SS) as the between-groups factor and Distance from Soma (in multiples of 25 μm) as the within-groups factor. Independent t-tests between SS and CTL rats were performed for the dendritic morphological measures of number of terminals, total dendrite length, highest branch order, and distance to the most distal dendrite terminal. Independent t-tests between SS and CTL rats were performed for each marker investigated using western blotting.

Results

Adolescent social instability increased dendritic arborisation in the anterodorsal subregion and reduced dendritic arborization in the anteroventral and posterodorsal subregions of the medial amygdala

Anterodorsal medial amygdala. The interaction between Distance from Soma and Stress Group was significant for the average number of intersections of dendrites in MeAD neurons ($F_{16,320} = 1.770$, $p = 0.034$). The interaction appeared to be driven by a higher average number of intersections of dendrites in the neurons of SS rats than in CTL rat at distances of 25 μm and 350 μm from the soma; nevertheless the post hoc comparisons were not significant ($p = 0.060$ and $p = 0.067$, respectively; other post-hoc t-tests, $p > 0.124$). Average dendrite diameter of MeAD neurons decreased based on Distance from Soma ($F_{16,320} = 45.193$, $p < 0.001$), but was not affected by Stress Group ($p = 0.298$). The interaction between Distance from Soma and Stress Group was not significant ($p = 0.596$) (see Figure 5-3).

Neurons in the MeAD had an increased number of dendrite terminals ($t_{20} = 2.119$, $p = 0.047$) and an increased highest branch order ($t_{20} = 2.138$, $p = 0.045$) in SS rats compared with CTL rats. The MeAD neurons of CTL and SS rats did not differ in total length of dendrite matter ($p = 0.121$) or in the distance to the most distal dendrite terminal ($p = 0.558$) (see Figure 5-3).

Anteroventral medial amygdala. The interaction between Distance from Soma and Stress Group was significant for the average number of intersections of dendrites in MeAV neurons ($F_{15,300} = 1.842$, $p = 0.029$). The interaction appeared to be driven by a lower average number of intersections in the MeAV neurons of SS rats compared with CTL rats from approximately 50 – 175 μm from the soma. Post-hoc t-tests found a lower average number of intersections in the MeAV neurons of SS rats compared with CTL rats at 50 μm ($t_{20} = 2.250$, $p = 0.036$), 75 μm ($t_{20} = 2.432$, $p = 0.025$), and 100 μm ($t_{20} = 2.311$, $p = 0.032$) from the soma. The lower average number of intersections of SS rats

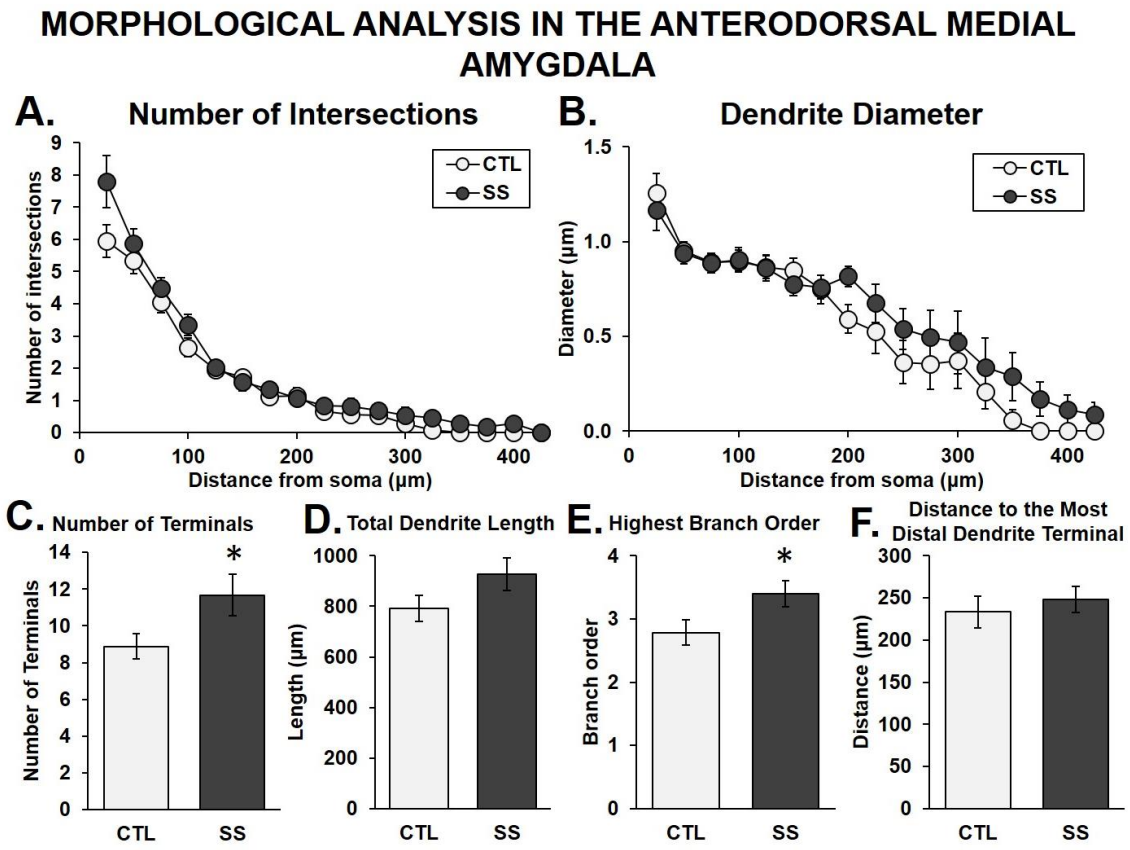

*Figure 5-3. Morphological analysis of stellate dendrites in the anterodorsal subregion of the medial amygdala in rats that underwent adolescent social instability (SS) and non-stressed controls (CTL); three-dimensional Sholl analyses measuring the number of dendritic intersections (A) and the average dendrite diameter (B) within concentric spheres radiating outward from the soma in 25 µm increments, the total number of dendrite terminals (C), the total length of dendrite matter (D), the highest branch order (E), and the distance to the most distal dendrite (F). (A) The three-dimensional Sholl analysis measuring the number of dendritic intersections revealed a significant interaction of Distance from Soma and Stress Group ($p < 0.05$). (B) Average dendrite diameter decreased based on Distance from Soma ($p < 0.001$). * indicates a significant increase in SS rats compared with CTL rats in (C) the total number of terminals ($p < 0.05$) and (E) the highest branch order ($p < 0.05$).*

compared with CTL rats at the distances of 150 μm ($p = 0.070$) and 175 μm ($p = 0.071$) did not meet statistical significance (other post-hoc t-tests, $p > 0.133$). Average dendrite diameter of MeAV neurons decreased based on Distance from Soma ($F_{15,300} = 29.667$, $p < 0.001$), but was not affected by Stress Group ($p = 0.827$). The interaction between Distance from Soma and Stress Group was not significant ($p = 0.995$) (see Figure 5-4).

Neurons in the MeAV had a reduced total length of dendrite matter in SS rats compared with CTL rats ($t_{20} = 2.400$, $p = 0.026$). The MeAV neurons of CTL and SS rats did not differ in the total number of dendrite terminals ($p = 0.556$), highest branch order ($p = 0.851$), or in the distance to the most distal dendrite terminal ($p = 0.157$) (see Figure 5-4).

Posterodorsal medial amygdala. The average number of intersections of dendrites in MePD neurons decreased based on Distance from Soma ($F_{21,441} = 145.765$, $p < 0.001$), but was not affected by Stress Group ($p = 0.301$). The interaction between Distance from Soma and Stress Group was not significant ($p = 0.973$). Average dendrite diameter of MePD neurons decreased based on Distance from Soma ($F_{21,441} = 57.297$, $p < 0.001$), but was not affected by Stress Group ($p = 0.508$). The interaction between Distance from Soma and Stress Group was not significant ($p = 0.918$) (see Figure 5-5).

Neurons in the MePD had a reduced number of dendrite terminals in SS rats compared with CTL rats ($t_{21} = 2.355$, $p = 0.028$). The MePD neurons of CTL and SS rats did not differ in total length of dendrite matter ($p = 0.382$), in highest branch order ($p = 0.139$), or in the distance to the most distal dendrite terminal ($p = 0.867$) (see Figure 5-5).

