Preconception parental predator stress induces brain and behavioral changes in offspring

By

© Sriya Bhattacharya, B.Sc., M.Sc., M.Sc.

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Department of Psychology, Faculty of Science

Memorial University of Newfoundland

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Abstract

Brain and behaviour are shaped by experience. Recent data suggest that experiences of the parents can be 'passed-on' to alter brain and behavior of future generations. Stress during the preconception period is a new topic of interest, as it involves both prospective parents. The current study examined how parental preconception predator stress impacts the development of offspring brain and behaviour throughout its lifespan. Male and female mice (F0) were exposed to a live rat (rat exposure test) or control condition (no live rat exposure) for five minutes. Two days later, all mice underwent the elevated plus maze to assess anxiety-like behavior. Eight days later (a total of 10 days post predator exposure), stressed males were mated to stressed females and control males were mated to control females. Behavior of the offspring (F1) was assessed during adolescence and again in adulthood (following a mild stressor). F0 and F1 brains were examined for stress-induced changes in glucocorticoid receptors (GR), FK506 binding protein 5 (FKBP5), doublecortin, and c-FOS. Following a mild stressor, preconception stressed offspring show increased anxiety-like behavior, hyperarousal, and deficits in spatial memory. These mice also show elevated plasma corticosterone and altered GR, FKBP5 and c-FOS expression in the hippocampus. Furthermore, following a mild stressor, offspring (F2 generation) with at least one set of grandparents who were predator stressed showed increased anxiety-like behavior, enhanced hyperarousal, and deficits in spatial memory compared to offspring whose both sets of grandparents were controls. Despite the high incidence and potentially tragic outcome, there is little research on neural mechanisms underlying stress-induced disorders such as posttraumatic stress disorder (PTSD). These results may contribute to

identifying *at-risk* individuals, as well as point to potential novel therapies for these devastating disorders.

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List of Abbreviations

PTSD	Post-Traumatic Stress Disorder
BEPMRF=	baseline EPM ratio frequency
BLDENT=	baseline LDB light side entries
BLDTIM=	baseline LDB light side time
BOFTIM=	baseline OF centre time
BOFDIS=	baseline OF distance travelled
BOFVEL=	baseline OF velocity
BSI=	baseline social interaction ratio time
BFSW=	baseline force swim immobile time
BPSAVG=	baseline mean peak startle amplitude) and adulthood (after 2min RET)
SEPMRT=	after 2min RET EPM ratio time
SEPMRF=	after 2min RET EPM ratio frequency
SLDENT=	after 2min RET LDB light side entries
SLDTIM=	after 2min RET LDB light side time
SOFTIM=	after 2min RET OF centre time
SOFDIS=	after 2min RET OF distance travelled
SOFVEL=	after 2min RET OF velocity
SSI=	after 2min RET social interaction ratio time
SFSW=	after 2min RET force swim immobile time
SPSAVG=	after 2min RET mean peak startle amplitude)
Adcyap1r1=	adenylate cyclase-activating polypeptide Type I receptor gene
BARX =	BARX homeobox 1
Bdnf=	brain-derived neurotrophic factor
Comt=	catechol-O-methyltransferase gene
CDV=	community and domestic violence
Corin=	corin, serine peptidase genes
Cftr=	cystic fibrosis transmembrane conductance regulator
Nr3c1=	nuclear receptor subfamily 3 group C member 1 gene
Nr3c2 =	nuclear receptor subfamily 3 group C member 2 gene
Smyd3=	SET and MYND domain containing 3
Slc6a4=	solute carrier family 6 member 4
Aims =	amplification of inter-methylated sites
Avp =	arginine vasopressin
Dlgap2=	disks large-associated protein 2
Fkbp5=	FK506 binding protein 5
GR =	glucocorticoid receptor
LH=	left hemisphere
RH=	right hemisphere
BLA=	basolateral amygdala
CeA=	central amygdala
mPFC=	medial prefrontal cortex
DEN=	dentate gyrus

Chapter 1: Introduction

Individuals exposed to highly traumatic experiences (*e.g.* physical assault, rape, natural disaster, kidnapping, combat, etc.) can develop Post-Traumatic Stress Disorder (PTSD). Though it has been called many different things, PTSD has existed since the times of ancient Greece. During the American Civil War, it was referred to as "soldier's irritable heart"(Trimble M.R., 1982), in the First World War, it was referred to as "shell shock"(Crocq and Crocq, 2000; Myers, 1916) and in the Second World War, it was referred to as "war neurosis''(Ellis, 1985). More recently, during the Vietnam War, it was coined "combat stress reaction"(Solomon et al., 1989) and finally, in 1980, the term PTSD was established in the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM) in 1980.

This introduction will begin with an examination of the criteria and symptoms associated with PTSD. Several preclinical paradigms used to model PTSD will be discussed. I will conclude with an examination of relevant behavioral, physiological, and molecular changes that occur in rodents after traumatic stress, and the recent literature that suggests these changes can persist into subsequent generations. I will conclude with a justification for the hypothesis that preconception predator stress results in lasting changes in brain and behavior in first and second generation offspring.

1.1 Diagnostic criteria for PTSD

The DSM-V classifies PTSD as a stress and trauma-related disorder, defined by a set of symptom clusters that appear for at least 30 days following severe trauma. Symptoms include *re-experiencing* of the trauma (unwanted intrusion of the memory in the form of thoughts, nightmares, and flashbacks, intense upset evoked by conditioned cues or environmental stimuli), *avoidance* of

cues related to the traumatic event (situations, places, activities), and *hyperarousal* (increased startle response, irritability, sleep problems). Negative disturbances in mood and cognition (dissociative amnesia, negative affect, and anhedonia) are also recognized as core symptoms (American Psychiatric Association, 2013). Not only can exposure to trauma affect the psychological status of an individual, but it can also have deleterious consequences on the individual's social, professional, and family life. As a result, the fallout from trauma carries a large burden for individuals and society (Kessler et al., 1995).

1.2 Prevalence of PTSD

Epidemiological studies have found that between 37 and 92% of people report past exposure to at least one traumatic event (Atwoli et al., 2015; Kessler et al., 2017). Of those, 25-35% go on to develop PTSD (Yehuda, 2001; Kessler et al., 2005). These figures contribute to the lifetime prevalence of the disorder which is currently estimated at 6.1% in the United States and 9.2% in Canada (Goldstein et al., 2016; Van Ameringen et al., 2008). PTSD is therefore one of the most common psychiatric disorders as most anxiety and affective-related disorders (e.g. generalized anxiety disorder, agoraphobia, panic attacks, obsessive compulsive disorder, major depressive disorder, bipolar disorder) have relatively lower lifetime prevalence, ranging from 0.7 to 5% (Statistics Canada, 2015). Among "highly exposed" groups (e.g. low-income, low social support, urban populations), lifetime rates of PTSD can be as high as 40% (Breslau et al., 1998). Moreover, in the wake of large-scale disastrous events, incidence and prevalence of PTSD can be shifted sharply upward, like those affected in the New York City area following the 9/11/2001 terrorist attacks or the Mississippi Delta following Hurricane Katrina in 2005 (Galea et al., 2002, 2008).

Trauma event type confers differences in risk for PTSD. Direct interpersonal victimization related to rape, physical abuse, and kidnapping have the highest conditional risk, while other trauma event categories such as automobile accident or natural disaster confer a lower risk (Benjet et al., 2016; Kessler et al., 2017; Kilpatrick, 2013; Kilpatrick et al., 2013). Women are twice as likely to develop PTSD as men (Kessler et al., 1995). In addition, a dose-response relationship exists between symptom severity and frequency of trauma experience: the more traumatic events a person experiences, the greater the intensity of their PTSD symptoms (Binder et al., 2008). Finally, younger adults and adolescents appear more susceptible to PTSD than older adults. In a national survey, Kilpatrick and Saunders (1997) found that 8.1% of adolescents met criteria for PTSD during their lifetime and 4.9% currently met criteria. The survey also showed that the rates of lifetime and current PTSD increased significantly with age; by age 17, the rates of lifetime and current PTSD were 13.1% and 8.4%, respectively.

1.3 Comorbidities

Nearly 80% of women and 90% of men with a lifetime history of PTSD develop at least one other disorder (Kessler et al., 1995). For instance, depression accompanies PTSD almost half of the time (Davidson & Froa, 1993). Substance abuse develops frequently among men, whereas women are more prone to psychologically determined physical complaints (Najavits et al., 1997; Testa et al., 2012). Anxiety disorders (i.e. generalized affective disorder, panic disorder, simple phobia, social phobia, agoraphobia) are common among both sexes (McLean et al., 2011). Co-morbidity with PTSD can be due to the overlap in symptom criteria; for example, criteria C and D

PTSD symptoms (e.g., irritability, hypervigilance, exaggerated startle) overlap with symptoms that characterize generalized anxiety disorder (Bandoli et al., 2017; Flory and Yehuda, 2015).

Children with PTSD also have fairly high rates of psychiatric co-morbidity such as major depression, substance abuse, panic disorder, attention-deficit hyperactivity disorder (ADHD), oppositional defiant disorder, conduct disorder (McClellan and Stock, 2013). Depression and anxiety disorders (e.g. agoraphobia, separation anxiety, and generalized anxiety disorder) are quite common in children who have been traumatized (Beesdo et al., 2009). In addition, children may respond to trauma by displaying behavioural problems. Disruptive behaviour disorders, like Conduct Disorder and Oppositional-Defiant Disorder, are not uncommon among children with PTSD, and are most often associated with physical abuse, exposure to violence, or coercive family dynamics (Cohen et al., 2009, 2018; Reebye et al., 2000; Thabet et al., 2013). Young children often present anxiety-related responses manifested as hyperactivity, distractibility, and impulsivity which are hallmarks of ADHD (Krull, 2019; Riccio et al., 2005; Spencer et al., 2007; Wilens and Spencer, 2010). Some authors have even suggested that ADHD in traumatized children is misdiagnosed PTSD (Antshel et al., 2013).

Although many individuals who present with the symptoms of PTSD actively seek out psychotherapy, psychopharmacology, or both (Van Ameringen et al., 2008) only about 60% are responsive to interventions (Önder et al., 2006), with only about a third of patients achieving full remission (Berger et al., 2009). The disparity in those achieving full remission, coupled with epidemiological studies estimating the lifetime prevalence of PTSD at 5-10% for the general population (Van Ameringen et al., 2008), suggests a desperate need for understanding the mechanisms that contribute to the vulnerability, development, and maintenance of PTSD

ultimately leading to the identification of novel, more effective, candidate treatments (Hauger et al., 2012; Reul and Nutt, 2008; Steckler and Risbrough, 2012).

1.4 Cognitive Deficits in PTSD

Memory and other cognitive processes gradually diminish as we grow older, but research in the last several decades shows that those who experience persistent or high levels of stress are especially vulnerable (Lavretsky and Newhouse, 2012; Salleh, 2008; Schneiderman et al., 2005). The most recent DSM (5th ed.; DSM-5) added "negative alterations in cognitions and mood" as a symptom cluster in the diagnostic criteria for PTSD. The inclusion was based on studies indicating that individuals with PTSD have deficits in some forms of learning and memory (Blechert et al., 2007; Brown et al., 2014; Careaga et al., 2016; Levy-Gigi et al., 2014). For example, combat veterans with PTSD tend to retrieve autobiographical memories and imagine future events with less episodic specificity than veterans without PTSD (Brown et al., 2013, 2014, 2016). Additionally, Levy-Gigi et al. (2012) found that, while individuals with PTSD were not impaired in the learning of a stimulus-outcome association in an acquired equivalence task, they showed deficits when required to generalize previous knowledge. Furthermore, patients with PTSD have impaired extinction of fear conditioned associations (Blechert et al., 2007; Milad et al., 2009; Orr et al., 2000; Careaga et al., 2016). Patients with PTSD from Vietnam combat and childhood abuse showed deficits on neuropsychological measures that have been validated as probes of hippocampal function (Orcutt et al., 2003; Regan et al., 2007). In addition, magnetic resonance imaging (MRI) studies show a reduction in volume of the hippocampus in both combat veterans and victims of childhood abuse (Bremner, 1999; Orcutt et al., 2003; Regan et al., 2007). In combat veterans, the hippocampal volume reduction was correlated with deficits in verbal memory on

neuropsychological testing. Indeed, reduced hippocampal volume appears to be a risk factor for PTSD as indicated by monozygotic twin studies (Careaga et al., 2016; Gilbertson et al., 2002; Pitman et al., 2006). These studies introduce the possibility that traumatic stressors can have long-term effects on the structure and function of the brain (Bremner, 1999; Koenigs et al., 2008).

1.5 Stress hormones and PTSD

Stress hormones appear to play a key role in the development of, and vulnerability to PTSD. Cortisol (principal human stress hormone; corticosterone is the main glucocorticoid in most other species) coordinates and prepares the body to respond to environmental demands and stressors to achieve systems homeostasis. Selye (1956) was the first to demonstrate a common pathway of physiological activity in response to stress. This pathway was later dubbed the hypothalamic-pituitary-adrenal (HPA) axis. During a stressful event, cells of the paraventricular nucleus (PVN) of the hypothalamus respond by secreting corticotropin-releasing hormone (CRH) into capillaries in the median eminence of the hypothalamus. CRH released into this portal capillary system stimulates neurosecretory cells in the anterior pituitary which in turn release adrenocorticotropin hormone (ACTH). From there, ACTH travels through the blood stream and acts on the cortex of the adrenal gland where it stimulates secretory cells to release glucocorticoids, particularly cortisol, into the general circulatory system. Cortisol prepares the body to adapt to current stressors by suppressing immune system activity, counteracting insulin, supporting increased glucose availability (e.g. gluconeogenesis), and regulating water retention and electrolytic balance in the kidneys (Dunlop and Wong, 2019; Khani and Tayek, 2001). Cortisol also decreases the activity of PVN and anterior pituitary, negatively influencing its own release. This regulator mechanism limits the stress response helping return the body to homeostasis and is

often referred to as the negative feedback loop (Sapolsky, 1985). As the term stress is ambiguous and not well defined, it is important to note that various daily events that many people might interpret as innocuous or pleasurable, such as exercise or sex, evoke this canonical HPA axis stress response (Finke et al., 2018; Jokinen et al., 2017; Stranahan et al., 2008). Nevertheless, unlike sex and exercise, the stressors that satisfy Criteria A of PTSD in the DSM-5, while quite varied, are traumatic, sudden, unexpected, involuntary, and uncontrollable (American Psychiatric Association, 2013; Kessler et al., 2017; Kilpatrick, 2013; Kilpatrick et al., 2013).

Dysregulated or aberrant HPA axis activity, especially in terms of cortisol, is often postulated as part of the etiology and pathophysiology of PTSD. The direction of this altered neuroendocrine activity that might confer a susceptibility to developing and maintaining PTSD, however, has yielded inconsistent findings (Zoladz and Diamond, 2013). Mason and colleagues (1986) were the first to report lower mean 24-hour basal urinary cortisol levels from inpatient PTSD combat veterans (Mason et al., 1986). Although this study only compared different psychiatric groups, subsequent studies have shown similar low cortisol levels relative to healthy controls (Kanter et al., 2001; Rohleder et al., 2004; Yehuda et al., 1993). Conversely, other studies have reported increased basal cortisol levels (Carrion et al., 2002; Lemieux and Coe, 1995; Young and Breslau, 2004b, 2004a) or no difference in individuals with PTSD compared to healthy controls (Duval et al., 2004; Otte et al., 2007; Yehuda et al., 2004). Discrepancies in reported cortisol levels concern issues around, but not limited to, sex, length of combat exposure in veterans, differences in trauma type, childhood trauma (Carpenter et al., 2011), current PTSD status, differences in naturally fluctuating levels of cortisol throughout the day (at awakening or peak, nadir, fastening state) (Ljubijankić et al., 2008), comorbidity with other psychiatric disorders such as major depressive disorder and substance use disorders, plasma vs. urinary vs. cerebral spinal

fluid cortisol levels, and lack of statistical power (Dunlap et al., 2019; Zoladz et al., 2013) . Given such inconsistencies, these findings suggest that basal cortisol abnormalities may only represent a subset of the manifestation of PTSD. As such, basal cortisol is a rather dubious biomarker for PTSD. Zoladz and Diamond (2013) suggest investigating a more comprehensive role for cortisol in multiple physiological processes.

Similar to the heterogenous findings of baseline cortisol activity, mixed evidence linking altered cortisol levels in the immediate aftermath of an acute traumatic experience to PTSD development have been reported. Specifically, one study found a negative correlation between diminished cortisol levels peri-trauma and the development of PTSD (Ehring et al., 2008) , whereas other studies have found no correlation (Heinrichs et al., 2005; Resnick et al., 1995), or a positive correlation, especially in relation to childhood trauma and the development of PTSD months later (Delahanty et al., 2005; Lipschitz et al., 2003; Pfeffer et al., 2007). Here it is probable that the ontogeny of HPA axis during childhood contributes differently to the development of PTSD compared to maladaptive adulthood responses. In adults, administration of hydrocortisone to patients undergoing very invasive surgeries show fewer PTSD symptoms in follow-up sessions (Schelling et al., 2004, 2006), providing credence for perturbed cortisol functioning in adults' response to acute traumatic stress that go on to develop PTSD. Moreover, studies investigating cortisol levels in the dexamethasone suppression test and trier social stress test of individuals with PTSD have shown rather consistently robust inhibition of cortisol in response to synthetic glucocorticoids or psychosocial stress (Wichmann et al., 2017; Yehuda, 2002; Yehuda et al., 2004; Zoladz and Diamond, 2013).

1.6 Stress effects across generations in humans

As noted above, traumatic stress can have deleterious effects on an individual's mental health. However, recent data suggests that the harmful effects of traumatic stress during one's lifetime can propagate into future generations (Blaze and Roth, 2015), perhaps leading to an increased vulnerability to the development of psychopathology. There is a variety of research showing intergenerational effects of stress. Briefly, intergenerational transmission involves direct stress exposure to the parental (F0) generation and subsequent offspring generation (F1) by means of either the developing germ cell or fetus. If the stress exposure occurred while the fetus (F1) was developing in utero, intergenerational transmission occurs in the F2 generation. This is in contrast to transgenerational whereby the germ cells have not been exposed to stress.

In a landmark study by Solomon and colleagues in 1988, Israeli veterans of the 1982 Lebanon War who were offspring of Holocaust survivors were more likely to develop PTSD than other Israeli soldiers following their military experiences who did not have parents interned in Nazi concentration camps (Solomon et al., 1988a, 1988b, 1989). Likewise, PTSD in former Israeli soldiers captured and held as prisoners of war during the 1973 Yom Kippur War positively correlated with offspring PTSD (Zerach et al., 2017). Furthermore, in a study of Cambodian refugees living in the United States, parental PTSD predicted higher rates of child PTSD for older children. A gradient effect was found such that when neither parent had PTSD, 12.9% of the children had PTSD; when one parent had PTSD, 23.3% of the children had PTSD; and when both parents had PTSD, the percentage of children with PTSD jumped to 41.2% (SACK et al., 1995). In a more recent report, offspring of parents suffering from PTSD that survived the 1994 Tutsi genocide in Rwanda showed greater secondary traumatization symptoms and were less resilient (Shrira, 2019; Shrira et al., 2019). In addition to intergenerational effects, transgenerational effects

of stress have also been found. For example, offspring of Israeli Holocaust survivors had a higher incidence of PTSD, mood disorders, anxiety disorders, and substance abuse disorders three generations downstream (Solomon et al., 1988b; Zerach et al., 2017). Overall, these data suggest that parental traumatic stress make offspring more vulnerable to mental illness.

Interestingly, alterations in stress hormone release have been observed in the offspring of individuals with PTSD. Yehuda and colleagues (2000) found lower serum cortisol levels and greater cortisol suppression in the offspring of Holocaust survivor's suffering from PTSD compared to healthy subjects who were not offspring of Holocaust survivors (Yehuda et al., 2000). Further, pregnant women who developed PTSD in response to the September 11, 2001, World Trade Center collapse had infants with lower cortisol levels compared with infants of mothers who did not develop PTSD (Yehuda et al., 2005). Similarly, Yahyavi et al., (2015) found that offspring of Iranian combat veterans from the Iran-Iraq war with a current or past history of PTSD had significantly lower serum cortisol levels than offspring of combat veterans without a history of PTSD (Yahyavi et al., 2015). Moreover, beyond diurnal changes in stress hormones, youth offspring of mothers with PTSD also show a blunted, maladaptive, cortisol saliva response to an acute laboratory stressor (Danielson et al., 2015), a response seen likewise in female PTSD patients (Pierrehumbert et al., 2009; Wichmann et al., 2017; Zaba et al., 2015).

While prenatal stress appears to produce brain and behaviour changes in offspring (as described above), there is little research investigating the effects of stress prior to conception on future generations. As adults, the children of Holocaust survivors had higher rates of mood, anxiety, and substance abuse disorders compared to Jewish controls (Yehuda et al., 2008). While lifetime rates of PTSD did not differ between groups, there was an association between offspring with PTSD and maternal PTSD. The effect of maternal PTSD was present regardless of paternal

PTSD, but the effect was strongest when both parents had PTSD (Yehuda et al., 2008). These findings provide support for the possibility of a predisposition for PTSD that is influenced by the experiences of previous generations.

Both ethical limitations and the logistical constraints associated with human research limit full understanding of the mechanisms underlying these generational effects of stress. For this reason, recent efforts have been made to assess generational effects of stress in rodents.

1.7 Animal models of PTSD

Animals have been used to study human disease over the last 100 years, increasing our knowledge on the causes, susceptibility, prevalence, and treatments of many life-threatening disorders. Animal models provide an important avenue for studying the pathophysiology of PTSD because they overcome ethical limitations associated with human research. Furthermore animal models permit: 1) exposure to a severe stressor in a controlled fashion; 2) study of the effect of stress on affect as it develops; and 3) study of pharmacological and other treatments which may be difficult to test in humans, but can be easily evaluated in animals. Although it is not possible to model all aspects of PTSD in animals, several experimental paradigms have been developed which demonstrate PTSD-like symptoms. Described below are the most common animal models of PTSD.

1.7.1 Immobilization/restraint stress

Both restraint and immobilization stress involve placing a rodent in an enclosed chamber thereby reducing or preventing movement acutely (single session) or chronically (several sessions). Total immobilization is considered the most severe of the restraint methods. Indeed, two hours of complete immobilization increases 1) anxiety-like behaviour in the elevated plus maze

and open-field tests (Andero et al., 2013; Mitra et al., 2005), 2) compulsive-like behaviour in the marble-burying test (Kedia and Chattarji, 2014), 3) increased fear learning (Andero et al., 2013), 4) REM sleep (Meerlo and Turek, 2001) yet reduces declarative memory performance in the water maze task (Ozbaki et al., 2016) . Moreover, restraint stress (30-120 min) results in autonomic responses including arterial pressure (MAP) and heart rate (HR) increases, skeletal muscle vasodilatation and cutaneous vasoconstriction (Crestani, 2016; Lopes-Azevedo et al., 2020; Reis et al., 2011; Scopinho et al., 2013). Furthermore, 24-hour-continuous restraint stress induces long-term depressive-like phenotypes in mice (Chu et al., 2016). During chronic restraint stress, mice are generally subjected to complete immobilization (2-6 hr/d, for approximately 10 consecutive days) in rodent immobilization bags without access to either food or water. This type of stress leads to increased anxiety-like behavior (Vyas et al., 2002) and changes in neuronal morphology within brain regions that mediate fear (Miller and McEwen, 2006). Importantly, morphological and numerical alterations in dendritic spines changes are mostly manifested after chronic immobilization stress-exposure (i.e., 10 days) (Andero et al., 2013; Kedia and Chattarji, 2014; Mitra et al., 2005). Finally, restraint stress induces a long-term desensitization of the hypothalamic-pituitary-adrenal (HPA) axis activity. This causes excess cortisol release and leads to increased activity of the pro-inflammatory immune mediators and disturbances in neurotransmitter transmission (Armario et al., 2004, 2008, Belda et al., 2008, 2015).

Restraint/immobilization stress paradigms are commonly used for several reasons. First, the duration of the stressor can easily be controlled and size of the chamber regulated, and hence, variability across studies can be reduced. Second, it is convenient for taking blood samples from the tail for hormonal measurements (especially in pregnant dams). Finally, both males and females respond to this type of stressor.

1.7.2 Single prolonged stress

Single prolonged stress (SPS) paradigms typically involve three stressors: 2-hour restraint-immobilization stress followed by forced swimming for 20 minutes, and ending with exposure to diethyl ether until loss of consciousness (Eagle et al., 2013; George et al., 2013; Han et al., 2014; Zhe et al., 2008). These stressors induce psychological, physiological, and endocrine responses. Rats exposed to this SPS procedure exhibit increased anxiety-like behaviour in the elevated plus maze, enhanced fear acquisition, and reduced fear extinction learning (Imanaka et al., 2006; Knox et al., 2012; Takahashi et al., 2006; Wang et al., 2008; Yamamoto et al., 2009). Similar effects have been observed in mice (Perrine et al., 2016). Morphological changes following SPS models include apoptotic volume loss in the hippocampus and the dorsal raphe nucleus (Han et al., 2014; Liu et al., 2012). One major limitation of the SPS model is that despite PTSD being more likely in women than men (Kessler et al., 2005), SPS-induced deficits in extinction are only seen in male rats (Keller et al., 2015; Lisieski et al., 2018; Souza et al., 2017). Despite this, the SPS model stands out as a well-characterized, translationally controllable paradigm to study of the neurobiology of traumatic stress-induced adaptations in brain and behaviour.

1.7.3 Unpredictable variable stress

Unpredictable variable stress (UVS) models expose animals to a variety of chronic stressors, such as restraint stress, social isolation, over-crowding, tank change, cold stress, chasing, heat stress, dorsal body exposure, predator stress and alarm pheromone stress over many days (commonly, 2 stressors/day for 1-8 weeks). Exposure to UVS induces anxiety- and depressive-like behaviors (Barnum et al., 2012; Biala et al., 2018; Chakravarty et al., 2013; Monteiro et al., 2015;

Sequeira-Cordero et al., 2019), reduces exploratory behavior (Kazlauckas et al., 2011; Sequeira-Cordero et al., 2019; Sturman et al., 2018), and causes subtle stress-related changes in the thymus and the spleen (Monteiro et al., 2015). In addition, chronic UVS impairs recognition memory in the novel object recognition task (Kazlauckas et al., 2011). Furthermore, chronic UVS impairs circadian rhythmicity, sociability, and contextual discrimination performance in rodents (Bondi et al., 2008; Monteiro et al., 2015). These stress-induced behavioral impairments are accompanied with reduced expression levels of brain derived neurotrophic factor (BDNF) in the different brain regions (i.e. hippocampus and prefrontal cortex) (Cieślik et al., 2011; Larsen et al., 2010; Molteni et al., 2016) and enhanced plasma corticosterone levels (Biala et al., 2018; Cox et al., 2011; Gaelle et al., 2019; Matuszewich et al., 2014; Monteiro et al., 2015). There are advantages to the UVS model. For example, UVS paradigms have high etiological validity for repeated, uncontrollable and unpredictable traumatic events, such as deployment stress. UVS paradigms also produces long-lasting behavioural and physiological alterations similar to those observed in PTSD patients. Little information, however is known about sex differences and due to the use of several stressors, it is not clear which variations reliably induce long-term changes in brain and behavior.

1.7.4 Social defeat stress

Social defeat stress (SDS) typically involves an exposure (or chronic exposure) of an animal to a socially and physically dominant resident animal (Golden et al., 2011; Hammels et al., 2015). SDS produces long-lasting anxiety- and depressive-like behaviors (Berton et al., 2006; Campos et al., 2013a; Der-Avakian et al., 2014; Ieraci et al., 2016; Pulliam et al., 2010; Wei et al., 2018), increases amygdala activity (Abe et al., 2019; Fujii et al., 2019; Narayanan et al., 2011; Vasconcelos et al., 2015; Yang et al., 2016), and suppresses negative feedback of HPA axis activity 1 to 3 weeks after stress (Herman et al., 2016; Stranahan et al., 2008). Moreover, mice experiencing

social defeat stress display increased rapid eye movement in sleep (Fujii et al., 2019; Henderson et al., 2017; Wells et al., 2017). Unlike males, females do not show aggressive behaviors and hence, SDS is not used in females. This limits the utility of this model.