Posteroventral medial amygdala. The average number of intersections of dendrites in MePV neurons decreased based on Distance from Soma ($F_{22,462} = 84.088$, $p <$

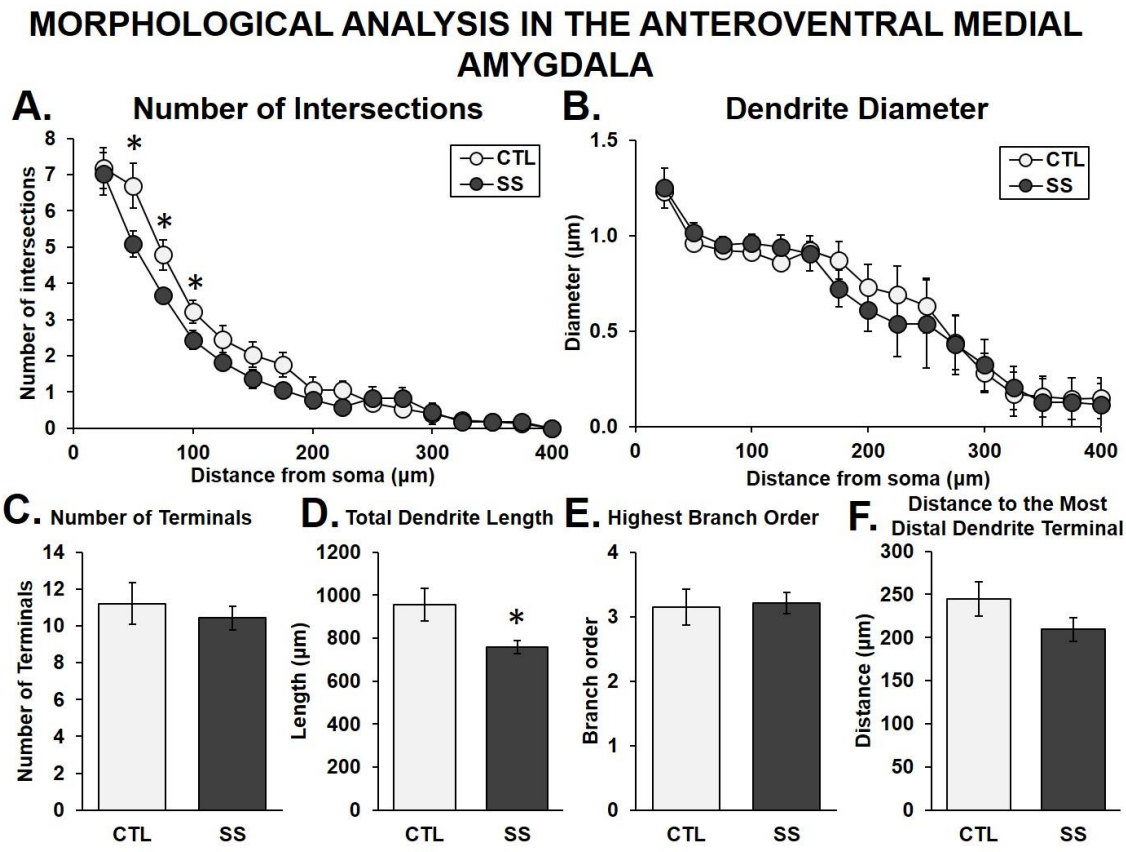

*Figure 5-4. Morphological analysis of stellate dendrites in the anteroventral subregion of the medial amygdala in rats that underwent adolescent social instability (SS) and non-stressed controls (CTL); three-dimensional Sholl analyses measuring the number of dendritic intersections (A) and the average dendrite diameter (B) within concentric spheres radiating outward from the soma in 25 µm increments, the total number of dendrite terminals (C), the total length of dendrite matter (D), the highest branch order (E), and the distance to the most distal dendrite (F). (A) The three-dimensional Sholl analysis measuring the number of dendritic intersections revealed a significant interaction of Distance from Soma and Stress Group ($p < 0.05$). (B) Average dendrite diameter decreased based on Distance from Soma ($p < 0.001$). * indicates a significant reduction in SS rats compared with CTL rats in (A) the number of intersections ($p < 0.05$) and (D) in the total length of dendrite matter ($p < 0.05$).*

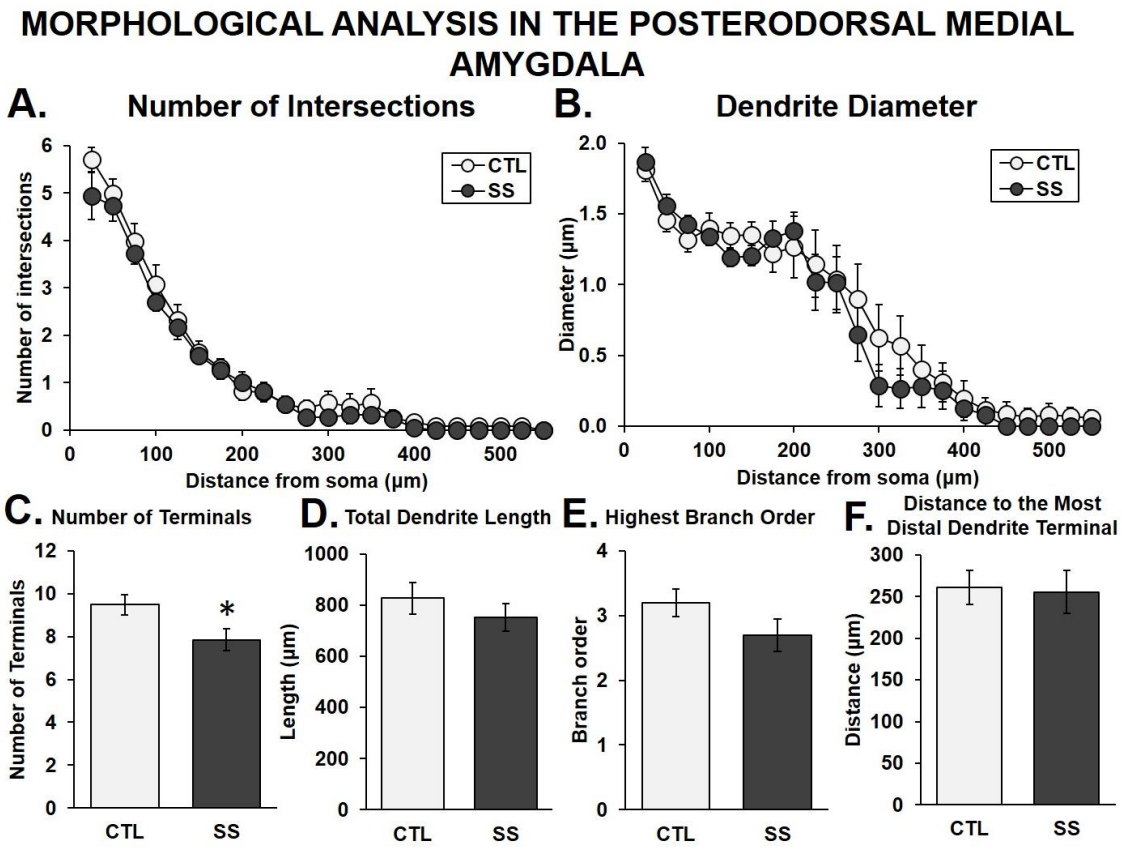

*Figure 5-5. Morphological analysis of stellate dendrites in the posterodorsal subregion of the medial amygdala in rats that underwent adolescent social instability (SS) and non-stressed controls (CTL); three-dimensional Sholl analyses measuring the number of dendritic intersections (A) and the average dendrite diameter (B) within concentric spheres radiating outward from the soma in 25 µm increments, the total number of dendrite terminals (C), the total length of dendrite matter (D), the highest branch order (E), and the distance to the most distal dendrite (F). (A) The number of dendritic intersections and (B) the average dendrite diameter both decreased based on Distance from Soma (p's < 0.001). * indicates a signification reduction in SS rats compared with CTL rats in (C) the total number of dendrite terminals (p < 0.05).*

0.001), but was not affected by Stress Group ($p = 0.591$). The interaction between Distance from Soma and Stress Group was not significant ($p = 0.788$). Average dendrite diameter of MePV neurons decreased based on Distance from Soma ($F_{22,462} = 37.613$, $p < 0.001$), but was not affected by Stress Group ($p = 0.590$). The interaction between Distance from Soma and Stress Group was not significant ($p = 0.909$) (see Figure 5-6).

The MePV neurons of CTL and SS rats did not differ in the total number of dendrite terminals ($p = 0.431$), total length of dendrite matter ($p = 0.867$), in highest branch order ($p = 0.676$), or in the distance to the most distal dendrite terminal ($p = 0.850$) (see Figure 5-6).

Dorsal lateral septum. The average number of intersections of dendrites in dLS neurons decreased based on Distance from Soma ($F_{13,273} = 132.178$, $p < 0.001$), but was not affected by Stress Group ($p = 0.897$). The interaction between Distance from Soma and Stress Group was not significant ($p = 0.999$). Average dendrite diameter of dLS neurons decreased based on Distance from Soma ($F_{13,273} = 102.596$, $p < 0.001$), but was not affected by Stress Group ($p = 0.788$). The interaction between Distance from Soma and Stress Group was not significant ($p = 0.575$) (see Figure 5-7).