1.7.5 Fear conditioning

The fear conditioning paradigm is most often used to model the intrusive fear memories associated with PTSD. Fear conditioning occurs when a neutral stimulus (i.e., tone or context) elicits defensive behaviours (i.e., freezing) if the neutral stimulus was previously paired with an aversive stimulus (i.e., shock; (LeDoux, 2000)). Though the freezing response is a species-specific defensive behaviour, the percentage of freezing behaviour depends on the strength of the aversive stimulus (i.e. the voltage of the shock), the number of conditioned stimulus-unconditioned stimulus (CS-US) pairings, and the degree of learning achieved by the animal. Several rodent studies reveal progressive development and persistence (3–5 weeks following single or multiple shocks, with or without subsequent reminders) of PTSD-like symptoms, including social withdrawal or avoidance, defensive behaviour, hypervigilance, sleep disturbances, and generalization of fear (Cullen et al., 2015; Louvart et al., 2006; Mikics et al., 2008; Philbert et al., 2012; Pryce et al., 1999; Siegmund and Wotjak, 2007b). Note, these studies applied somewhat higher amperage (0.8–3.0 mA) compared to most conditioned fear paradigms that focus on fear learning (~0.5–1.5 mA). In addition, some studies report that delivery of numerous (2–10), high intensity (~1.5 mA) footshocks help to increase the levels of ACTH and CORT hormones (Cordero and Sandi, 1998; Pugh et al., 1997). Significant advantages of fear conditioning paradigm are (1) reliable delivery and precise control of the number and amperage of the shocks, length of the session, and inter-shock intervals; (2) well-defined and reproducible context and environmental cues by using

standard boxes with lights and speakers; (3) adjustable changes in contextual modalities, cues, and reminders; and (4) compared to restraint stress, there is less habituation (Siegmund and Wotjak, 2007b, 2007a). One of the major concerns with shock-induced models is that compared to human subjects and other animal trauma models, there is relatively little individual variability in terms of sensitivity and resiliency; the vast majority of subjects exposed to electric shock will go on to display behavioural consequences to stress (Bali and Jaggi, 2015) . Indeed, fear conditioning produces similar behaviours (freezing) across all animals and individual differences are not usually reported (individual differences in freezing to the conditioned cue or context are rarely noted). This is in contrast with human PTSD, where only a proportion of trauma-exposed individuals develop the disorder (Kessler et al., 2005; Lewis et al., 2019; Santiago et al., 2013; Sareen, 2016).

1.8 Predator stress as an animal model of PTSD

While there is no one ideal animal model of PTSD that recapitulates all symptoms of the disorder, predator stress paradigms are arguably one of the most comprehensive approaches used by researchers (Deslauriers et al., 2018). Predator stress typically involves acute or chronic exposure of a prey species (mouse or rat) to a predator or predator cue (typically a cat, rat, or ferret). This "traumatic" event is more ecologically valid than other trauma-induced models (i.e. fear conditioning) as it presents the animal with an event (exposure to a predator or predator cues) that they could possibly encounter in nature (Adamec et al., 1998; Cohen and Zohar, 2004; Muñoz-Abellán et al., 2008). Predator stress is a life-threatening experience which precipitates changes in brain and behaviour minutes to days post-exposure, and more recently, in subsequent generations (Ahmadzadeh et al., 2011; St-Cyr et al., 2017, 2018; St-Cyr and McGowan, 2015; Thayer et al., 2018). A considerable volume of literature exists demonstrating that predator stress causes

anxiety-like behaviour, avoidance of trauma-related cues, hyperarousal, and impaired spatial memories (Adamec, 1997; Adamec et al., 1998, 2005a; Adamec and Shallow, 1993; Blundell et al., 2005; Cohen et al., 2009, 2018; Diamond et al., 2006; Fifield et al., 2013, 2015; Goswami et al., 2013; Lau et al., 2016; Lebow et al., 2012; Schöner et al., 2017; Zoladz et al., 2015). Similar to PTSD, female mice appear more susceptible to predator stress (Adamec et al., 2006) and common pharmacological treatments of PTSD (e.g. sertraline) are efficacious in reducing anxiety-like behaviours and hyperarousal following predator stress (Adamec et al., 2004; Matar et al., 2006). In addition, predator stress-induced anxiety-like behaviors are long-lasting (up to four weeks post stress (Adamec and Shallow, 1993; Burgado et al., 2014; Chen et al., 2014; Miura et al., 2011) which further supports its usefulness as a model of PTSD. Compared to humans, rodents live short and accelerated lives. Rats live up to 3 years compared to humans' approximately 80 years (developed countries average, UN World Population Prospects). Generally speaking, rats live approximately 27 times faster, meaning that one rat day is around 27 human days (Agoston, 2017; Andreollo et al., 2012; Quinn and Cresswell, 2005). Therefore, since anxiety-like behaviours are increased for at least three weeks after predator stress (Adamec and Shallow, 1993; Miura et al., 2011), this equates to a rodent experiencing anxiety for the equivalent of 18 months of the human life span. This timeline meets the criterion for chronic PTSD described in the DSM-V (APA, 2013). Furthermore, predator stress results in high individual variability in terms of sensitivity and resiliency; only about one-third of predator-exposed rats display extreme anxiety-like responses (Dopfel et al., 2019; Pettorelli et al., 2015). Finally, predator stress paradigms reliably induce hyperarousal (enhanced acoustic startle response) which closely parallels symptoms seen in human patients with PTSD (Adamec et al., 2004, 2006, 2008a, 2008b; Cohen and Zohar, 2004; Dopfel et al., 2019; Pettorelli et al., 2015).

1.8.1 Predator stress methodology

While the diversity in predator stress paradigms represents how effective this paradigm is at modelling PTSD-like symptoms in animals, there is no one agreed upon methodology. We and others (de Lima et al., 2017; Rorabaugh et al., 2015; Zoladz et al., 2015) have used a predator stress model which involves direct, unprotected exposure of a rodent (mouse or rat) to a cat. While Adamec, Kent, Anisman, Shallow, and Merali (1998) and Fifield et al., (2014) have reported robust changes in associative and non-associative fear memories, many other published studies were unable to replicate these effects (Adamec et al., 2004, 2006, 2008a; Adamec and Shallow, 1993; Clay et al., 2011; Fifield et al., 2013, 2015). One potential explanation for these discrepancies is fatigue in the predator. This can lead to differential behavioral responses from the predator, which can ultimately affect the reliability of the model. To avoid this confound, other researchers have used predator cues (i.e. predator odor) instead of the predator (Cohen et al., 2014; Fenchel et al., 2015; Manjoch et al., 2016; Mayer et al., 2014). Predator odors can be either obtained naturally (feline's fur, bedding or litter for instance) or created synthetically (TMT, for example, is a component of fox feces). In the presence of cat odors and TMT, rats elicit different patterns of responses. Normally, cat odor triggers anxiety-like behaviours whereas TMT evokes avoidance responses (Adamec et al., 2004; Dielenberg et al., 2001; Dielenberg and McGregor, 2001; Sheriff et al., 2009; Wallace and Rosen, 2000). Other "protected" exposures to predator stress include exposure to predator fur (Blanchard et al., 2003a, 2003b) or a collar that a cat wore (Berardi et al., 2014; Dielenberg et al., 2001; Dielenberg and McGregor, 2001). Similar to the direct, unprotected predator exposures, the results from these methodologies are not always replicable. Differences may be due to the type of predator used (e.g., domestic or feral cat), or the

state of the predator (well feed or food deprived (Adamec et al., 1980; Herron and Buffington, 2010).

We have recently used a modified version of the Rat Exposure Test (RET) in which a mouse undergoes a brief (typically 5 min) protected exposure to a live rat. Mice are afraid of rats because rats kill and eat mice. This behavior is called muricide. Mice avoid rat odor and show fear reactions when presented with a rat (Rylov, 1985). The RET was first developed by Yang et al. (2004) to evaluate mouse defensive behaviours. In response to a live rat, mice demonstrate defensive behaviours, including freezing and avoidance (Yang et al., 2004). Follow–up studies from different laboratories have successfully replicated these findings (Amaral et al., 2010; Blanchard and Blanchard, 1989; Campos et al., 2013b, 2013a) . We, and others, have also shown that rat-exposed mice show increased anxiety-like behavior, hyperarousal, and cued fear memory, lasting at least 10-15 days (Yang et al., 2004). We have found that the rat/mouse interaction paradigm is less expensive (than housing a cat), more consistent, and robust than other types of predator stress paradigms.

1.9 Cognitive deficits and predator stress

As described above, the DSM-V has included cognitive deficits as one of the symptoms of PTSD (Friedman, 2013; Friedman et al., 1995; Zoellner et al., 2013). Cognitive deficits have also been shown following predator stress (Cohen et al., 2009; Diamond et al., 1999; Park et al., 2008). For example, unlike naïve controls, predator stressed rats were impaired on a contextual odor discrimination task (Cohen et al., 2009). In addition, predator stressed rats showed spatial memory impairment in the radial-arm water maze (Diamond et al., 1996, 1999, 2006; Park et al., 2006; Sandi et al., 2005; VanElzakker et al., 2011; Woodson et al., 2003), a hippocampus-dependent

spatial memory task (Diamond et al., 1999). Finally, Park et al. (2008) showed that a 30 min cat exposure between acquisition and recall produced deficits in the Morris Water Maze (MWM). It is not known, however, if mice exposed to the RET show cognitive deficits similar to those described above.

1.10 Sex differences and predator stress

As described above, prevalence rates of PTSD differ across men and women (Kessler et al., 1995, 2005). While there have only been a handful of predator stress studies examining sex differences, there is variability in the results. Some studies report elevated responses in females to predator stress. For example, Adamec et al (2006) showed that female mice exposed to a cat had a higher startle amplitude (hyperarousal) than males (Adamec et al., 2006). In addition, spatial memory following a predator stress 'reminder' was impaired in female rats only (Burke et al., 2013). Furthermore, female rats had higher CORT levels than males following exposure to a predator odor (Hubbard et al., 2004). Despite the differential corticosterone response, both male and female rats displayed similar levels of conditioning and subsequent avoidance (memory) of the place that contained the cat odor (Hubbard et al., 2004). A lack of sex effect was also reported by Pooley and colleagues who showed that predator exposure led to comparable sex differences in startle response and negative feedback control of corticosterone (Pooley et al., 2018). Only males, and not females, show an enhanced startle response enhanced HPA negative feedback after exposure. Dexamethasone (DEX), a pituitary GR agonist, blocked the stress induced increase in CORT levels only in predator stress exposed males, indicating an enhanced sensitivity to DEX in this group and not in predator stress exposed females. A lack of sex effects was also reported by Falconer and Galea (2003) which showed that males and females exhibit a strong and equivalent

increase in defensive behaviors in the presence of predator odor (Falconer and Galea, 2003). The is consistent with Park and colleagues (Park et al., 2008) who showed that predator stress impairs short-term memory, as well as processes involved in memory consolidation and retrieval, in male and female rats (Park et al., 2008). Furthermore, it is not known if sex differences exist in response to the RET. Hence, additional work is necessary to fully understand the effects of predator stress across sex.

1.11 Brain areas activated by predator stress

Electrophysiological, molecular, and lesion studies have identified specific brain areas, particularly those of the limbic system[1], critical to predator stress. Electrophysiological studies in rodents suggest that predator stress (exposure to live cat) leads to long-term potentiation (LTP)-like changes in amygdala afferent and efferent pathways. In particular, predator stress causes potentiation in neural transmission from hippocampus via the ventral angular bundle to the basolateral amygdala (BLA) and from central amygdala (CN) to the lateral periaqueductal gray (lPAG)(Adamec et al., 2004, 2005b, 2005a, 2008b). Although the functional significance of this characteristic electrophysiological response is not clear, lesion studies suggest that the amygdala is important in predator stress-induced behavioral responses. Specifically, lesions (neurotoxic or ibotenic-acid) of the BLA reduce freezing and avoidance behaviors in rats in response to cat fur or cat odor (Takahashi et al., 2006; Vazdarjanova et al., 2001), but have no effect in rats exposed to

[1] Briefly, the ***limbic system*** consists of the phylogenetically old limbic lobe and other subcortical structures and their connections. The limbic system is a complex set of structures that lies on both sides of the thalamus, just under the cerebrum. It includes the hypothalamus, the hippocampus, the amygdala, and several other nearby areas. It appears to be primarily responsible for our autonomic or endocrine function in response to emotional stimuli and is involved in reinforcing behavior(RajMohan and Mohandas, 2007).

TMT (Fendt et al., 2003; Wallace and Rosen, 2000). Surprisingly, TMT and fox urine (Day et al., 2004; Funk and Amir, 2000), but not cat odor (Dielenberg et al., 2001; Dielenberg and McGregor, 2001), increases FOS expression in the BLA. Furthermore, 30 min exposure to a cat increases c-FOS expression in the BLA (VanElzakker et al., 2011). c-FOS, the immediate early gene, and its protein product FOS are expressed in very low amounts basally, but are quickly produced (within <1 h (Armario et al., 2008)) in response to a stimuli (Herdegen et al., 1998; Piechaczyk and Blanchard, 1994) and rapidly down-regulated (Staples et al., 2008). Thus, FOS expression provides a way of assessing neuronal activation in response to discrete stimuli (Adamec et al., 2010; Hoffman et al., 1993). Whether these behavioural and molecular differences in unconditioned fear behaviour stem from fundamental differences in the type of predator odor (TMT vs. cat odor) or perhaps the magnitude of the presented odor stimulus are issues requiring further investigation.

In contrast to the electrophysiology results, several research groups have concluded that the CN *does not* play an essential role in predator odor-induced unconditioned fear behaviour. Indeed, neither neurotoxic lesions nor chemical inactivation of the CN dampens fear-related behaviours including freezing and avoidance during exposure to either TMT (Fendt et al., 2003; Rosen, 2004) or cat odor (Li et al., 2004). In addition, there was no change in FOS expression in the CN in rats exposed to predator odor (Dielenberg et al., 2001; Dielenberg and McGregor, 2001). This is in contrast to shock-induced conditioning studies which report that damage to the CN impairs the expression of conditioned behaviors (Fanselow, 1994; Phillips and LeDoux, 1992; Weisz et al., 1992), autonomic (Kapp et al., 1979; Zhang et al., 1986), and endocrine responses (Champagne et al., 2003; Champagne and Meaney, 2007; Kapp et al., 1979; Prewitt and Herman, 1997; Zhang et al., 1986).

In addition to the amygdala, the hippocampus appears to play a key role in the response to predator stress. For example, exposure of rats to weasel gland secretions or TMT elicits a pattern of fast wave bursts in the hippocampal dentate gyrus (Heale et al., 1994; Heale and Vanderwolf, 1994). In addition, lesions of the dentate gyrus disrupt the development of freezing in rat pups exposed to odors of an unfamiliar and potentially threatening adult male rat (Takahashi, 1995, 1996). Furthermore, exposure to a predator increases c-FOS expression in the hippocampus (Baisley et al., 2011; Bepari et al., 2012; Pantazopoulos et al., 2011; VanElzakker et al., 2011; Zanette et al., 2019). There may be a differential contribution of the ventral and dorsal hippocampus as elevated c-FOS immunostaining data suggest that while both the dorsal and ventral hippocampus are required for forming a contextual representation, the ventral region also modulates defensive behaviors associated with predators (Wang et al., 2013). Other studies (Chen et al., 1997; Tulchinsky, 2000) suggest predator odors induce elevated delta-FOSB expression in the dorsal hippocampus DG region 5 days after stress exposure (Hawley et al., 2012). FOSB or delta-FOSB is a useful neuronal activity marker for its unique stability (a half-life of weeks) property (Tulchinsky, 2000; Wallace et al., 2008). In wild animals, exposure to a natural cue of predator danger (predator wild bird's vocalizations) also elevates neuronal activity detected by delta-FOSB expression in both the amygdala and hippocampus (Zanette et al., 2019).