The dLS neurons of CTL and SS rats did not differ in the total number of dendrite terminals ($p = 0.557$), total length of dendrite matter ($p = 0.957$), in highest branch order ($p = 0.747$), or in the distance to the most distal dendrite terminal ($p = 0.835$) (see Figure 5-7).

Adolescent social instability reduced dendritic spine density in the dorsal lateral septum

SS rats had lower spine density per 10 μm of dendrites in the dLS than did CTL

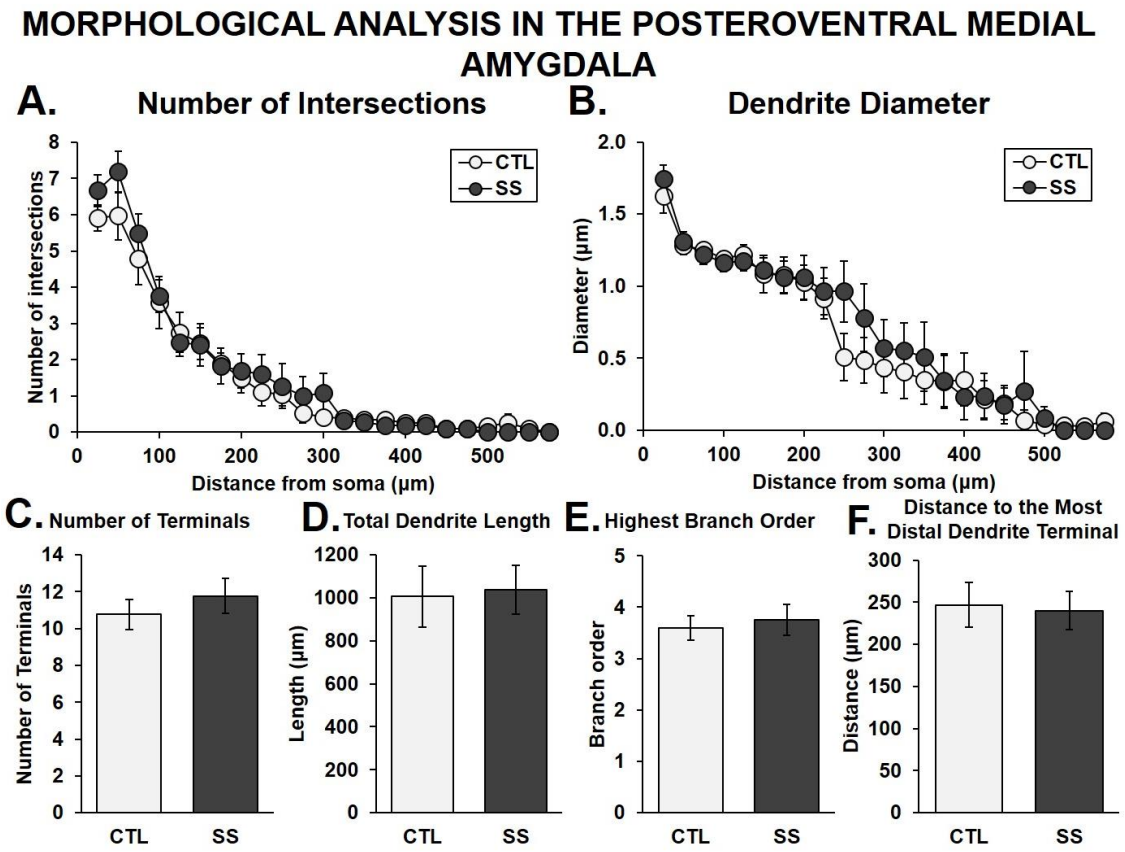

Figure 5-6. Morphological analysis of stellate dendrites in the posteroventral subregion of the medial amygdala in rats that underwent adolescent social instability (SS) and non-stressed controls (CTL); three-dimensional Sholl analyses measuring the number of dendritic intersections (A) and the average dendrite diameter (B) within concentric spheres radiating outward from the soma in 25 µm increments, the total number of dendrite terminals (C), the total length of dendrite matter (D), the highest branch order (E), and the distance to the most distal dendrite (F). (A) The number of dendritic intersections and (B) the average dendrite diameter both decreased based on Distance from Soma (p's < 0.001).

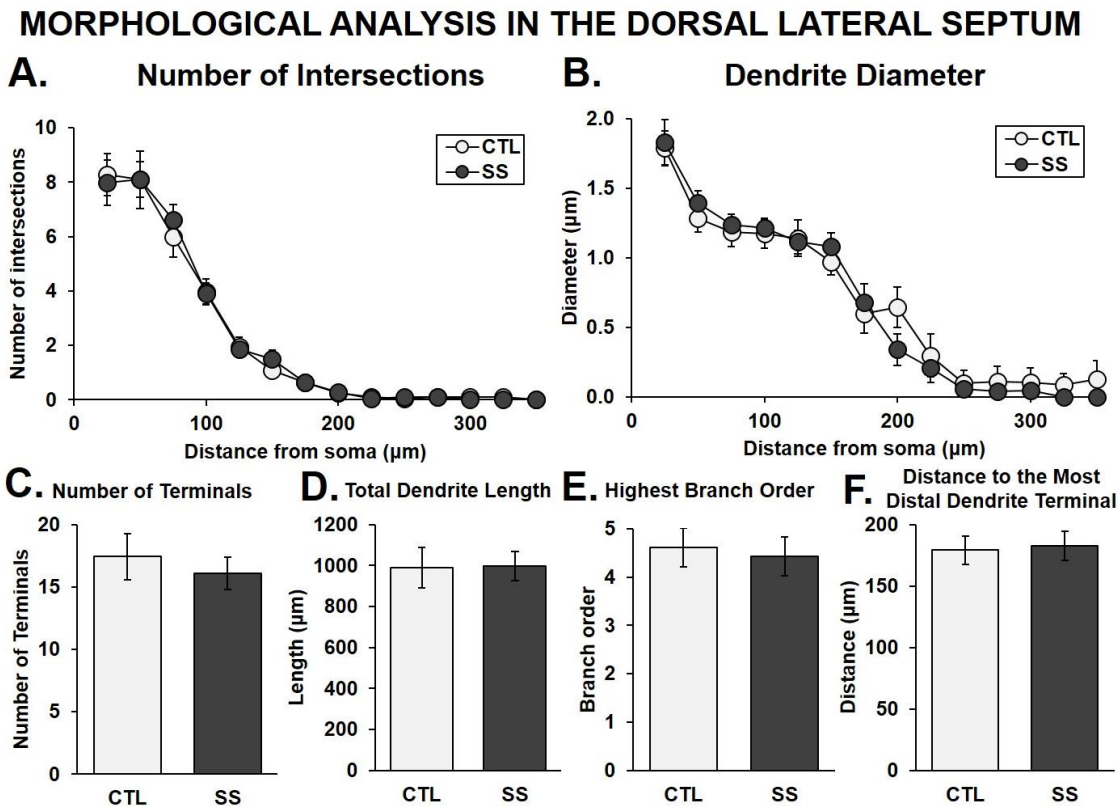

Figure 5-7. Morphological analysis of stellate dendrites in the dorsal lateral septum in rats that underwent adolescent social instability (SS) and non-stressed controls (CTL); three-dimensional Sholl analyses measuring the number of dendritic intersections (A) and the average dendrite diameter (B) within concentric spheres radiating outward from the soma in 25 µm increments, the total number of dendrite terminals (C), the total length of dendrite matter (D), the highest branch order (E), and the distance to the most distal dendrite (F). (A) The number of dendritic intersections and (B) the average dendrite diameter both decreased based on Distance from Soma (p's < 0.001).

rats (t_{18} = 2.181, p = 0.043). CTL and SS rats did not differ in spine density in any subregion of the medial amygdala (all p > 0.463) (see Figure 5-8).

Adolescent social instability reduced synaptophysin in the medial amygdala and increased calcium/calmodulin-dependent protein kinase II in the lateral septum

In the medial amygdala, SS rats had lower synaptophysin protein expression than did CTL rats (t_{12} = 2.464, p = 0.03), and protein expression for all other markers of interest did not differ between CTL and SS rats (all p > 0.357). In the lateral septum, SS rats had higher CaMKII (α and β subunits combined) protein expression than did CTL rats (t_{14} = 2.680, p = 0.018), and protein expression for all other markers of interest did not differ between CTL and SS rats (all p > 0.506) (see Figure 5-9).

Discussion

Repeated SS in adolescence altered dendritic structure in the medial amygdala, with the direction of difference between SS and CTL rats dependent on the subregion of the medial amygdala, and reduced dendritic spine density in the dorsal lateral septum compared with CTL rats. SS rats also had reduced synaptophysin in the medial amygdala and increased CaMKII in the lateral septum compared with CTL rats, but did not differ in spinophilin, postsynaptic density protein 95 (PSD95), or GR protein expression in the medial amygdala or lateral septum.

Dendritic morphology in the medial amygdala

Adolescent SS increased the number of dendritic intersections in the MeAD and reduced the number of dendritic intersections in the MeAV in regions close to the soma compared with CTL rats. SS rats also had an increased number of dendrite terminals and higher average branch order in the MeAD and reduced total length of dendrite matter in

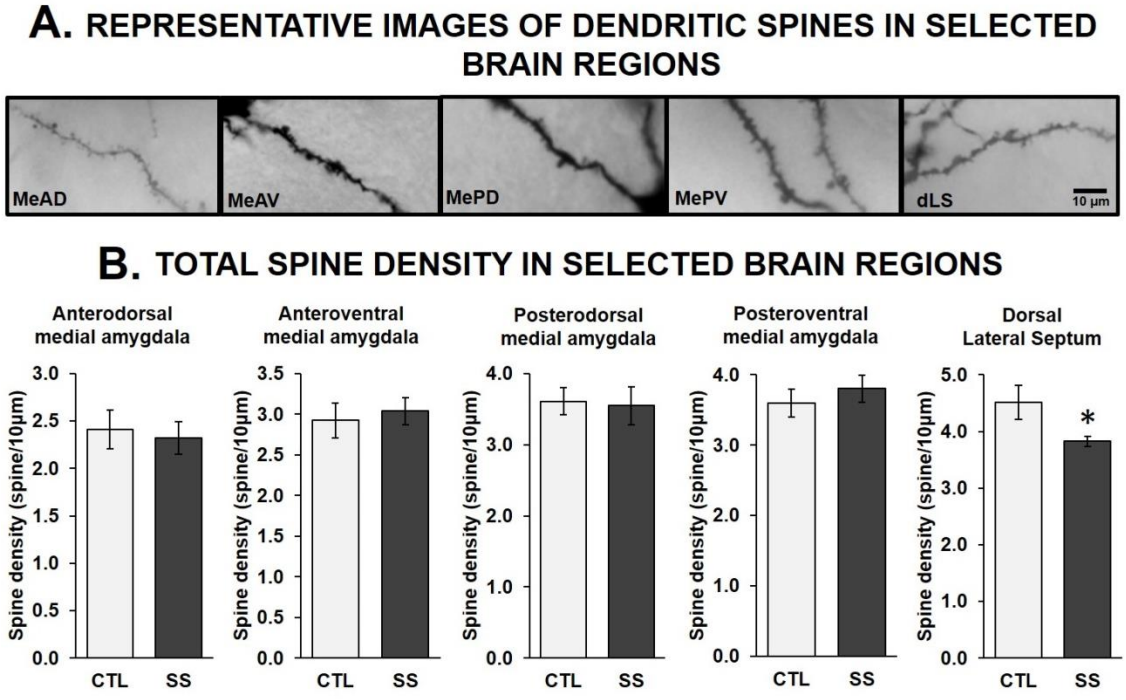

*Figure 5-8. Images depicting dendrites (A) and mean total spine density (B) in the anterodorsal medial amygdala (MeAD), the anteroventral medial amygdala (MeAV), the posterodorsal medial amygdala (MePD), the posteroventral medial amygdala (MePV), and in the dorsal lateral septum (dLS) of rats that underwent adolescent social instability (SS) and non-stressed controls (CTL). * indicates an effect of Stress Group ($p < 0.05$).*

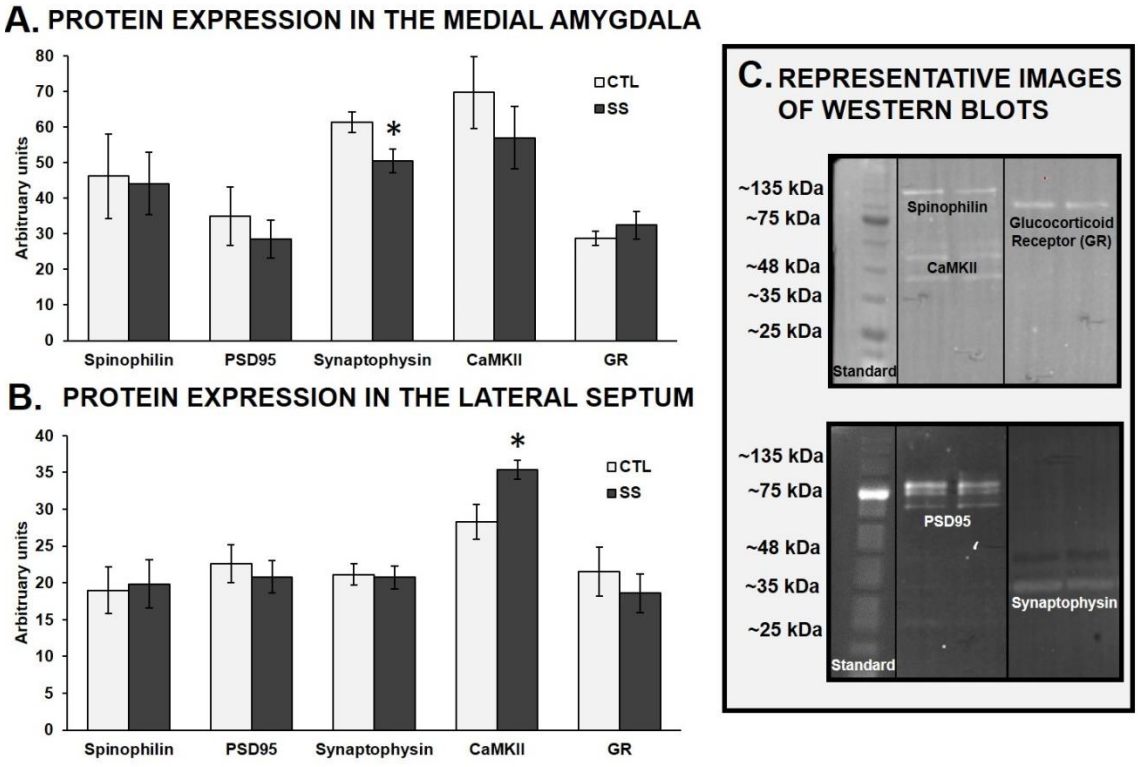

*Figure 5-9. Mean (± S. E. M.) protein expression in the medial amygdala (A) and in the lateral septum (B) of rats that underwent adolescent social instability (SS) and non-stressed controls (CTL), and representative western blot bands for each marker of interest and standard protein ladders (C). * indicates an effect of Stress Group ($p < 0.05$). PSD95 = postcynaptic density protein 95; CaMKII = calcium/calmodulin-dependent protein kinase type II.*

the MeAV. In the MePD, SS rats had a reduced number of dendritic terminals compared with CTL rats. No differences in dendritic arborisation were observed in the MePV and no differences in dendrite diameter were found in any subregion between SS and CTL rats. These structural differences in the medial amygdala may underlie some of the functional differences we have observed in the medial amygdala between SS and CTL rats. SS rats had reduced Zif268 (an immediate-early gene used as a marker of neural activity, reviewed in Knapska and Kaczmarek, 2004) immunoreactive cell counts in the MePD on PND 45 in response to their 16^{th} time returning to an unfamiliar peer after 1 h isolation (the final day of the SS procedure) compared with rats paired with an unfamiliar peer for after 1 h isolation for the first time (Hodges and McCormick, 2015). Further, Fos immunoreactivity in the posterior subregions of the medial amygdala was positively correlated with time spent in social interaction in a test arena with an unfamiliar peer in CTL rats and negatively correlated in SS rats (the opposite pattern of correlations were found in the lateral septum) (Hodges et al., 2018).

No other study has investigated how social stressors in adolescent rats influences dendritic morphology in the medial amygdala, but one study in mice found reduced length of dendritic matter and number of intersections in medial amygdala stellate neurons after daily 2 h restraint in late adolescence into adulthood (PND 49 to 70) (Lau et al., 2017). Others have reported effects of adolescent stressors in other amygdalar nuclei. For example, a reduced number of dendritic intersections in the basolateral amygdala within the range of 20 μm to 90 μm from the soma was found in male rats that underwent a different form of social instability stress in adolescence (1 h immobilization stress and return to different (2 of 5) cagemates daily from PND 28 to 63) compared with non-

stressed controls (Tsai et al., 2014). Daily 20 min restraint from PND 25 to 31 increased the total length of dendrite matter in the lateral amygdala (Padival et al., 2015) and exposure to repeated restraint for 6 h daily from PND 20 to 41 increased total length of dendrite matter in the basolateral amygdala (Eiland et al., 2012) compared with controls. Our lack of findings of changes in dendritic spine density in the medial amygdala of SS rats is in contrast to previous findings of reduced dendritic spine density in the MePD after acute stressor exposure in adult rats (Marcuzzo et al., 2007) and in the total medial amygdala after repeated stressor exposure in adult mice (Bennur et al., 2007). Thus, the changes observed in spine density likely depend on the neural structure, the type of stressor, and on the ages for which the stressor is administered.