A study comparing the effects of cat odor and TMT on FOS expression found that exposure to cat odor, but not TMT, induced significant FOS expression in medial hypothalamus (Staples et al., 2008). This result suggests the medial hypothalamic defensive system is not broadly activated by all predator odors to modulate fear behavior. Finally, studies have reported that the medial prefrontal cortex (mPFC), including the prelimbic and infralimbic cortex, is involved in predator odor-induced unconditioned fear. One study reported that temporary inactivation of the prelimbic

region increased freezing in rats exposed to TMT (Fitzpatrick et al., 2011). However, another study showed that in 38–42 days old adolescent rats, inactivation of the prelimbic cortex impaired freezing induced by cat odor (Chan et al., 2011). Studies indicate that exposure to cat odor activates c-FOS expression (Chan et al., 2011; Staples et al., 2008) in the mPFC, whereas no significant increases in mPFC c-FOS were found after exposure to TMT (Asok et al., 2013; Day et al., 2004; Staples et al., 2008). The mPFC of rats also showed elevations in expression of delta-FOSB several days after exposure to cat odor (Mackenzie et al., 2010). In this study, expression of delta-FOSB in the mPFC was associated with long-term effects of predator odor on conditioned fear. Like the hypothalamus, mPFC was also activated by some but not all predator odors. Therefore, research will be required to determine how distinct predator odors such as TMT and cat odor activate brain structures like hypothalamus and mPFC to modulate unconditioned and conditioned fear behavior. Overall, it seems that brain regions activated by predator exposure depend on the type of predator stress, testing paradigm, testing condition, and the behavioural responses assessed.

1.12 Stress hormones and predator stress

Hormones influence the behaviour and physiology of prey in response to predators (Adamec et al., 1998; Adamec and Shallow, 1993; Armario et al., 2004, 2008, Blanchard et al., 2003a, 2003b, 2005; Bowen et al., 2014; Campeau et al., 2008; Clinchy et al., 2013, 2011; Dielenberg et al., 2001; Dielenberg and McGregor, 2001; Lau et al., 2016; Roseboom et al., 2007; Rosen, 2004; Schulkin et al., 2005; Takahashi, 1995, 1996, Takahashi et al., 2005, 2006, 2007; Wang et al., 2012; Zhang et al., 1986; Zoladz et al., 2012; Zoladz and Diamond, 2013). Predator and predator scent are evidenced to be graded stress to rodents (Adamec et al., 2008a). Unlike the strong stress resulting from close contact with the predator, mild predator scent presumably

contains only the pheromones comprised of both volatile and non-volatile molecules from urine and sebaceous gland secretion, and odors emitted from the non-present predator (Kelliher et al., 1999). Previous studies have found that the effect of predator scent-exposure on rodent anxiety and risk assessment fall between controls and those exposed to a predator (Adamec et al., 2004, 2006). Characterized by mouse-killing behavior (muricide), the rat is often regarded as a mouse predator (Beekman et al., 2005; Molina et al., 1987). Hence, rat- and rat scent-exposure are graded predator stress to mice.

Generally, stress increases hypothalamic–pituitary–adrenal (HPA) functioning and provoke hyperactivity of the neuropeptide-secreting systems, which eventually lead to the release of stress hormones (Beekman et al., 2005; Creel, 2001). The elevated stress hormones alter metabolic pathways, which exert profound and diffusive effects, such as on reproduction competition ability, including aggressive and defensive levels, pheromone production, sexual attractiveness of urine odor, and related modulations of the neural systems (Creel, 2001; De Kloet and Reul, 1987; Sands and Creel, 2004; Zhang et al., 1986). In rodents, released stress hormones induced by predator stimuli lead to reduced aggression levels, as well as less attractiveness to female conspecifics, which might weaken male–male competition, resulting in a reduction of reproductive success (Creel, 2001; Zhang et al., 1986).

Predator stress increases glucocorticoid levels, alters gene expression, and the release of CRH (described about CRH in the section 1.5) in the amygdala in rodents similar to that seen in PTSD. Global gene expression has been assessed in response to predator exposure using complementary DNA (cDNA) microarrays (gene chips) in rats and chickens. Roseboom et al. (2007) euthanized rats 3 h after predator exposure and found increased CRH-binding protein gene expression in the

amygdala, consistent with previous studies (Schulkin et al., 2005). Jöngren et al. (2010) euthanized chickens 2 week after predator exposure and identified 13 significantly differentially expressed genes in the midbrain (Jöngren et al., 2010). Roseboom et al.'s (2007) findings confirm that cDNA microarrays can be used to identify the expression of genes expected to be upregulated in response to fear, and Jöngren et al.'s (2010) study shows that this approach can be used to detect long-lasting effects, even in non-mammalian subjects (Jöngren et al., 2010; Roseboom et al., 2007). Moreover, it is well-established that glucocorticoids are involved in the induction of long-lasting changes in anxiety-like behaviour after predator exposure (Clay et al., 2011; Clinchy et al., 2011, 2013). This positive correlation between corticosterone concentrations and scores on anxiety-like behaviour during cat exposure also demonstrated in humans (Baig et al., 2006; BROOKS et al., 1986). Together, these data suggest that corticosterone concentration can be used as an indicator for stress activation in rodents.

1.13 Stress across generations in laboratory rodents

1.13.1 Stress across generations in other (non-predator stress) PTSD animal models

The effects of stress on future generations has been examined for many years. In one of the first studies by Thompson (Thompson, 1957), rat dams were trained before pregnancy in a conditioned avoidance test and then subjected to the stimulus daily throughout pregnancy. This maternal stress induced anxiety-like behaviors in the offspring. Since then, there has been many studies showing that prenatal stress, usually using restraint stress, increases anxiety-like behaviors (Nakhjiri et al., 2017; Soares-Cunha et al., 2018; Vallée et al., 1997; Xu et al., 2014) and impairs spatial learning (Gué et al., 2004; Holubová et al., 2018; Kapoor et al., 2009; Lemaire et al., 2000; Lordi et al., 1997; Modir et al., 2014; Sun et al., 2017; Szuran et al., 2000; Yang et al., 2006) in

offspring. In addition to behavior, prenatal stress alters HPA activity in offspring. Rat offspring born to mothers exposed to restraint stress during pregnancy exhibited a phase advance in the evening increase in corticosterone levels (Koehl et al., 1997). This finding is consistent with other prenatal stress studies in rats, guinea pigs, and primates (Clarke et al., 1994; Clarke and Schneider, 1993; Henry et al., 1994; Kapoor et al., 2009; Kapoor and Matthews, 2005). The elevated and prolonged plasma cortisol likely results from decreased glucocorticoid receptor feedback sensitivity (De Kloet and Reul, 1987). Finally, prenatal stress also dampens adult neurogenesis in offspring (Fenoglio et al., 2006; Henry et al., 1994; Lemaire et al., 2000; Mandyam et al., 2008).

Although a relatively recent finding in rodents, *preconception* stress also appears to alter offspring behaviour. Dias and Ressler (2014) found that odor fear conditioning of male F0 mice resulted in a behavioural sensitivity in the naïve F1 generation specific to the conditioned odor. This effect persisted in the F2 generation, when in-vitro fertilization was used, and in a cross-fostering study, suggesting that the behavioural changes were the result of inheritance rather than social transmission. To our knowledge, this was the first published study to assess lasting behavioural changes in offspring in response to preconception stress in the parental generation (at least in laboratory studies). The idea that preconception stress can alter future generations is in line with the human research which reports that Holocaust survivors have child and grandchildren with a higher incidence of psychiatric illness (Yehuda, 2002; Yehuda et al., 2000, 2004, 2008, 2015, 2016). Hence, further research assessing the role of preconception stress as a risk factor for psychiatric disease is critical.

1.13.2 Predator stress effects across generations

While the deleterious effects of predator stress are well known (described above), there is growing evidence that predator stress during pregnancy can alter offspring brain and behavior. Exposure to predator rat urine during the first week of pregnancy leads to a decrease in the litter size and survival of mice offspring (de Catanzaro, 1988). Predator odor exposure also induces heightened levels of circulating corticosterone in pregnant female rodents and alters offspring development (Weinstock et al., 1988). In rats, exposure to a live predator prenatally leads to a predisposition to seizures associated with alterations in hippocampal plasticity in the F1 generation offspring (Ahmadzadeh et al., 2011; Saboory et al., 2011). More recently, adult F1 offspring (postnatal day 90) of mice exposed to a predator odor during the last half of pregnancy display increased predator-avoidance behaviour, alterations in social behaviour, novelty-induced anxiety-like behaviours, and increased corticosterone levels (St-Cyr et al., 2017, 2018; St-Cyr and McGowan, 2015; Thayer et al., 2018). In contrast, male F1 offspring of voles exposed to cat odor spent more time exploring and showed a decrease in corticosterone compared with the control group. Interestingly, both FOSB/delta-FOSB mRNA protein expression were downregulated in the hypothalamus of male, but not female, vole offspring in response to cat urine of parents who were predator stressed (Chen et al., 2018).

While these data suggest that predator stress in the parental generation impacts offspring, many questions are left unanswered. For example, does preconception predator stress alter offspring brain and behavior? Are these changes due to maternal social environment, or are they a result of genetic (epigenetic) transmission to the offspring? Are these changes persistent (into F2

generation)? Are there sex differences in offspring as a result of parental predator stress? These questions are addressed in the current study.

1.14 Current Study

The goal of the current study was to examine the lasting effects of preconception predator stress on brain and behavior. We show that a single, five minute exposure to a rat (RET) prior to conception produces increased anxiety-like behavior, hyperarousal, and impaired social behaviors in adolescent offspring. In adulthood, following a mild stressor, preconception stressed mice show increased anxiety-like behavior, impaired social behavior, hyperarousal, and impaired spatial memory, yet normal depressive-like behaviors. These effects do not differ across sex. The behavioral phenotype in adulthood is similar, albeit to a lesser extent, in the absence of the milder stressor. We also show that the experience of the biological parents, and not the maternal social environment, drives the behavioral phenotype of the offspring. We see changes in the glucocorticoid system (corticosterone levels, GR and FKBP5 receptor expression) and dampened adult neurogenesis in response to a mild stressor in adult offspring from preconception stressed parents. Finally, we show that the effects of preconception stress are persistent – lasting at least into the second generation. To our knowledge, this is the first demonstration that preconception predator stress produces inter- and trans-generational effects on brain and behavior in offspring. Ultimately, this knowledge will contribute to understanding the development and advancing the treatment of these devastating disorders.

Chapter 2: Methods

2.1 Ethical Approval

Protocol and procedures for all experiments were followed according to the guidelines of the Canadian Council on Animal Care and Memorial University of Newfoundland Animal Care Committee.

2.2 Animals

Male and female C57BL/6 mice (see Table 9) were used in all experiments (except only males were used for molecular assays). In this thesis, data revealed no sex differences in all behavioural studies. Therefore, only male mice were used for molecular assays. All mice were given *ad libitum* access to food and water in standard laboratory conditions (i.e. temperature and humidity) on a twelve-hour light-dark cycle (lights on at 7:00AM). Male long-Evans rats (approximately 150-200 mg/kg) were used as stimulus animals for the rat exposure test (RET). Rats were kept on a reverse light/dark cycle (lights off at 7:00AM) and food restricted to 85% of expected body weight to increase activity and interaction rate with mice. All animals purchased from Charles River Laboratories (St. Constant, QC) were left undisturbed in their home cages for at least one week after arrival prior to experimentation.

2.3 Experiments

2.3.1 Experiment 1. The effects of preconception predator stress on behaviour in offspring following 2 min mild stress exposure.

Sexually inexperienced male and female C57BL/6 mice (see Table 9), aged 7-8 weeks, were divided into two groups: Predator Stressed (PS) and Control (C). All mice were habituated to the exposure chamber for 5 min per day for five days. On day six, mice in the predator stressed

group were exposed to a live rat in the exposure chamber while mice in the control group were exposed to an empty chamber (similar to habituation days). Full details of the habituation and rat exposure test (RET) can be found in section 2.4.1. Two days after the RET, all mice were tested on the elevated plus maze (EPM) to evaluate anxiety-like behaviour. A description of the EPM can be found in section 2.4.2. Ten days after the RET (or 8 days after the EPM), male control mice were bred to female control mice and male predator stressed mice were bred to female predator stressed mice. Breeding pairs were housed together for seven days. A schematic of the F0 procedure can be found in Figure 1.

All first filial offspring (F1 generation) offspring were left undisturbed, except when ear notched for identification, with their mothers until weaning. Mother-pup behaviour was recorded for 40 min on alternate days from post-natal (PND) day 5-12 (see Table 2). All mice were weaned on approximately PND 21. Male and female offspring were housed with their same-sex littermates in groups no larger than five. Behavioural testing for offspring began at PND 24. All offspring underwent a behavioural battery for six days (one test/day) starting with the EPM, followed by the open field (OF), light/dark (LD) box, acoustic startle (AS), forced swim (FS), and the social interaction (SI). A detailed description of the behavioural battery can be found in section 2.4.

Seventeen days later (PND 55), mice began habituation for the RET. After five days of habituation, all F1mice were exposed to a rat for two minutes (2 min RET exposure). Note that this was the first exposure to a rat by F1 mice. Two days later (PND 62), all offspring underwent a second behavioural battery identical to the first one. A schematic of the F1 procedure can be found in Figure 2.

2.3.2 Experiment 2. The effects of preconception predator stress on spatial learning following a mild stressor in offspring

The exact same protocol was followed for the F0 and F1 mice as described in experiment 1 except that following the 2 min RET, F1 mice were tested in the Morris Water Maze (MWM, described in section 2.4.8) only.

2.3.3 Experiment 3. The role of the maternal social environment on offspring behaviour

The exact same protocol was followed for the F0 and F1 mice as described in experiment 1 except for the fostering procedure for the F1 mice. Fostering was performed initiated 3–4 h after parturition. For each litter, the biological mother was removed, offspring were thoroughly mixed with the foster mother's bedding, and the bedding was placed in a clean warm cage. All mice from the same litter were placed with a foster mother. Recipient and donor mother's behaviour were observed for at least 10 min to ensure acceptance of the new litter. All offspring were fostered to either a control mother or a predator stressed mother. This provided four groups: offspring from PS biological parents fostered to a PS mother (PS biological parents-PS fostered mother: BS-FS), offspring from PS biological parents fostered to a C mother (PS biological parents-C fostered mother: BS-FC), offspring from C biological parents fostered to a PS mother (C biological parents-PS fostered mother: BC-FS), offspring from C biological parents fostered to a C mother (C biological parents-C fostered mother: BC-FC). Foster mother-pup behaviour was recorded for 1 hr on alternate days from PND 5-12 (see Table 2). After the 2 min RET, all F1 mice underwent the MWM immediately following the behavioural battery. See Figure 3 for procedure.