Our focus on the medial amygdala is because of its importance in social function (reviewed in Knapska et al., 2007). All subregions of the medial amygdala receive fibers from the main and accessory olfactory bulbs and the vomeronasal organ (Pro-Sistiaga et al., 2007; Mohedano-Moriano et al., 2007) and are involved in processing social stimuli from opposite-sex and same-sex conspecifics (Kang et al., 2009; Meredith and Westberry, 2004). Nevertheless, the subregions of the medial amygdala send distinct efferent projections to other social brain regions to influence social behaviours (Canteras et al., 1995) and differ in how they process social stimuli. For example, both male and female odors increase Fos-ir in the MePD and only female odors increase Fos-ir in the MeAD in male rats (Donato Jr. et al., 2010). Sex hormones in the medial amygdala during puberty are necessary for the expression of adult male behaviour (Sano et al., 2016; reviewed in Sisk et al., 2016) and blocking sex hormone receptors in the MePD (the subregion with the highest density of androgen and estrogen receptors; Scordalakes

et al., 2002), but not in other subregions, influences the social preferences of both males and females (Hosokawa and Chiba, 2010; Fujiwara et al., 2016). Our findings of subregion-dependent changes in dendritic morphology in SS rats thus may separately influence, or interact to influence, the function of several social behaviours.

There are sex differences in the neuronal dendritic spine density, neuronal somatic volume, and astrocytes in all subregions of the medial amygdala in rats (Rasia-Filho et al., 2002; 2004; Hermel et al., 2006; Rocha et al., 2007) and they are modulated by sex hormones (reviewed in Cooke, 2006). For example, the estrous cycle in female rats influences the size of neuronal somatic volume and dendritic spine density in the MePD, MePV, and MeAV (Carrillo et al., 2007; Zancan et al., 2015), and castration reduces dendritic spine density and soma size in the MePD in male rats (Cooke et al., 2003; Zancan et al., 2017). Female rats that undergo adolescent SS are exposed to higher amounts of corticosterone after the procedure compared with females that were repeatedly isolated and returned to their cagemate daily (McCormick et al., 2007) and we have recently found that adolescent SS female rats also have reduced social interaction with unfamiliar peers compared with CTL females when tested soon after the procedure in adolescence or in adulthood (Asgari et al., unpublished data). Changes in dendritic arborisation may also be occurring in the medial amygdala of SS females, but we are only just beginning to examine social brain regions in females after adolescent SS.

Dendritic morphology in the dorsal lateral septum

To our knowledge, our finding of decreased spines in the lateral spetum is the first report of stressors in adolescence reducing spine numbers in this brain region. Nevertheless, others have reported reduced dendritic spine density in the hippocampus

and prefrontal cortex and subregion dependent changes in spine density in the amygdala after stressor exposures in adolescence (reviewed in Radley and Morrison, 2005). For example, exposure to social defeat in adolescent mice (PND 35 to 45) reduced the number of stubby spines in the CA1 part of the hippocampus compared with non-stressed controls (Iniguez et al., 2016) and 9 days of repeated restraint in adult rats increased spine density in the basal amygdala (Padival et al., 2013). In contrast, daily 20 min restraint from PND 25 to 31 reduced dendritic spine density in the lateral amygdala in male rats (Padival et al., 2015).

The lateral septum is involved in the exploration of novel stimuli (reviewed in O'Connell and Hofmann, 2011), and the neuropeptide oxytocin in the lateral septum has been found to regulate time spent in social interaction (Veenema et al., 2012; Bredewold et al., 2014), the social recognition of familiar and novel peers (Lukas et al., 2013), and social anxiety (Guzmán et al., 2013; Zoicas et al., 2014). In addition to a reduction in social interaction, SS rats had increased oxytocin receptor binding in the dorsal lateral septum compared with CTL rats (Hodges et al., 2017). Further, Fos-ir in the dorsal lateral septum was negatively correlated with time spent in social interaction with an unfamiliar peer in CTL rats and positively correlated in SS rats (the opposite pattern of correlations was found in the medial amygdala) (Hodges et al., 2018). Our present finding of altered dendritic morphology gives another example of how adolescent SS influences the development of the lateral septum.

Markers of synaptic plasticity

The proteins synaptophysin, spinophilin, PSD95, and CaMKII were measured in the medial amygdala and lateral septum. Synaptophysin is found in the presynaptic small

vesicles of neurons and in neuroendocrine cells, and can be used as an indirect measure of synapse number (Navone et al., 1986; Wiedenmann et al., 1986). Spinophilin (highly enriched in dendritic spines and scaffolding protein) and PSD95 (a major scaffolding protein in the postsynaptic density) have prominent roles in glutamatergic signalling, and also are used as markers of synapse numbers (Allen et al., 1997; reviewed in Kennedy, 1998). CaMKII is an enzyme highly concentrated in the postsynaptic membrane at glutamatergic synapses and is involved in the induction of long-term potentiation (LTP) (reviewed in Fukunaga et al., 1996; Lisman and Zhabotinsky, 2001). The only differences found between SS and CTL rats in these proteins was a reduction in synaptophysin in the medial amygdala and an increase in CaMKII in the lateral septum in SS rats relative to CTL rats.

Others have reported changes in the amount of synaptophysin after social manipulations in adolescence. Adult rats that were isolation-housed from PND 30 to 35 had reduced synaptophysin in the prefrontal cortex (Leussis et al., 2008), and adult rats that were isolation-housed from PND 21 to 28 had reduced synaptophysin in the hippocampus of adult rats (Arcego et al., 2017) compared with socially-housed controls. In those studies, however, changes in synaptophysin were found in adulthood after stressor exposure in adolescence and it remains unknown if the changes we demonstrated here in synaptophysin in adolescent SS rats would still be evident in adulthood. In a previous study, no differences were found in synaptophysin in the hippocampus between adolescent SS and CTL rats investigated as adults, but the medial amygdala was not investigated (McCormick et al., 2012). Higher protein expression of CaMKII in the lateral septum of adolescent SS rats compared with CTL rats is in keeping with our past

finding of higher protein expression of the alpha subunit of CaMKII in the dorsal hippocampus of SS rats compared with CTL rats when tested in adulthood (McCormick et al., 2012). Greater differences between SS and CTL rats in the proteins may have been found if the subregions of the medial amygdala were investigated; the Golgi-Cox measures indicated that the direction of difference in dendritic morphology varied across subregions of the medial amygdala, thus gross dissections of the medial amygdala may obscure regional differences.

SS and CTL rats did not differ in GR protein expression in the medial amygdala and lateral septum. Changes in GR protein expression after repeated stressor exposure have been reported for other brain structures with an increase or decrease dependent on brain region and stressor-type. For example, daily 6 h restraint for 28 days reduced GR protein expression in the prefrontal cortex (Chiba et al., 2012) and daily 2 h immersion in a water bath for 28 days reduced cytosolic GR in the prefrontal cortex and increased nuclear GR in the hippocampus of adult rats (Mizoguchi et al., 2003). Further, 10 days of immobilization stress in adult rats increased GR protein expression in the prelimbic cortex and ACC compared with non-stressed controls, and these effects were dependent on the activation of the basolateral amygdala (Tripathi et al., 2018). The medial amygdala is responsive to stress exposures (Kellogg et al., 1998; Martinez et al., 1998) and is involved in the activation of the paraventricular nucleus (Dayas et al., 1999) and glucocorticoid release in response to stressor exposure (Solomon et al., 2009; reviewed in Herman et al., 2005). The SS procedure, although known to elevate glucocorticoid concentrations (McCormick et al., 2007; Hodges and McCormick, 2015), may not be sufficient to produce effects on GR densities. In adulthood, adolescent SS rats do not

differ from CTL rats in corticosterone release to a number of different stressors (McCormick et al., 2005; 2008; Mathews et al., 2008).

Conclusion

The interaction of all subregions of the medial amygdala are required to evaluate social odors and influence aggressive, reproductive, and other behaviour output (e.g. in hamsters, Maras and Petrulis, 2010a; 2010b; in mice, Cádiz-Moretti et al., 2016), and connections between the lateral septum and medial amygdala are essential for the recognition of familiar and novel individuals (reviewed in Maroun and Wagner, 2016). Thus, the structural differences observed in the present experiment in the medial amygdala and lateral septum may underlie the differences observed between SS and CTL rats in a variety of social behaviours (reviewed in Hodges and McCormick, 2018 chapter), but this possibility remains to be tested. In addition, we have yet to test if these changes in dendritic morphology are lasting, but we have effects of adolescent SS several weeks after the last stress exposure on social interaction (Green et al., 2013; Hodges et al., 2017; 2018) and sexual behaviour (McCormick et al., 2013) in adulthood, which may indicate that the structural changes induced by adolescent social stress in social brain regions are also lasting.