2.3.4 Experiment 4. The effects of predator stress on physiological and molecular substrates

Mice underwent the RET (as described in section 2.4). Briefly, mice were habituated to the rat exposure chambers (without the rat) for 5 min per day for five days (Fallon 2017; Whiteman 2017). On day six, half of the mice were exposed to a live rat (PS group) while the other were exposed to an empty chamber (C group).

To assess alterations in the F1 mice from PS and C mice were generated using the exact same protocol as described in experiment 1. All F1 mice underwent the behavioural battery starting at PND 24 and at PND 60, all mice were exposed to the 2 min RET.

The following procedures were completed on the F0 and F1 mice:

1. To measure corticosterone, mice were decapitated 30 min after the 5 min RET (F0 mice; n=5 control animals, n=5 stressed animals) or 2 min RET (F1 mice; n=5 control animals, n=5 stressed animals), trunk blood (about 500 ul) was collected. All mice were sacrificed between 15:00–16:00 pm to avoid circadian rhythm effects.
2. To measure neural activation, mice were anesthetized and perfused 90 min (for c-FOS) after the 5 min RET (F0 mice; n=4 control animals, n=4 stressed animals) or 2 min RET (F1 mice; n=4 control animals, n=4 stressed animals).
3. To measure GR (F0 mice; n=5 control animals, n=5 stressed animals, F1 mice; n=5 control animals, n=5 stressed animals) and FKBP5(F0 mice; n=5 control animals, n=5 stressed animals, F1 mice; n=5 control animals, n=5 stressed animals) activation, mice were anaesthetized and perfused two days after the 5 min RET (F0 mice) or 2 min RET (F1 mice).

A full description of the anaesthetic, perfusion, corticosterone assay, immunohistochemistry (c-FOS) and immunofluroscence (GR and FKBP5) can be found in sections 2.5.

2.3.5 Experiment 5: The effects of a 2 min exposure to a rat on behaviour

To determine if a two min exposure to a rat produces lasting changes in behaviour, sexually inexperienced male C57BL/6 mice, aged 7-8 weeks, were divided into two groups: Predator Stressed (PS) and Control (C). All mice were habituated to the exposure chamber for 5 min per day for five days. On day six, mice in the predator stressed group were exposed to a live rat in the exposure chamber while mice in the control group were exposed to an empty chamber (similar to habituation days) for two minutes. Full details of the habituation and RET can be found in section 2.4. Two days after the 2 min RET, all mice were assessed for anxiety-like behaviour (EPM, OF, LD box), depressive-like behaviour (FS), hyperarousal (AS), social interaction (SI), and spatial learning (MWM) as described in section 2.4.

2.3.6 Experiment 6: The effects of preconception predator stress on adult offspring behaviour

To assess whether preconception stress produces anxiety-like behaviours in adult offspring, we used the exact same protocol for the F0 and F1 mice as described in experiment 1 except that at PND 60, offspring were not exposed to the 2 min RET. Instead, the behavioural battery and MWM was carried out from PND 60-73 (behavioural tests are described in section 2.4).

2.3.7 Experiment 7: The effects of preconception predator stress on second filial generation offspring behavior

Second filial generation (F2) animals are the offspring from the mating of F1 mice. F1 mice were generated as described in experiment 1. At weaning, however, F1 mice did not undergo the behavioral test battery; instead the mice were left undisturbed. At PND 60, mice were bred to produce four F2 groups: (1) male F1 mice (from control F0 parents) were bred with female F1 mice (from control F0 parents) to generate F2 offspring labelled FC-MC (father control-mother control); (2) male F1 mice (from predator stressed F0 parents) were bred with F1 females (from control F0 parents) to generate F2 offspring labelled FS-MC (father stressed-mother control); (3) male F1 offspring (from control F0 parents) were bred with female F1 offspring (from predator stressed F0 parents) to produce F2 offspring labelled FC-MS (father control-mother stressed); (4) male F1 mice (from predator stressed F0 parents) were bred with F1 females (from predator stressed F0 parents) to generated F2 offspring labelled FS-MS (father stressed-mother stressed). The F2 generation followed the exact same protocol as the F1 generation described in experiment 1. Mice were weaned and separated on PND 21 behavioral testing was completed on days PND 24-30, mice underwent the 2 min RET on PND 60, and behavioral testing was again carried out starting on PND 62 (behavioral battery plus MWM).

2.4 Behavioural Tests

2.4.1 Rat Exposure Test (RET)

The exposure chamber was a standard Plexiglas rat cage (47 cm x 26 cm x 20 cm) containing a clear Plexiglas divider with small holes (not large enough to allow the mouse to pass through to

the rat side or vice versa but intended to allow free olfactory flow). The duration of the exposure was either 5 minutes or 2 minutes depending on the specific experiment.

RET Habituation: Both rats and mice were habituated to the exposure chamber once a day for five days prior to exposure day. Mice were habituated to the 'mouse' side of the chamber while rats were exposed to the 'rat' side. Appropriate care was taken before any RET such as no mouse cage ever attached to cage that ever contained a rat to avoid rats smell. During mouse habituation trials, the 'rat' side of the chamber was empty. During rat habituation trials, the 'mouse; side of the chamber was empty. ***RET Exposure:*** Similar to habituation trials, the mouse was placed in the left (or 'mouse') side of the exposure chamber. The right ('rat') side of the chamber contained either a live rat (predator exposed mice) or was left empty (control mice). All control mice were run before predator exposed mice to reduce rat scent exposure. Following exposures, all animals were returned to their home cages. All exposures were video recorded and hand-scored for mouse freezing duration and frequency as an index of fear behaviour. Freezing was defined as immobility except for respiration. All chambers were wiped down with 40% ethanol between exposures.

2.4.2 Elevated plus maze (EPM)

The EPM was made of white Plexiglas (0.6 cm thick). It had four 29.0 x 5.1 cm arms with a square, 10.2 cm^2 centre platform connecting the arms at right angles. Two closed arms were on opposite sides of the platform and were enclosed by three 14 cm high walls, leaving the ceiling and the entrance to the square platform open. The two open arms had neither walls nor a ceiling, but had a 0.5 cm high lip on all sides. The maze was elevated by four 45 cm high legs positioned at the end of each arm. Mice were placed facing away from the center platform and allowed to

explore for 5 min. Time in the open and closed arms, and number of entries into the open and closed arms were calculated with the EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands). Ratio time was calculated as the total time in the open arms divided by total time in all arms. Ratio frequency was calculated as the total number of entries into the open arms divided by total entries in all arms (Adamec et al., 2004, 2006).

2.4.3 Open field (OF)

The OF test was carried out in a 48x48x48 cm grey Plexiglas box. A square was marked off by tape 10 cm from the walls of the box to determine the amount of time the mice spent in the centre of the box relative to the perimeter. Mice were placed in the centre of the box and allowed to explore for 5 min. Duration in the center and distance traveled was calculated with the EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands; Fallon 2017;(Adamec et al., 2006; Adamec and Shallow, 1993).

2.4.4 Light/dark box (LD)

The LD boxes were made from two grey Plexiglas (0.5 cm thick) boxes (length: 20.3 cm, width: 20.3 cm, height: 14.9 cm) connected by a central alleyway corridor (length: 10.2 cm, width: 6.4 cm, height: 7.6 cm). The light box had a clear Plexiglas removable lid with twenty-five evenly placed ventilation holes. The dark box had a grey, opaque, acrylic Plexiglas removable top. 9W, 550 lm light was positioned 5.1 cm above the light box. Mice were placed into the light side of a light dark box and were allowed to explore for 5 min. Video recordings were taken from above the light box for later analysis. The number of entries and total time in the light side of the box was measured from video by a researcher blind to treatment (Adamec et al., 2006; Fifield et al., 2013, 2015).

2.4.5 Acoustic startle (AS)

In the AS test, mice were placed into a 12.7 cm long cylindrical animal enclosure with a diameter of 3.7 cm. An electric transducer below the enclosure records activity throughout the experiment. All enclosures were placed inside a San Diego Instruments startle chamber. Mice habituated to a background white noise of 50 DB for 5 min before receiving 120 DB pulses of white noise every 30 seconds for 15 min. Analysis compared the maximum activity during the pulse to the activity immediately prior to the pulse for a measure of peak startle amplitude (Adamec et al. 2006, 2009). For each trial, peak startle amplitude was determined by subtracting the transducer output at the beginning of the noise burst (Vstart) from the maximum transducer output (Vmax) during the recording window (Vmax–Vstart).

2.4.6 Force swim (FS)

In the FS test, a 66 cm tall cylindrical Plexiglas container of diameter 24.5 cm was filled with water to a height of approximately 15 cm. Water was kept at a temperature of 22 ± 1 °C. Mice were placed into the pool for 6 min and allowed to swim freely. The first 2 min allowed the mice to habituate and were not analyzed. Video recordings were taken from the front of the chamber. The total amount of time the mice spent immobile and the number of times the mice were immobile was scored by a researcher blind to treatment (Can et al., 2011; Yankelevitch-Yahav et al., 2015).

2.4.7 Social interaction (SI)

The SI test took place in the OF chamber. In the first 150 seconds, an empty cage with metal bars was placed against the centre of one wall. The subject was removed briefly and a novel male C57BL6 mouse was placed inside of the cage. The subject was then returned to the box for an additional 150 seconds. Video recordings were taken from above and scored by a researcher

blind to treatment. Ratios were calculated to determine the frequency and amount of time spent interacting with the cage in the initial trial compared to that of the second trial (Golden et al., 2011).

2.4.8 Morris Water Maze (MWM)

The MWM utilized a metal circular pool (diameter: 120 cm, height: 31 cm) surrounded by Plexiglas walls that extend 31 cm above the tank. The escape platform, attached to a Plexiglas base to be kept in a fixed location, was constructed out of white, polyvinyl pipe, and was filled with sand (height: 26.5 cm, diameter: 26.5 cm). The pool was filled with water to 3 cm above the platform. To hide the platform, white, non-toxic, tempera paint was added to the water. Cues were placed on the walls around the room.

For twelve consecutive days, all mice underwent four training trials/day (intertrial interval = 1 hour). During training, the platform remained fixed between quadrants Q1 and Q2. Each mouse was given a maximum of 60 seconds to climb onto the platform and remained there for 5 seconds. If a mouse failed to reach the platform, an experimenter guided the mouse to the platform. On day 13, a probe trial was carried out in which the platform was removed and the mice were given a free 60 second swim. Video recordings were taken during the training trials and the probe trial and analyzed with EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands; Blundell et. al., 2010).

2.5.1. Hormone assay

Corticosterone concentrations were measured with an ELISA kit purchased from Arbor Assays Company (Catalog Number K014-H1). The minimum level of detection of the kit was 16.9

pg/ml corticosterone. The kit measures total corticosterone in serum including the corticosterone combined with corticosteroid-binding globulin (CBG). The cross reactivity of the corticosterone kit for cortisol is 0.38% (tested at the 50% binding). Briefly, 50 µl of standards or samples were added in duplicate to wells of the microtiter plate. 75-µl of assay buffer was added to the non-specific binding (NSB) wells and 50 µl of assay buffer was added to wells to act as maximum binding wells. Then, 25 µl of the DetectX Corticosterone Conjugate and 25 µl of the DetectX Corticosterone antibody (except the NSB wells) were added to each well and the titer plate was shaken for 1 h at room temperature. After the plate was washed using the wash solution and blot dried by hitting plate onto paper towels, 100 µl of the TMB substrate was added to each well and incubated for 30 min at room temperature. The optical density (OD) of corticosterone was read at 450 nm wavelength using a plate reader within 15 min after the reaction was terminated by adding 50 µl of the stop solution. The concentration of corticosterone was calculated according to the standard curves.

2.5.2 Immunostaining for F0 and F1 generation mice

2.5.2.1 Brain collection for immunostaining

Brain collection occurred at 90 min (for c-FOS; (Bullitt, 1990; Figueiredo et al., 2003), or 2 days (GR and FKBP5) after exposure (Asada et al., 2011; Garrett et al., 2015), animals were anaesthetized and perfused for staining procedure.

Transcardial perfusion was used to maintain tissue, using Urethane (15%, prepared in distilled water) as an anesthetic. The animals were perfused with ice cold 4% paraformaldehyde (PFA; Fisher Scientific, Hampton, New Hampshire, USA; in 0.1M phosphate buffer, pH 7.4), after

a 1-minute saline (0.9%) pre-flush to remove all blood. The brains were removed from the skull and post-fixed individually, in a 4% PFA solution. Twenty-four hours prior to slicing, brains were immersed in 20% sucrose. All brains were stored in a 4°C environment, with all solutions at ice cold temperatures to prevent the melting of tissues.

2.5.2.2 Slide preparation for analysis

A Leica CM3050 S cryostat machine was used to section all brain samples, with a D-profile tungsten knife. The specimen temperature (OT) of the cryostat was set to -19 ± 3°C, with the temperature of the chamber (CT) set to -17 ± 3°C. All slicing was conducted manually, with section and trimming thickness set to 30µm for all sections. Coronal sections were collected, prioritizing the cortex, ventricles, and hippocampus. All slides were transferred to slide storage boxes and kept in a -80°C environment until staining procedures.

2.5.2.3 Immunohistochemistry (for c-FOS)

Immunostaining followed previously published procedure(Bhattacharya et al., 2017). During staining, slides containing sections were removed from the -80c storage and thawed in a 4C room for 10 minutes, followed by 8 minutes in room temperature to further dry. The border of the slides was marked with a PAP hydrophobic pen to create a barrier for the antibody. Primary antibodies rabbit c-FOS (1/1000, Cell Signaling), rabbit delta-FOSB (1/2000, Cell Signaling) were diluted in phosphate buffered saline with PBS + 0.2% TritonX-100, and 2% normal goat serum. 1mL primary antibody was applied to each slide and incubated for 48 hours at 4C. After the initial incubation period, slides were washed for 20 minutes with PBS (phosphate buffer saline) and the secondary antibody was then applied. The secondary antibody was a biotinylated goat anti-rabbit: [PBS, 0.2%triton x-100 and 2% normal goat serum] and was incubated for 2 hours. Washing

procedures were conducted again for 20 minutes, and the slides were then incubated with the Vectastain avidin/biotinylated enzyme (A+B) solution for one hour. Washing procedures were repeated and the reaction product was visualized by adding 0.05% DAB with 0.01% hydrogen peroxide (H_2O_2) for 5 minutes. Sections were washed with distilled water, air dried for 24 hours. Next day, sections were finally dehydrated with alcohol/xylene, and cover slipped with permount (Fisher Scientific).

2.5.2.3.1 c-FOS staining analysis

Images of sections were captured with a CCD camera connected to the Olympus bright field microscope at 10x, 20x and 40x magnifications. Olympus cellSens imaging software was used to process and store the images. Representative fields consisted of eight regions within the dentate gyrus and CA1 area. a) dentate gyrus: dorsal right hemisphere (DENTRH), dorsal left hemisphere (DENTLH), ventral right hemisphere (VDRH), and ventral left hemisphere (VDLH) and b) CA1 area: dorsal right hemisphere (CA1RH), dorsal left hemisphere (CA1LH), ventral right hemisphere (VCA1RH), ventral left hemisphere (VCA1LH), basolateral amygdala (BLA) and central amygdala (CeA). The light intensity of the microscope was kept at the same level for all the sections from all animals analyzed. The intensity of c-FOS staining were normalized with background optical density (OD) and analyzed using ImageJ analysis software. To analyze the sections, OD of the negative control slide was used as a background OD for the section. Six slides were randomly selected from each mice. For each slide, 5 randomly selected visual fields in the region of interest (ROI) were chosen. We recorded the relative optical density (ROD) by drawing four 100 um diameter circles on region of interest layer in each section and averaged to obtain the OD reading. The ROD of the region of interest (ROI) was obtained by using the following formula: (OD of background − OD of ROI)/OD of background.

2.5.2.4 Immunofluorescence (for GR, FKBP5)

For immunofluorescence staining, primary antibody incubation process was similar with immunohistochemistry procedure. However, secondary antibody incubation was quite different from DAB immunohistochemistry procedure. Here, sections were incubated with fluorophore conjugated secondary antibodies. Slides containing sections were removed from the -80°C storage and thawed in a 4°C for 10 minutes, followed by 8 minutes in room temperature to further dry. The border of the slides was marked with a PAP hydrophobic pen to create a barrier for the antibody. The primary antibodies mouse GR (1/1000, Santa Cruz) and rabbit FKBP5 (1/500, Thermo Scientific) were diluted in phosphate buffered saline with PBS + 0.2% TritonX-100, and 2% normal goat serum. Primary antibody was applied to each slide and incubated for 48 hours at 4^{o}C. 48 hours later, slides were washed for 20 minutes with PBS and the secondary antibody was then applied. The secondary antibody was a alexa Fluor® 555 goat anti-rabbit (for FKBP5 primary antibody) or alexa Fluor® 555 goat anti-mouse (for GR primary antibody): [diluted in PBS, 0.2% triton x-100 and 2% normal goat serum] and was incubated for 2 hours in dark condition to protect from direct light exposure. Washing procedures were conducted again for 20 minutes, and cover slipped via fluoroshield mounting medium with DAPI (Abcam) which is commonly used as a nuclear counterstain in fluorescence microscopy.

2.5.2.4.1 GR, FKBP5 staining analysis

Images of sections (for GR, FKBP5 staining) were captured with a CCD camera connected to the Olympus fluorescence microscope at 10x, 20x and 40x magnifications. Olympus cellSens imaging software was used to process and store the images. The light intensity of the microscope was kept at the same level for all the sections from all animals analyzed. Representative fields

consisted of eight regions: dentate gyrus [dorsal right hemisphere (DRH), dorsal left hemisphere (DLH)], CA1, CA2 and CA3 [right hemisphere and left hemisphere], basolateral amygdala (BLA), central amygdala (CeA), thalamus (Thla), hypothalamus (Hypoth), medial prefrontal cortex (mPFC). Six slides were randomly selected from each mice. For each slide, 5 randomly selected visual fields in the region of interest were chosen. We recorded the relative optical density of positive cells in each field to evaluate the average ROD. The ROD of GR or FKBP-immunopositive cells were analyzed using ImageJ analysis software.

2.6 Statistical analysis

In behavioural experiments, a 2 (control vs stress) x 2 (male vs female) ANOVA was carried out for each measure (EPM, OF, LDB, SI, AST, and FST) in this study for F0, F1 and F2 mice. For F1 and F2 MWM test, during training duration to the platform and distance travelled to reach the hidden platform were analyzed using a 2 (stressed vs control) x 2 (male vs female) x 12 days repeated measures ANOVA, and the percentage of time spent in each quadrant during probe trial was analyzed using a 2 (stressed vs control) x 2 (male vs female) x 4 (quadrant: target, adjacent1, adjacent2, opposite) ANOVA. Latency to the platform location and number of platform location crosses during probe trial was analyzed using a 2 (stressed vs control) x 2 (male vs female) ANOVA. Two tailed t test were carried out to analyze biochemical (plasma corticosterone assay) and staining data (c-FOS, GR and FKBP) for F0 and F1 mice. p value was adjusted for staining data by using Benjamini-Hochberg procedure to control for false discoveries when we looked at the differences between stressed and control animals over a large number of brain areas(Benjamini and Hochberg, 1995). For F1 cross-fostering experiment, a 2 (biological parents: stressed or control) x 2 (foster mother: stressed or control) x 2 (sex: male or female) ANOVA was carried out

for each measure (EPM, OF, LDB, SI, AST, and FST), and F1 cross-fostering MWM data analysis were identical with F1 and F2 MWM test. Differences between groups (control vs stressed) were considered significant when p values were < 0.05. Moreover, non-significant (NS, $p > 0.05$) statistical results were not represented in the result section (see Table 3 for NS statistical results).

Chapter 3: Results

3.1 Changes in behavior and brain following a single, five minute exposure to a rat

3.1.1 Five min RET produces lasting changes in anxiety-like behavior in F0 mice

Consistent with a previous study (Yang et al., 2004), acute exposure to a live predator (rat) increased species-typical defensive and anxiety-like behaviors. During the RET, mice exposed to the rat showed increased freezing duration [$F(1,98)=136.54$, $p=0.001$] and frequency [$F(1,98)=34.76$, $p=0.001$] compared to controls (Figure 7A,B, Table 3). Two days later when tested in the EPM, predator stressed mice spent less time [ratio time: $F(1,98)=317.75$, $p=0.001$] and entered the open arms [ratio frequency $F(1,98)=174.16$, $p=0.001$] less often than controls (Figure 7C,D, Table 3). There were no significant effects of sex in the RET or EPM (see Table 3).

3.1.2 A single, 5 min exposure to a rat increases plasma corticosterone levels

Consistent with previous studies (Amaral et al., 2010; Blanchard and Blanchard, 1989), a 5 min exposure to a rat increased plasma corticosterone levels compared to controls [F0 mice; $t(8) = 3.63$, $p=0.007$, Figure 14A].

3.1.3 A single, 5 min exposure to a rat increases c-FOS activation in several brain regions

Consistent with previous studies (Dielenberg et al., 2001; Hawley et al., 2012; VanElzakker et al., 2011; Wang et al., 2013; Zanette et al., 2019), we show that acute predator stress increases c-FOS expression in the hippocampus. As shown in Figure 12A,13A (See Table 4), a five minute exposure to a rat increased c-FOS expression in the dentate gyrus [dorsal right hemisphere: DENTRH; $t(6)=20.43$, $p=0.0001$, dorsal left hemisphere: DENTLH; $t(6)=10.14$,

p=0.0001, and ventral left hemisphere: VDLH; t(6)=10.35, p=0.0001] and CA1 [dorsal right hemisphere: CA1RH; t(6)=8.36, p=0.0001, ventral right hemisphere: VCA1RH; t(6)=8.40, p=0.0001, and ventral left hemisphere: VCA1LH; t(6)=3.58, p=0.012] compared to controls.

3.1.4 A single, 5 min exposure to a rat alters GR and FKBP5 expression in several brain regions

Previous studies report that exposure to a traumatic stress increases glucocorticoid receptor (GR) expression (Benjamini and Hochberg, 1995; Xuan et al., 2014). Thus, we examined GR expression following predator stress (Figure 15A,B, 16A, Table 4). A 5 min exposure to a rat increased GR expression in the dentate gyrus [DENTRH; t(8)=6.44, p=0.0001, DENTLH; t(8)= 10.78, p=0.0001)] and CA1 [CA1RH; t(8)=11.92, p=0.0001, CA1LH; t(8)=7.11, p=0.0001] compared to controls. In contrast, stressed mice showed lower GR expression in CA2 [CA2RH; t(8)=15.29, p=0.0001, CA2LH; t(8)= 34.30, p=0.0001] and CA3 [CA3RH; t(8)=11.80, p=0.0001, CA3LH; t(8)=11.75, p=0.0001] compared to controls (See Figure 15A, 16A). GR expression was elevated in the hypothalamus (Hypotha; t(8)=11.28, p=0.0001), lowered in the thalamus (Thla; t(8)=3.08, p=0.015) and BLA (t(8)=10.31, p=0.0001), and did not differ in the medial prefrontal cortex (mPFC; t(8)=0.187, p=0.856) in predator stressed mice compared to controls (Figure 15B, 16A, Table 4).

FKBP5 is a co-chaperone that modulates glucocorticoid-dependent responses by regulating the activity of their receptor, the GR (Blaze and Roth, 2015; Dias and Ressler, 2014; Jakovcevski et al., 2008). Therefore, FKBP5 is critical for GR *in vivo* function. We report that a single, 5 min exposure to a rat increases FKBP5 expression in four different brain areas (Figure 17A,B,18A and Table 4) [DENTRH; t(8)=4.87, p=0.0049, CA2RH; t(8)=7.98, p=0.0002, CA3LH; t(8)=3.97,

p=0.0123 and BLA; t(8)=14.97, p=0.0001] compared to controls (Figure 17A, B, 18A and Table 4).

3.2 Preconception predator stress-induced changes in F1 behavior and brain

3.2.1 Preconception predator stress increases anxiety-like behaviour and hyperarousal in first filial (F1) adolescent mice.

We assessed anxiety-like behaviour, hyperarousal, social interaction and depressive-like behaviours in adolescent offspring (F1 mice) from stressed or control parents (Figure 8A-H, Table 3). Adolescent offspring from preconception stressed parents avoided the open arms of the EPM [ratio time: F(1,74)=223.47, p=0.0001; ratio frequency: F(1,74)=142.20, p=0.0001], spent less time in the light side of the light dark box [F(1,74)=13.28, p=0.0001], spent less time in the center [F(1,74)=96.52, p=0.0001] and travelled less distance [F(1,71)=48.76, p=0.0001] in the OF, interacted less with a social target in the SI test [F(1,74)=14.53, p=0.0001], and displayed an enhanced acoustic startle response [mean peak startle amplitude: F(1,73)=73.12, p=0.0001] compared to offspring from control parents. There were no sex differences on any behavioral measure. There were no other significant differences across groups (see Table 3).

3.2.2 Preconception predator stress increases anxiety-like behaviour and hyperarousal in response to a mild stressor in adult F1 mice.

Around PND 60, all F1 offspring were exposed to the mild stressor (2 min RET). Offspring from preconception stressed parents froze longer [F(1,64)=31.36, p=0.001] and more often

[F(1,64)=24.22, p=0.001] than offspring from control parents after 2 min mild stress exposure (Figure 9A-H, Table 3).

Next, we assessed anxiety-like behaviour, hyperarousal, social interaction and depressive-like behaviours following a mild stressor (2 min RET) in adult offspring (F1 mice) from stressed or control parents. Offspring from stressed parents avoided the open arms of the EPM [ratio time: F(1,27)=169.71, p=0.0001; ratio frequency: F(1,27)=51.33, p=0.0001], spent less time in the light side of the light dark box [light side entries: F(1,27)=8.82, p=0.006; light side duration: F(1,27)=7.86, p=0.009], spent less time in the center [F(1,27)=53.09, p=0.0001] and travelled less distance [F(1,27)=26.86, p=0.0001] in the OF, and displayed an enhanced acoustic startle response [mean peak startle amplitude: F(1,26)=518.07, p=0.0001] compared to offspring from control parents (Figure 9A-H, Table 3). There were no other significant differences across groups (Table 3). Furthermore, there were no sex differences across behavioral tests except in the acoustic startle test [sex*stress: F(1,26)=5.74, p=0.024)]. Note that a single, 2 min exposure to a rat (2 min RET) did not produce anxiety-like behaviours in naïve mice (data not shown, see Table 3).

3.2.3 Preconception predator stress dampens spatial memory following a mild stressor in adult F1 mice

We assessed spatial learning and memory using the Morris Water Maze (MWM) in offspring from stressed or control parents two days after they were exposed to a mild (2 min RET) stressor. There were no differences across groups during training. While offspring from stressed and control parents travelled less distance [distance (F(11,198)=11.25, p<0.0001)] and took less time [duration: (F(11,198)=10.36, p<0.0001) to reach the platform, there were no significant group or group interactions (all Fs<1, all p>0.05) across days. There was significant sex effect only for

distance travelled ($F(1,18)=6.179$, $p=0.023$) over trials but there were no significant sex interactions (All $Fs < 1$, NS).

During the probe test (day 13, platform removed), control offspring spent significantly more time in the target quadrant [$F(1,19)=9.50$, $p=0.006$] compared with stressed offspring. In addition, offspring from stressed parents took longer to get to the platform location [platform latency: $F(1,17)=34.05$, $p=0.0001$] and crossed the platform location less often [platform frequency: $F(1,17)=23.84$, $p=0.0001$] than control offspring (Figure 10, see Table 3). Note that a single, 2 min exposure to a rat (2 min RET) *did not* alter spatial learning and memory in the MWM in naïve mice (data not shown, see Figure 10, Table 3).

3.2.4 Biological parent stress experience, and not maternal social environment, determines anxiety-like behaviour and hyperarousal in the adolescent F1 mice.

Offspring from predator stressed and control parents were fostered to either a control mother or a predator stressed mother. This provided four groups: offspring from PS biological parents fostered to a PS mother (PS biological parents-PS fostered mother: BS-FS), offspring from PS biological parents fostered to a C mother (PS biological parents-C fostered mother: BS-FC), offspring from C biological parents fostered to a PS mother (PS biological parents-C fostered mother: BC-FS), offspring from C biological parents fostered to a C mother (C biological parents-C fostered mother: BC-FC). We assessed anxiety- and depressive- like behaviours, social behaviour, and hyperarousal in the cross-fostered F1 generation mice in adolescence (Figure 19 A-H, Table 3). There was a significant main effect of biological parent in the EPM [ratio time: $F(1,102)=121.24$, $p=0.0001$; ratio frequency: $F(1,102)=22.25$, $p=0.0001$], OF [distance travelled: $F(1,102)=7.85$, $p=0.0001$], LDB [light side entries: $F(1,102)=6.26$, $p=0.014$)], and AST [mean

peak startle amplitude: F(1,102)=5.12, p=0.026]. There was a significant main effect of foster mom in time in center in the open field [F(1,102)=8.08, p=0.005]. A small, but significant sex effect was observed in light side entries of LDB [sex*biological mother: F(102)=4.20, p=0.043]. These data suggest that experience of the biological parents drives the offspring behavioral phenotype.