Chapter 6: General Discussion and Conclusion

My research findings demonstrate that SS in adolescent males alters the functioning and structure of brain regions involved in social novelty preference (medial amygdala, lateral septum, nucleus accumbens) and in the negative feedback of corticosterone release (arcuate nucleus), and these changes are evident within days of the termination of the SS procedure. My findings from chapters 2 and 4 provide evidence that adolescent SS changes how social brain regions processes social behaviour compared with age-matched CTL rats. My findings from chapters 3 and 5 provide evidence that adolescent SS increases oxytocin receptor binding and alters dendritic structure and markers of synaptic plasticity in the lateral septum, medial amygdala, and nucleus accumbens. In chapter 2, potentiated corticosterone was only found in adolescent SS rats and not in adults undergoing the same procedure or in adolescents that underwent repeated isolation and return to a familiar peer, which suggests that the effects on endocrine function are specific to adolescence and the quality of social experience. I also demonstrated altered social behaviour (reduced social interactions with unfamiliar peers, more time spent in social approach, impaired social recognition) soon after the termination of the adolescent SS procedure in chapters 3 and 4. All of my thesis results for endocrine, behavioural, and neural differences between adolescent SS and CTL rats are summarized in Table 6-1.

The effect of adolescent social instability on hypothalamic-pituitary-adrenal function

I replicated previous findings of reduced corticosterone in SS rats after the 16th isolation compared with rats undergoing isolation for the first time and added new

Table 6-1. Summary of all thesis results: adolescent social instability (SS) vs. non-stressed control (CTL) rats.

	ADOLESCENT SS RATS VS. CTL RATS		
	ENDOCRINE MEASURES	BEHAVIOURAL MEASURES	NEURAL MEASURES
CH 2	• ↑ **CORT** in response to being isolated and paired with an unfamiliar peer in the homecage for 1 h on PND 45		• ↓ **Zif268** in the arcuate nucleus and posterodorsal medial amygdala in response to being isolated and paired with an unfamiliar peer in the homecage for 1 h on PND 45
CH 3		• ↓ **time interacting with an unfamiliar peer** on PND 46 • ↑ **time in social approach with unfamiliar peers** on PND 46 and 47 • **No difference in time spent investigating familiar and novel peers** on PND 46 and 47, CTL rats spent more time investigating novel peers	• ↑ **oxytocin receptor binding density** in the dorsal lateral septum and nucleus accumbens on PND 46
CH 4		• ↓ **time interacting with an unfamiliar peer** on PND 46	• ↓ **Fos** in the paraventricular and arcuate nuclei in response to social interaction with an unfamiliar peer on PND 46 • **Fos** in the arcuate nucleus and dorsal lateral septum positively correlated with time spent in social interaction with an unfamiliar peer on PND 46, opposite pattern in CTL rats • **Fos** in the posterior medial amygdala negatively correlated with time spent in social interaction with an unfamiliar peer on PND 46, opposite pattern in CTL rats
CH 5			• ↑ **dendritic intersections, dendritic terminals, average branch order** in the anterodorsal medial amygdala on PND 46 • ↓ **dendritic intersections and total length of dendrite matter** in the anteroventral medial amygdala on PND 46 • ↓ **number of dendritic terminals** in the posterodorsal medial amygdala on PND 46 • ↓ **dendritic spines** in the dorsal lateral septum on PND 46 • ↓ **synaptophysin** in the medial amygdala on PND 46 • ↑ **calcium/calmodulin-dependent protein kinase II** in the lateral septum on PND 46

↑ indicates that SS > CTL, $p < 0.05$; ↓ indicates that SS < CTL, $p < 0.05$; PND = postnatal day.

knowledge by measuring corticosterone during the recovery period after isolation on the last day of the procedure and by measuring corticosterone in both adolescent and adult rats. My main finding of adolescent SS on endocrine function was that adolescent SS rats had higher corticosterone when paired with an unfamiliar cage partner for 1 h after isolation on the last day of the SS procedure (PND 45) than did adolescent rats that underwent 1 h isolation and were returned to an unfamiliar cagemate for the first time (described in chapter 2). This effect on corticosterone was age-dependent (only occurred in adolescence) and dependent on social context after isolation (only occurred in rats exposed to social instability). Habituation is more likely to occur after repeated exposures to the same stressor (homotypic stressor) such as daily isolation/confinement compared to exposures to a variety in stressors (heterotypic stressor). Thus, for adolescents, new cage partners may represent a heterotypic stressor, whereas the same experience may represent a homotypic stressor for adults. These findings demonstrate a vulnerability to quality of social experiences in adolescence.

The change in HPA function appears to be short-lived; past research that tested corticosterone release in adults that underwent adolescent SS and found no difference in corticosterone compared with controls (Mathews et al., 2008, McCormick et al., 2005; 2008). Nevertheless, changes in HPA function may be specific to social contexts, and corticosterone release in response to a social stressor or a social environment in adulthood after adolescent SS has yet to be tested. Adolescent SS might only affect HPA function in adulthood when the rats are placed in social situations.

I originally predicted that because adolescent SS rats had potentiated corticosterone on the last day of the procedure (one hour after being paired with an

unfamiliar peer for the 16th time) that they might have higher corticosterone compared with CTL rats in response to interacting with unfamiliar peers in a novel environment and this would in turn reduce their time spent in social interaction. However, I recently found that CTL rats have increased corticosterone in response to social interaction with an unfamiliar peer in a novel environment on PND 46 and SS rats do not (unpublished findings). These findings provide more evidence that high corticosterone is not the reason why adolescent SS rats have reduced social interaction with unfamiliar peers.

The sensitized corticosterone in SS rats in response to being paired with an unfamiliar cagemate for on PND 45 may be linked to the exposure to 1 h isolation beforehand, the context (homecage vs. novel environment), and/or the length of the social interaction (15 min vs. 1 h). High exposure to corticosterone may play a greater role in the effects of adolescent SS on the structure and function of social brain regions (described in chapters 2 to 5) and on adolescent social behaviour (described in chapters 3 and 4) than on the functioning of the HPA axis into adulthood.

The effect of adolescent social instability on behavioural measures

My findings provided novel information about age differences in social behaviour in the home cage after exposure to isolation and being returned to their home cage partner or an unfamiliar partner. Age differences in home cage behaviour were found, such that, in adolescents, isolation/confinement increased affiliative behaviour, irrespective of partner familiarity, whereas in adults, the same procedures led to decreased affiliative behaviour (described in chapter 2). I provided novel information about social behaviour in adolescence after SS that gave evidence of lasting behavioural effects. My finding of less time spent interacting with unfamiliar peers in adolescent SS rats compared with

CTL rats on PND 46 (described in chapters 3 and 4) combined with previous findings in social interaction in adults that underwent adolescent SS (Green et al., 2013) provides evidence that adolescent SS causes a lasting deficit in social interaction. In addition, a recent study from the laboratory of Dr. McCormick found that daily 1 h isolation and pairing with a familiar peer (their cagemate) after each isolation from PND 30 to 45 did not result in a reduced time spent interacting with an unfamiliar peer on PND 46 compared with CTL rats (Simone et al., 2018). Thus, the reduction in social interaction with unfamiliar peers after adolescent SS is unique to the experience of being repeatedly paired with unfamiliar peers.

Adolescent SS does not reduce the preference for social interaction (they displayed increased social approach and found social interactions with unfamiliar peers just as rewarding as CTL rats), but changing the quality of social experiences in adolescence may have resulted in SS rats treating all conspecifics as familiar in physical interactions. My findings in chapter 3 suggest that adolescent SS rats may have reduced social interactions with unfamiliar peers because they have a reduced preference for social novelty or because they are less able to differentiate familiar from novel peers. Adolescent SS rats displayed no difference in the time spent investigating familiar (homecage) partners versus unfamiliar partners in a novel environment, and they showed impaired social recognition of unfamiliar peers after intervals of 30 or 90 min. A second possibility could be that adolescent SS impairs behavioural sequencing and social interaction is reduced because SS rats are less able to predict and coordinate their behaviour with that of their partner. Adolescent SS did not affect generalized anxiety (adolescent SS predicted less anxiety on an elevated plus maze compared with CTL rats

on PND 46; see chapter 4), but a third possibility could be that adolescent SS increases social anxiety with unfamiliar peers, which may be expressed as a more generalized anxiety in adulthood (see McCormick et al., 2008; Green et al., 2013).