3.2.5 Biological parental stress, and not maternal social behavior, determines anxiety-like behaviour, social behavior, hyperarousal, and spatial memory deficits in adult offspring exposed to a mild stressor.

During the mild stressor (2 min RET), there was a significant main effect of biological parents [freezing duration: F(1,102)=73.49, p=0.0001, freezing frequency: F(1,102)=29.18, p=0.0001]. Similarly, following the mild stressor, there was a significant main effect of biological parent in the EPM [ratio time: F(1,102)=983.21, p=0.0001; ratio frequency: F(1,102)=231.25, p=0.0001], OF [time in center: F(1,102)=75.77, p=0.0001], LDB [light side duration: F(1,102)=17.28, p=0.0001, light side entries: F(1,102)=18.54, p=0.0001], SI test [social interaction ratio: F(1,102)=4.40, p=0.0001], and AST [mean peak startle amplitude: F(1,102)=458.38, p=0.0001]. There were no sex effects or other significant differences across groups in other behavioural measures (Figure 20 A-I and Table 3).

Across the twelve days of water maze training, all mice (BC-FC, BC-FS, BS-FC, BS-FS) learned the location of the hidden platform at a similar rate. A linear trend analysis revealed that the mice travelled significantly shorter distances (F(1,65)=496.11, p=0.0001) and took less time (F(1,65) =258.44, p = 0.0001) to reach the hidden platform over trials (Figure 21, Table 3).

On probe day (day 13), offspring whose biological parents were stressed, regardless of who their foster mother was (stressed or control), showed significantly higher latency to reach the platform location [$F(1,65)=52.27$, $p=0.0001$), crossed the platform location less often [$F(1,65)=57.50$, $p=0.0001$], and spent significantly more time in the target quadrant ($F(1,65)=71.73$, $p=0.0001$) than offspring whose biological parents were controls (Figure 21, Table 3).

3.2.6 In the absence of a mild stressor, F1 adult mice from stressed parents showed increased anxiety-like behaviour and hyperarousal but normal spatial memory and social behaviors.

In a separate set of animals, we wanted to determine if the preconception stressed behavioral phenotype in the F1 persisted into adulthood *in the absence of the mild stressor*. We assessed anxiety-like behaviour, social behaviour, acoustic startle and depressive-like behaviours in adult offspring (~PND 60) from preconception stressed or control parents. Consistent with our previous results (see result section 3.2.1), adult offspring from stressed parents avoided the open arms of the EPM [ratio time: $F(1,23)=17.21$, $p=0.0001$; ratio frequency: $F(1,23)=28.64$, $p=0.0001$], spent less time in light side of the LDB [$F(1,23)=6.04$, $p=0.022$], spent less time in the center [$F(1,23)=14.62$, $p=0.001$] and travelled less distance [$F(1,23)=17.06$, $p=0.0001$] in the OF, and displayed an enhanced startle response [mean peak startle amplitude: $t(25)=2.77$, $p=0.01$] compared to offspring from control parents (Figure 11A-E, Table 3). There were no other significant differences across groups or sex differences (Table 3). The results of these data suggest that preconception stress behavioral effects persist into adulthood in the F1 generation.

3.2.7 In response to a mild stressor, preconception stressed offspring show increased plasma corticosterone levels.

We assessed plasma corticosterone levels following a mild stressor (2 min RET) in adult offspring (F1 mice) from stressed or control parents. Following the 2 min RET, offspring from preconception stressed parents had higher plasma corticosterone levels than offspring from control parents [t(8)=4.16, p=0.003, Figure 14B]. However, in the absence of a mild stressor (without the 2 min RET), there were no differences in plasma corticosterone levels across adult F1 mice groups (Figure 14C, p >0.05).

3.2.8 Exposure to a mild stressor induces c-FOS activation in several brain regions of offspring from stressed parents.

In response to a mild stressor (2 min RET), preconception stressed offspring showed increased c-FOS expression in the dentate gyrus [DENTRH; t(6)=8.24, p=0.0001, DENTLH; t(6)=20.25, p=0.0001, VDRH; t(6)=15.25, p=0.0001, and VDLH; t(6)=10.44, p=0.0001] and CA1 [CA1RH; t(6)=32.84, p=0.0001, CA1LH; t(6)=16.65, p=0.0001, VCA1RH; t(6)=16.22, p=0.0001, VCA1LH; t(6)=10.62, p=0.0001] compared to offspring from control parents (Figure 12B, 13B). There were no significant differences observed across groups in the BLA or CeA. See Figure 13B and Table 4].

3.2.9 In response to a mild stressor, preconception stressed adult offspring show altered GR and FKBP5 expression in several brain regions.

Following a mild stressor (2 min RET), we measured GR expression in preconception stressed and control offspring (F1 mice). Preconception stress increased GR expression in the dentate gyrus [DENTRH; t(8)=9.51, p=0.0001, DENTLH; t(8)=16.10, p=0.0001], CA1 [CA1RH; t(8)=14.07, p=0.0001, CA1LH; t(8)=10.41, p=0.0001], CA3 [CA3LH; (t(8)=3.49, p=0.013], the thalamus [Thla; t(8)=15.48, p=0.0001] and hypothalamus [Hypotha; t(8)=15.92, p=0.0001]

compared to controls (Figure 15C-D, 16B and See Table 4). There were no significant differences across groups in (CA2RH; CA2LH; CA3RH; BLA and mPFC, see Table 4).

In addition, following a mild stressor (2 min RET), we measured FKBP5 expression in preconception stressed and control offspring (F1 mice). Preconception stress increased FKBP5 expression in the dentate gyrus [DENTRH; $t(8)=5.77$, $p=0.0006$, DENTLH; $t(8)=39.44$, $p=0.0001$], CA1 [CA1RH; $t(8)=4.99$, $p=0.001$, CA1LH; $t(8)=11.35$, $p=0.0001$], thalamus [$t(8)=8.29$, $p=0.0001$] and BLA [$t(8)=8.55$, $p=0.0001$] compared to controls (Figure 17C-D and 18B). Interestingly, in offspring from control parents, a mild stressor increased FKBP5 in CA2 [CA2RH; $t(8)=2.92$, $p=0.02$, CA2LH; $t(8)=14.32$, $p=0.001$] and CA3 [CA3RH; $t(8)=8.73$, $p=0.0001$, CA3LH; $t(8)=6.32$, $p=0.0001$] compared to offspring from stressed parents (Figure 17C-D and 18B). There were no significant differences across group observed in the hypothalamus and mPFC (See Table 4).

3.3. Preconception predator stress-induced changes in second filial (F2) mice

3.3.1 Preconception predator stress increases anxiety- and depressive-like behaviours, decreases social interaction, and increases hyperarousal in F2 adolescent mice.

We assessed anxiety-like behaviour, hyperarousal, social interaction, depressive-like behaviours and spatial learning and memory in F2 mice. From the F1, we generated 4 groups of F2 mice: FC-MC, FC-MS, FS-MC, FS-MS (as described in section *2.3.7*).

In the EPM, there were significant main effects of F1-mother [$F(1,71)=17.81$, $p=0.0001$], F1-father [$F(1,71)=60.43$, $p=0.0001$], and F1-mother*F1-father interaction [$F(1,71)=5.18$, $p=0.026$] for ratio time and significant main effects of F1-mother [$F(1,71)=22.25$, $p=0.0001$] and

F1-father [F(1,71)=13.30, p=0.0005] for ratio frequency. In the LDB, there was a significant main effect of F1-father [F(1,71)=28.95, p=0.0001] for light side duration. For OF, there was a significant main effect of F1-father [F(1,71)=24.66, p=0.0001] and a F1-mother*F1-father interaction [F(1,71)=8.42, p=0.005] for center time and significant main effects of F1-mother [F(1,71)=21.67, p=0.0001], F1-father [F(1,71)=6.15, p=0.0155], and a F1-mother*F1-father interaction [F(1,71)=7.68, p=0.007] for distance travelled. In the SI test, there was a significant main effect of F1-father [F(1, 71)=17.99, p=0.0001] for social interaction ratio. In the FST, there was a significant main effect of F1-mother [F(1,71)=4.32, p=0.041] for time immobile. In the AST, there were significant main effects of F1-mother [F(1,71)=5.13, p=0.026] and F1-father [F(1,71)=17.73, p=0.0001] for peak startle amplitude (Figure 22 A-E, see Table 3).

To determine if there was a grandparental stress effect, planned comparisons were done comparing all three stressed groups (FC-MS, FS-MC and FS-MS) to the control group (FC-MC) across all behavioural measures (see Table 5A). Offspring who had at least one grandparent that was stressed (FC-MS, FS-MC, FS-MS) showed increased anxiety- and depressive-like behaviours, decreased social interaction, and hyperarousal compared to offspring with both grandparents who were not stressed (FC-MC). In the EPM, FC-MC mice spent more time in the open arms [ratio time: F(1,71)=68.54, p=0.0001] and entered the open arms more often [ratio frequency: F(1,71)=34.39, p=0.0001] than the three stressed groups. FC-MC mice spent more time in light side of the LDB [F(1,70)=5.59, p=0.0208], spent more time in the center of the OF [F(1,71)=27.35, p=0.0001] and travelled more in the OF [F(1,71)=33.90, p=0.0001. DC-MC mice spent more time with the social target [SI ratio: F(1,71)=5.23, p=0.0252] in the SI test, spent less time immobile [F(1,70)=5.23, p=0.0252] in the FST, and showed a lower peak startle amplitude [F(1,71)=7.16,

p=0.0092] in the AST compared to the three stressed groups. The results of these data suggest that preconception stress can have transgenerational effects on behavior.

To access whether there was a differential contribution from the grandmother and the grandfather to the grandchild's behavioural phenotype, planned comparisons were done comparing the FS-MC group to the FC-MS group (see Table 5B). The experience of the F1-paternal grandparents (if the grandparents were stressed) appears, in some cases, to drive the behavioural phenotype in the F2 generation. The FS-MC group spent less time in the open arms [ratio time: F(1,71)=5.50, p=0.0218] of the EPM, less time in the light side [F(1,70)=10.34, p=0.0020] of the LDB, less time in the center [F(1,71)=7.72, p=0.007] of the OF, and less time interacting with the social target [F(1,70)=9.39, p=0.0031] in the SI test than the FC-MS group. Finally, there was only one sex effect in light side duration in LDB [F(1,70) = 8.58, p=0.005].

3.3.2 Preconception predator stress alters behavior in the F2 mice following a mild stressor.

Around PND 60, all F2 offspring were exposed to the mild stressor (2 min RET). There were significant main effects of F1-mother [F(1,70)=11.80, p=0.001], F1-father [F(1,70) = 34.49, p=0.0001] and a F1-mother*F1-father interaction [F(1,70)=5.39, p=0.023] for freezing duration and a signification main effect of F1-father [F(1,70)=11.28, p=0.001] and a F1-mother*F1-father interaction [F(1,70)=4.68, p=0.034] for freezing frequency.

We next assessed anxiety-like behaviour, hyperarousal, social interaction depressive-like behaviour, and spatial learning and memory in adult offspring (F2 mice) from stressed or control grandparents. In the EPM, there were significant main effects of F1-mother [F(1,70)=77.72, p=0.0001], F1-father [F(1,70)=176.97, p=0.0001], and a F1-mother*F1-father interaction [F(1,70)=7.07, p=0.010] for ratio time and significant main effects of F1-mother [F(1,70)=47.16,

p=0.0001], F1-father [F(1,70)=93.19, p=0.0001], and a F1-mother*F1-father interaction [F(1,70)=17.74, p=0.0001] for ratio frequency. In the OF, there were significant main effects of F1-mother [F(1,70)=26.89, p=0.0001], F1-father [F(1,70)=78.72, p=0.0001] and a F1-mother*F1-father interaction [F(1,70)=6.87, p=0.011] for time spent in center and significant main effects of F1-mother [F(1,70)=34.92, p=0.0001], F1-father [F(1,70)=35.89, p=0.0001] for distance travelled. In the LD box, there was a significant main effect of F1-father [F(1,70)=75.51, p=0.0001] for light side duration. In the SI test, there was a significant main effect of F1-father [F(1,70)=21.56, p=0.0001] for SI ratio. In the FST, there was a significant main effect of F1-mother [F(1,70)=4.98, p=0.029] for immobile time. For AST, there were significant main effects of F1-mother [F(1,70)=22.09, p=0.0001], F1-father [F(1,70)=40.07, p=0.0001] for peak startle amplitude (Figure 23 A-J, see Table 3).

To determine if there was a grandparental stress effect, planned comparisons were done comparing all three stressed groups (FC-MS, FS-MC and FS-MS) to the control group (FC-MC) across all behavioural measures (see Table 5A). Offspring who had at least one grandparent that was stressed (FC-MS, FS-MC, FS-MS) displayed more species typical defensive behaviors (increased freezing frequency and duration) during the mild, 2 min RET than FC-MC mice [freezing duration: F(1,70)=47.34, p=0.001; freezing frequency: F(1,70)=197.97]. In the EPM, F2 offspring from control grandparents (FC-MC) spent more time in the open arms [F(1,70)=218.11, p=0.0001] and entered the open arms [F(1,70) =1806.40, p=0.0001] more often than mice from the other three stressed groups (FC-MS, FS-MC, FS-MS). In the OF, FC-MC F2 offspring spent more time in the center [F(1,71)=27.35, p=0.0001) and travelled more distance (F(1,71) =33.90, p=0.0001) than three stressed groups. In addition, FC-MC F2 offspring spent more time in light side [F(1,70) = 18.01, p=0.0001] of the LDB, showed increased social interaction [SI ratio:

F(1,70)=5.16, p=0.0261] in the SI test, spent less time immobile [F(1,70)=6.09, p = 0.0160], and showed decreased hyperarousal [peak startle amplitude: F(1,71)=33.34, p=0.0001] compared to offspring in the three stressed groups.

To access whether there was a differential contribution from the grandmother and the grandfather to the grandchild's behavioural phenotype, planned comparisons were done comparing the FS-MC group to the FC-MS group (see Table 5B). The experience of the F1-paternal grandparents (if the grandparents were stressed) appears, in some cases, to drive the anxiety-like behavioural phenotype in the F2 generation. In the EPM, FS-MC mice spent less time in the open arms than FC-MS mice [F(1,70)=7.97, p=0.0062]. Similarly, FS-MC mice spent less time in the center of the OF [F(1,70)=4.92, p=0.0297], spent less time in the light sight of the LD box [F(1,70)=31.52, p=0.0001], and spent less time in social interaction [social interaction ratio: F(1,70)=7.05, p=0.0098] than FC-MS mice. Note that there were two significant sex effects observed in ratio time measure of EPM [F(1,70)=10.580, p=0.002] and SI [F(1,70)=8.34, p=0.005].