My findings in chapter 4 suggest that specific types of social interaction are reduced in adolescent SS rats compared with CTL rats; lower social interactions in adolescent SS rats are found in social behaviours other than anogenital sniffing (as shown in figure 4-8, and replicated in adolescent SS rats in experiment 1 of chapter 4, unpublished findings). My findings suggest that changing the quality of social experiences in adolescence can reduce specific types of social behaviours or impair the correct pattern of social behaviour that is normally displayed with unfamiliar peers. Adolescent SS rats may display social behaviours meant for familiar peers with unfamiliar peers. More tests are required to discover which patterns of social interaction with unfamiliar peers may be changed in adolescent SS rats.

In experiment 2 of chapter 4, low novelty-seeking in early-life was found to be a predictor of susceptibility to the effects of SS on social interactions with an unfamiliar peer in adolescent rats. CTL and SS rats that were higher in novelty-seeking on PND 30 did not differ in social interaction, but SS rats spent less time in social interaction with unfamiliar peers than did CTL rats if they were lower in novelty-seeking on PND 30. These data suggest that high novelty-seekers are resistant to some of the behavioural effects of altered social experience in adolescence, but whether or not high novelty seekers are resistant to the other behavioural changes or to the endocrine and neural changes described here remains to be investigated.

The effect of adolescent social instability on brain structure and function

My findings provided additional evidence to the adolescent development literature of brain regions that underlie the development of social behaviour in adolescent males. I also provided novel knowledge about functional and structural changes in the adolescent brain after changing the quality of social experiences. My findings suggest specific subregions of the medial amygdala, lateral septum, and the nucleus accumbens as targets in future studies aimed at relieving the deficits in social behaviour after disrupted social experiences in adolescence. Moreover, my findings in chapter 3 suggest that reduced social interaction, increased social approach, and impaired social recognition after SS may be linked to changing the oxytocin system in the lateral septum and nucleus accumbens. Nevertheless, the effects of activating or blocking the activation of specific social brain regions during social behaviours in adolescent SS rats remain to be tested. These studies would be required to investigate if the altered social behaviour in adolescent SS rats is actually dependent on the activation of the medial amygdala, lateral septum, and nucleus accumbens.

The medial amygdala plays major roles in behaviours that have been previously found to be changed in adulthood after adolescent SS (e.g., impaired sexual behaviour with females, McCormick et al., 2013; increased aggression in food competition with a conspecific, Cumming et al., 2014). Thus, changes in social brain regions soon after adolescent SS may endure and/or continue on an altered developmental trajectory into adulthood to affect future social behaviour, but this remains to be tested.

The medial amygdala also plays a role in olfactory processing, and changes in the structure and function of the medial amygdala may indirectly suggest changes in the olfactory system in adolescent SS rats. The piriform cortex (a brain region with major

involvement in smell perception), however, did not differ in Fos-ir in response to social interaction with an unfamiliar peer between adolescent SS and CTL rats (unpublished data). Nevertheless, adolescent SS rats did have lower doublecortin (a marker of immature neurons) in the subventricular zone compared with CTL rats (unpublished data), and newborn neurons from the subventricular zone migrate to the olfactory bulb. Thus, adolescent SS rats may have an impairement in olfactory processing that could be interfering with social recognition. Experiments that target olfactory memory have yet to be performed in adolescent SS rats and would elucidate if an impaired olfactory system underlies their reduced social interaction, increased social approach, and impaired social recognition.

My main focus was on social brain regions involved in social novelty preference and social interactions with unfamiliar peers, but my conclusions of impaired social coordination with unfamiliar peers in adolescent SS rats suggest there may be changes in the hippocampus and in the prefrontal cortex that were not detected in my experiments. As described in chapter 1, the hippocampus and prefrontal cortex are critical for performing appropriate sequencing of behaviour with peers. In chapter 2, I found an interaction of stress group and age in Zif268-ir in the hippocampus, such that adolescent SS rats had higher and adult SS rats had lower Zif268-ir in the hippocampus after being paired with an unfamiliar peer for the 16th time compared with CTL rats paired with an unfamiliar peer for the 1st time (as described in chapter 2). Past research found higher doublecortin (McCormick et al., 2012) and I found reduced glutamic acid decarboxylase isoform 67 (a marker of GABA neurons) in the hippocampus of adolescent SS rats compared with CTL rats (unpublished data). Future studies should investigate other

functional and structural changes in the hippocampus and prefrontal cortex after adolescent SS.

The arcuate nucleus was also indicated as a brain region altered by adolescent SS and in response to social interaction with unfamiliar peers; adolescent SS rats had reduced Zif268-ir in response to interacting with an unfamiliar peer in the home cage and reduced Fos-ir in response to interacting with an unfamiliar peer in a novel environment compared with CTL rats (see chapter 2 and 4). The arcuate nucleus acts to reduce corticosterone release in response to glucocorticoid receptor activation in the region (negative feedback of the hypothalamic-pituitary-adrenal axis) (Leon-Mercado et al., 2017). In chapter 2, reduced Zif268-ir in the arcuate nucleus may underlie the potentiated corticosterone in response to being paired with an unfamiliar cagemate for 1 h. Nevertheless, my findings in chapter 4 of lower Fos-ir in the paraventricular nucleus and arcuate nucleus in response to social interaction with an unfamiliar peer could also fit with my most recent finding that corticosterone increases in response to social interactions with an unfamiliar peer in a novel context in CTL rats but not in SS rats (unpublished findings). Future studies will be required to elucidate the significance of reduced neural activity in the arcuate nucleus of adolescent SS rats in different social contexts.

Other implications of my findings

A heightened development of several brain regions that also have high GR densities, prolonged exposure to corticosterone in response to several stressors, and a greater responsiveness to corticosterone during adolescence may combine to explain why the brain is particularly vulnerable to environmental changes during this period (reviewed

in Romeo, 2017). More studies are beginning to investigate the changes in the HPA axis, the brain, and in behaviour after stressor exposure in the adolescent period, but very few studies include age comparisons. Age comparisons using the same stressor are important to determine what effects of the stressor exposure are specific to each period of development. My experiments in chapter 2 add to the lack of adolescent and adult comparisons in the developmental literature. I found novel differences in how adolescents and adults respond to changing the quality of social experiences. In addition, most studies that perform adolescent and adult comparisons only investigate differences between prepubertal adolescents and adults. Changes in the brain continue to develop from prepuberty to postpuberty or peripuberty (the time around puberty) in adolescence (Cimino et al., 1995; Morris et al., 1980), and changes in brain are found between postpubertal or periburtal adolescents and adults (Brenhouse et al., 2008; Choi et al., 1997; Levitt and Moore, 1979; Trauth et al., 1999). My thesis findings add to the lack of information in the literature concerning behavioural and neural differences between postpubertal or peripubertal adolescent and adult males.

The reorganization of several brain regions occur in both males and females around the time of puberty and gonadal hormones around the time of puberty and after puberty are found to influence adolescent neural and behavioural development (reviewed in Juraska and Willing, 2017). My finding of an enhanced vulnerability to SS in adolescence might be dependent on implementing the SS procedure around the time that puberty normally takes places in rats. A disruption of gonadal hormone function may contribute to the altered social behaviour and the functional and structural changes I found in social brain regions between adolescent SS compared with CTL rats. Moreover,

gonadal hormones and their receptors play a major role in social recognition (reviewed in Gabor et al., 2012). Future studies should focus on investigating if there are changes in gonadal hormone receptors in social brain regions, such as the medial amygdala, or the changes in how gonadal hormones affect social brain regions after adolescent SS. These findings would further elucidate the role of the HPG function in the social interaction, social approach, and social recognition of adolescent SS rats.

My focus on the period of adolescence soon after the SS procedure compared to past studies that have investigated the effects of adolescent SS on the brain and on behaviour when tested in adulthood are important first steps toward determining the effects that are long-lasting after adolescent SS versus the effects that emerge with time versus the effects that subside. If the effects of adolescent stressor exposure emerge early and last into adulthood, then the brain may have been reorganized to have long-term consequences on neural and behavioural responses to certain stimuli. If the effects of adolescent stressor exposure emerge only in adulthood, then the developmental trajectory of the brain may have been reorganized to cause neural and behavioural differences to lay dormant until a later time or until coming in contact with a stimulus during a specific stage of developmental. If the effects of adolescent stressor exposure subside soon after the stress procedure, then the brain was able to adapt and recover from certain consequences of the stressor. The effects of adolescent SS on social interaction are lasting from adolescence to adulthood, but changes in other behaviours are not long-lasting. For example, whereas I found impaired social recognition in adolescent SS rats in chapter 3, adolescent SS rats tested in adulthood did not differ in social recognition from CTL rats (unpublished findings). Moreover, whereas adolescent SS male rats spent less time on the

open arm of the EPM compared with age-matched CTL rats when tested in adulthood (higher anxiety-like behaviour), adolescent SS rats did not differ (McCormick et al., 2008) or had lower anxiety-like behaviour after controlling for individual differences in early-life novelty-seeking (see chapter 4) on PND 45 and PND 46, respectively, compared with adolescent CTL rats. Many of the changes in behaviour and in the brain that are present in adolescent SS rats tested in adulthood have not been investigated in adolescence, and many of my findings in adolescent SS social function have not been tested in adulthood. It will be important to continue investigating the onset and duration of adolescent SS effects.

Exposure to repeated social defeat, another social stressor, also sensitizes corticosterone release in adolescents and not in adults (Watt et al., 2009; Covington and Miczek, 2005). However, social defeat in adulthood has long-lasting behavioural and physiological effects (reviewed in Buwalda et al., 2011) and lasting effects after SS have only been found when the stressor was implemented in adolescence and not in adulthood (e.g. Morrissey et al., 2011). Thus, my thesis findings not only provided additional evidence that the functioning of the HPA axis is more susceptible to social stressors in adolescence, but also helped to characterize the effects of stressor that has the strongest effects in adolescence. Few stressors have effects specific to the adolescent period, thus it will be important to continue characterizing how adolescent SS alters the brain to elucidate what brain regions and types of behaviour are particularly vulnerable to experiences during adolescent development.

The use of rodents has proven to be of value for both translational and back-translational research (Insel et al., 2013; reviewed in Gee and Casey, 2015), but there is

much debate over the extent to which behavioural assessments in rodents reflect psychiatric disorders in humans (Stanford, 2017). Nevertheless, the general principles of adolescent social development that emerge from my investigations in rats facilitate the generation of testable hypotheses of adolescent social development and may provide answers to the question of how stressors perturb such development in humans. In humans, adolescents have also been found to be vulnerable to the effects of social stressors (e.g., giving a speech for an audience or being rejected by peers) compared with other age-groups (Gunnar et al., 2009; Stroud et al., 2009). In addition, there is evidence that stressor exposure in adolescence alters the development of social brain regions (reviewed in Chaby et al., 2017), and changes in the development of social brain regions are thought to contribute the onset of anxiety disorders and differently depending on sex (reviewed in Nelson et al., 2005). Social awkwardness and/or social restrictiveness have been described as symptoms of many anxiety disorders, and resemble my findings of reduced social interaction in adolescent SS rats. Thus, my findings provide brain regions of interest that may be involved in the behavioural symptomology of anxiety disorders and that are specific to environmental disturbances during the critical developmental period of adolescence.

In humans, adolescent females have been found to be experience higher amounts of stressors and were more likely to have depressive symptomology in response to chronic stressor exposure compared with adolescent males (Hankin et al., 2007; Shih et al., 2006; reviewed in Chaby et al., 2017). Similarly, in rats, females had higher corticosterone release in response to stressor exposure and displayed more depressive-like behaviours than did males after chronic mixed stressors (isolation, restraint, and

social defeat) or repeated social defeat in adolescence (Bourke and Neigh, 2011; Weathington et al., 2012). It remains unknown if many of the same changes in endocrine, behavioural, and neural measures after adolescent SS that I have found in chapters 2-5 are generalizable across the sexes. Nevertheless, the laboratory of Dr. Cheryl McCormick have noted the effects of adolescent SS on other endpoints (e.g., vulnerability to drugs of abuse, McCormick, 2010) and is in the early stages of investigation of the effects of adolescent SS in females on social behaviour and social brain regions. The laboratory of Dr. Cheryl McCormick and I recently found that adolescent SS female rats spent less time interacting with unfamiliar peers compared with CTL females when tested 24 h after the stress procedure and in adulthood, in keeping with findings in adolescent SS males (unpublished findings).

Conclusion

Humans and rats undergo important shifts in social behaviour and in HPA function in adolescence (McCormick et al., 2017b; McCormick, Hodges, and Simone, 2015). My findings highlight the unique plasticity of the adolescent period; the same stressor exposures in adulthood typically have little lasting effect. There has been evidence of a greater sensitivity to social experiences in adolescence compared to adulthood (reviewed in Burke et al., 2017), but the mechanisms that could underlie the effects of disrupting social experiences in adolescence on late-adolescent social behaviour have rarely been investigated. My findings and past findings give evidence that the experience of social stressors in adolescence can have immediate and enduring consequences for a broad range of social functions in males. Moreover, whereas other studies investigate the neural underpinnings of behavioural changes in adolescents after

chronic exposure to stressors that are more severe, my findings highlight the sensitivity of endocrine, behavioural, and neural development to mild alterations in social experiences during the adolescent period. Changing the quality of social experiences in adolescence alters the development of the lateral septum, medial amygdala, nucleus accumbens, and arcuate nucleus, and the direction of the changes in neural development are dependent on brain region and subregion. My findings add to increasing number of human and non-human animal studies that are attempting to yield new insights into functional and structural changes in social brain regions that underlie the development of adolescent behaviour (reviewed in Blakemore, 2012).

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Appendix A

AUP 12-12-02

Age differences in neural responding to acute and chronic stressors in rats.

AUP Renewal Application Teaching and Research Page 3 of 4

3. Describe any changes you plan to make to your procedures in the coming year that will recognize the CCAC's 3R Principle of *Replacement* of animals with alternative where possible, *Reduction* of numbers of animals used and *Refinement* of procedures to protect animals from pain, distress or mortality.

4. Please attach a brief report on the adequacy of the endpoint for this protocol.

SECTION IV PRINCIPAL INVESTIGATOR, TECHNICIAN AND/OR STUDENT INVESTIGATOR DECLARATION

Signatures reflect commitment to abide by the following principles for the duration of the animal project proposed:

a. All animals entered into this project will be treated in a humane manner, in accordance with the principles and guidelines of the CCAC such as are stated in the "Guide to the Care and Use of Experimental Animals".
b. This renewal accurately describes all proposed animal use. It will be kept current and will be modified only after obtaining approval of the Animal Care Committee (ACC).
c. All procedures will be carried out by well-trained and experienced personnel who are competent in the use of recognized techniques.
d. All procedures, which may cause pain or discomfort have been technically improved and/or minimized in such a way so that the expected results will be obtained with a minimum of discomfort to the animals.
e. The protocol number assigned by the ACC to this submission will be used when ordering animals and these animals will be used only for the project described.

Principal Investigator's Name and Signature — Date (dd/mm/yyyy): Nov 11 2013

Alternate Investigator's Name and Signature — Date (dd/mm/yyyy): Nov 11 2013

Lead Technician / Student Investigator's Name and Signature (if applicable) — Date (dd/mm/yyyy): / /

Technician / Student's Name and Signature (if applicable) — Date (dd/mm/yyyy): / /

Technician / Student's Name and Signature (if applicable) — Date (dd/mm/yyyy):

As Departmental Chair, I agree to fund animal-related expenses incurred by this teaching protocol up to a maximum amount of $

Departmental Chair's Name and Signature (Teaching Renewals Only) — Date (dd/mm/yyyy): / /

ACC APPROVAL

Fiona Hunter — 5/12 2013

ACC Chair's Name and Signature — Date (dd/mm/yyyy)

Comments:

Appendix B

Brock

Animal Care Committee (ACC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
Clinical Veterinarians – Dr. Alistair Ker and Dr. Susan Warren
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: December 4, 2014

Dear Dr. McCormick, Mr. Hodges and Ms de Lima Marcolin

Your "Animal Use Protocol (AUP)" entitled:

Stress in adolescents and effects on social behavior and alcohol intake in male rats

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 14 - 11 - 01**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 272 long evans rats, male

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Fiona Hunter, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Appendix C

Brock

Animal Care Committee (ACC)
Chair –Wendy Ward, PhD 905.688.5550 ext 3024
Clinical Veterinarians – Dr. Alistair Ker and Dr. Susan Warren
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: Dec 7, 2015

Dear Dr. McCormick and Mr. Hodges,

Your "Animal Use Protocol (AUP)" entitled:

Stress during adolescence and the effects on social memory and social conditioned place preference in male rats

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 15 - 11 - 01**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 148 male Long Evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

W. Ward

Wendy Ward, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Appendix D

Brock

Animal Care Committee (ACC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
Clinical Veterinarians – Dr. Alistair Ker and Dr. Susan Warren
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: August 17, 2016

Dear Dr. McCormick,

Your "Animal Use Protocol (AUP)" entitled:

Structural and Functional differences in the Brains Regions Supporting Social Behaviour after Social Stress in Adolescence in Rats

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 16 – 07 – 02**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 48 male long evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Jean Hampson
for Acting Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING (PAM).
Arrangements for PAM must be made, for one or more components of your study, when animals are ordered.
N.B. Dr. Ker is here most Wednesday afternoons at 2:30.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.