Across the twelve days of water maze training, each group of mice learned the location of the hidden platform at a similar rate. During training, all groups travelled less distance [distance: F(11,572)=33.79, p=0.0001] and took less time [duration: F(11,572)=32.37, p=0.0001] to reach the platform across days. There were no significant group or group interactions (All Fs<1, all p>0.05; Figure 24, see Table 3).

During the probe trial, there were significant main effects of latency [F1-father: F(1,53)=4.42, p=0.04], and number of platform location cross [F1-mother: F(1,53)=10.46, p=0.002, see excel Table 3].

To determine if there was a grandparental stress effect, planned comparisons were done comparing all three stressed groups (FC-MS, FS-MC and FS-MS) to the control group (FC-MC; see Table 6A). Offspring who had at least one grandparent that was stressed (FC-MS, FS-MC, FS-MS) showed spatial learning deficits in the probe trial compared to offspring with both grandparents who were not stressed (FC-MC). During probe trial, offspring labelled FC-MC spent significantly more time in the target quadrant [$F(1,53)=11.05$, $p=0.0016$] which had contained the hidden platform than three stressed conditions. Platform latency [$F(1,53)=10.56$, $p=0.002$] and number of platform location cross [$F(1,53)=14.63$, $p=0.0003$] of FC-MC were significantly higher than stressed groups.

To access whether there was a differential contribution from the grandmother and the grandfather to the grandchild's behavioural phenotype, planned comparisons were done comparing the FS-MC group to the FC-MS group (see Table 6B). There were no significant effects found in this comparison.

Chapter 4: Discussion

It is well known that traumatic stress can have deleterious effects on an individual's mental health. However, recent data in both humans and animals suggests that the harmful effects of traumatic stress during one's lifetime can propagate into future generations (Blaze et al., 2015; Blaze and Roth, 2015; Dias and Ressler, 2014; Yahyavi et al., 2015; Yehuda, 2002; Yehuda et al., 2000, 2008), perhaps leading to an increased vulnerability to the development of psychopathology. The goal of this thesis was to examine the 'inter' and/or 'trans' generational effects of predator stress using a rodent model. We show that a single, acute exposure to a predator *prior to conception* produces lasting changes in brain and behavior in both the F1 and F2 generations. To our knowledge, this is the first demonstration that preconception predator stress produces inter- and trans-generational alterations in anxiety-like behavior, hyperarousal, social interaction, and spatial memory. Moreover, these behavioral changes are driven by the experience of the biological parents, and not the maternal social environment. Finally, we show stress induced-increases in neuronal activity in stress-sensitive brain areas, elevated stress hormone levels, alterations in stress hormone receptor levels, and dampened adult neurogenesis in offspring from preconception predator stressed parents. Our findings suggest generational effects of stress and open a new avenue for future research investigating the mechanisms underlying these phenotypic changes. Ultimately, the goal is to better understand the risk and resilience factors associated with trauma in order to identify novel pharmacological interventions to improve mental health.

4.1 Effects of predator stress on F0, F1, and F2 behavior

4.1.1 Predator stress activates the stress response and induces lasting changes in anxiety-like behavior (F0 generation).

Consistent with previous studies (Jakovcevski et al., 2008; Yang et al., 2004), mice show species-typical defensive behaviors (manifested as increased freezing behavior) when exposed to a predator (in this case, a rat). Following a single five minute interaction, rat-exposed mice show increased plasma corticosterone levels compared to control (non-rat-exposed) mice. When tested in the elevated plus maze two days after exposure, rat-exposed mice spend less time and avoid the open arms more often than control mice indicating increased anxiety-like behavior. Our results are consistent with many studies showing both increased corticosterone (Figueiredo, 2002; Figueiredo et al., 2002; Park et al., 2008; St-Cyr and McGowan, 2015) and lasting anxiety-like behavior following predator stress (Adamec, 1997; Adamec et al., 1980, 1998, 2005a, 2005b, 2006, 2010; Adamec and Shallow, 1993; Blundell et al., 2005; Cohen et al., 2018, 2009; Diamond et al., 2006; Fifield et al., 2013, 2015; Goswami et al., 2013; Lau et al., 2016; Lebow et al., 2012; Schöner et al., 2017; Zoladz et al., 2015).

4.1.2 Preconception predator stress increases anxiety-like behavior and hyperarousal in naïve adolescent and adult offspring (F1 generation)

Many studies, including the current one, report alterations in behavior following a traumatic stress (Bremner, 1999, 2006; Bremner et al., 1995; Iribarren et al., 2005; Sareen, 2016). Less is known about the effects of stress on future generations. Hence, we examined a suite of behavioral tests measuring a variety of behaviors including anxiety-like behavior, depressive-like behaviors, hyperarousal, social behavior, and novel spatial learning and memory in the offspring of stressed and control parents. We show that a single, five minute exposure to a rat *prior to conception* causes behavioral changes in offspring. During adolescence, offspring from predator stressed parents entered the open arms less often and spent less time in the open arms

of the elevated plus maze compared to offspring from control parents. In addition, adolescent offspring from stressed parents spent less time in the center and traveled less distance in the open field, and both spent less time and entered the light side of the light/dark box less often than offspring from control parents. Across multiple measures from three different anxiety-like behavioral tests, offspring from stressed parents thus showed increased anxiety-like behavior compared to offspring from control parents. In addition to increased anxiety-like behavior, offspring from stressed parents spent less time with a social stimulus (social interaction test) and showed enhanced hyperarousal (elevated mean peak startle amplitude) compared to offspring from control parents. Preconception predator stress, however, had no effect on depressive-like behaviors (as measured in the forced swim test) in adolescent offspring.

Several studies have examined the transmission of prenatal acute stress on adolescent offspring (Clarke and Schneider, 1993; Mulder et al., 2002; Sobrian et al., 1997; Takahashi, 1995; Takahashi et al., 2005; Ward and Weisz, 1980). Although most of the research was done with different stressors (*e.g.* restraint stress, fear conditioning, variable stressor etc.), they do show alterations in adolescent behavior (Berghänel et al., 2017; Lerch et al., 2016; Mommer and Bell, 2013, 2013; O'Brien et al., 2017). For example, rhesus monkeys who were exposed to stress from mid- to late-gestation had offspring with impaired neuromotor development, attention deficits, and disturbed behavior (Clarke et al., 1994; Clarke and Schneider, 1993; Schneider, 1992) . These effects were long-term and persisted into the adolescent period of development (Clarke et al., 1994; Clarke and Schneider, 1993; Schneider, 1992). In humans, similar effects of maternal prenatal stress have been reported (Gutteling et al., 2005; Mulder et al., 2002).

We also found that, when tested several weeks later (as adults), offspring from preconception stressed parents showed a similar behavioral phenotype suggesting that stress in the parents produces persistent behavioral changes in the offspring. Adult offspring from stressed parents avoided the open arms of the EPM (decreased ratio time and ratio frequency in open arms of EPM), spent less time in light side of the light/dark box, spent less time in the center of the OF. Furthermore, offspring from stressed parents showed enhanced hyperarousal compared to offspring from control parents. Overall, we show that preconception predator stress produces anxiety-like behaviors, social deficits, and hyperarousal in adolescent and adult offspring.

4.1.3 Preconception predator stress alters behavior in response to a mild stressor in adult offspring (F1 generation)

Post-traumatic stress disorder develops in response to a traumatic event and patients often show sensitized reactions to mild stressors associated with the trauma, a response more suitable for the original traumatic event (Bremner et al., 1995; Dykman et al., 1997; Friedman et al., 1995). Furthermore, children of people with PTSD are more likely to have psychiatric conditions such as PTSD (Copeland et al., 2007; La Greca, 2007; Silva et al., 2000). In light of these factors, we assessed the behavioral response to a mild stressor in offspring from stressed or control parents. The mild stressor was a single, two minute exposure to a rat. Unlike the five minute interaction, a single two min exposure to a rat does not produce lasting anxiety-like behaviour or hyperarousal in naïve mice suggesting that this is a 'subthreshold' or 'mild' stressor (See Table 3). We chose a milder version of our RET (5 min RET) to increase the likelihood that the mild stressor would produce a significant behavioral phenotype in the offspring. Also, Dias et al., 2014 showed that exposure to the same conditioned stimulus (novel smell that was associated to the

shock for the parents) produced a fear potentiated startle response in the offspring. Future studies, however, should assess the effects of other mild stressors (*e.g.*. mild restraint stress) on offspring behaviors.

We show that offspring from preconception stressed parents demonstrate increased sensitivity to a mild stressor compared to offspring from control (non-stressed) parents. Specifically, we found that offspring from preconception stressed parents froze longer and more often during the two minute rat exposure than offspring from control parents. Moreover, plasma corticosterone levels were higher following the two minute stressor in the offspring from stressed parents compared to controls. Note that plasma corticosterone levels did not differ between preconception stressed and control offspring in the absence of a mild stressor. Hence, the increase in corticosterone is in response to a stressor, not simply an increase in baseline plasma corticosterone. Increased defensive behaviors and elevated corticosterone levels in response to a mild stressor indicate an increased fear response in the offspring from stressed parents. Following the mild stressor, offspring from preconception stressed parents show increased anxiety-like behavior as measured in the elevated plus maze (decreased ratio time and frequency), open field (decreased time in center, decreased distance travelled), and light/dark box (decreased duration and frequency in light side) compared to offspring from control parents. There were no differences across group, however, in depressive-like behaviors as measured in the forced swim test. Consistent with our findings, several studies report that exposure to predator odor during pregnancy causes increased predator-avoidance behavior, alterations in social behavior, novelty-induced anxiety-like behaviours, and increased corticosterone levels in offspring (de Catanzaro, 1988; St-Cyr et al., 2017, 2018; St-Cyr and McGowan, 2015; Weinstock, 2007; Weinstock et al., 1988). Unlike these studies, however, our mice were exposed to the stressor 10 days prior to

conception. To our knowledge, the effects of preconception predator stress on offspring behavior have not been assessed. There is at least one study, however, that examined the effects of preconception stress on offspring behavior. In this case, mice were conditioned to a novel odor with shock or control condition, 10 days later, mice were bred and offspring response to the conditioned odor was assessed (Dias and Ressler, 2014). Preconception conditioned offspring showed an increased startle response to the novel odor (odor that the parents were conditioned to). Our results are in line with this study, showing that preconception stress can significantly alter offspring behavior.

Given that predator stress alters spatial learning and memory (Park et al., 2008), we also assessed behavior in the MWM following the mild stressor in offspring from preconception stressed or control parents. Despite a normal learning curve (duration and distance travelled to reach the platform) during training, offspring from preconception stressed parents showed memory deficits during the probe trial. Relative to controls, offspring from stressed parents exhibited greater latency to reach the platform, crossed the platform zone less often, and spent less time in the target quadrant indicating a spatial memory deficit. Moreover, while offspring from control parents preferred the target quadrant over the other three quadrants, this preference was not apparent in the offspring from stressed parents. It is unclear why mice that had a normal learning curve had deficits during the probe trial. However, across most measures on the probe trial, offspring from stressed parents were impaired – suggesting a 'real' impairment. When we looked at the last day of training, there was a slight but insignificant impairment in our offspring from predator stressed parents which may explain the probe results. Our results are inconsistent with findings from Hayashi and colleagues (Hayashi et al., 1998), who showed that prenatally stressed rats did not differ from controls during the probe trial. However, they did not expose offspring to

a stressor after birth. It is possible that the preconception predator stress produced a vulnerability to a mild stressor that resulted in the greater impairment seen on the MWM probe trial. This is consistent with our data that *without* the mild stressor (2 min RET), there was no difference in the MWM behavior (training or probe) across offspring.

Our studies indicate that preconception predator stress leads to behavioral changes in the offspring. It is unknown, however, if there is a differential contribution from the mother and father to the offspring phenotype. In the current studies, we only crossed stressed males to stressed females and control males to control females. Future studies should include the two additional crosses (contol male to stressed female, stressed male to control female). This cross will help identify if the F1 phenotype is dependent on one parent more than another. In the study by Dias et a. 2014, they only fear conditioned F0 males mated with naive females. Future studies including the additional crosses following the RET are currently underway in the lab. Furthermore, in our studies, we tested generational effects of predator stress on offspring 10 days (similar to that used by Dias et al. 2014) after predator exposure. Studies suggest that offspring phenotypes vary depending upon the stressors utilized, the timing of maternal stress exposure and importantly timing intervals between stress exposures, and breeding procedure. Therefore, future studies including different time intervals (for example, immediate, 20 days or 2 month after predator exposure) before mating F0 mice would be very informative.

4.1.4 Biology, and not maternal social environment, drives the offspring (F1 generation) phenotype

Nature versus nurture is one of the oldest debates in psychology. In rodents, the mother–pup interaction prior to weaning is a highly regulated function depending on both maternal hormones and stimulation by the nursing offspring(Crabbe et al., 2016; Fleming et al., 1999). Variations in maternal behaviour and/or other elements of the early environment are thought to alter the development of individual differences in behaviour and physiology in offspring. In contrast, a growing body of evidence suggests that parental environmental experiences, imprinted on the genome, may be passed on to alter offspring phenotypes(Dias and Ressler, 2014; Franklin et al., 2010; Ho and Burggren, 2010; Weaver et al., 2007). To determine if maternal social environment or parental experience contributes to the behavioral phenotype in offspring, we ran a cross-fostering experiment. Cross-fostering is a simple and highly effective technique for separating the effect of rearing environment from genetic (or epigenetic) influences on behavior. Three to four hours after birth, all offspring were removed from their biological mothers and placed with foster mothers. Foster mothers were either predator stressed or control. All foster mothers accepted the new pups and there were no differences in maternal behavior across foster mothers. Similar to the previous experiment, behavior was assessed in the offspring during adolescence and again following a mild stressor (2 min RET) in adulthood. We show that adolescent offspring whose biological parents were predator stressed, regardless of the foster mother condition (stressed or control), have increased anxiety-like behavior as measured in the elevated plus maze (decreased ratio time and ratio frequency), light/dark box (less time in the light side), and open field (less time in the center and travelled less distance), deficits in social behavior (decreased SI ratio), and enhanced hyperarousal (acoustic startle response). As adults, following a mild stressor (2 min RET), a similar behavioural phenotype was observed in the offspring. Specifically, offspring whose biological parents were stressed showed increased anxiety-like behaviors, social deficits,

and enhanced hyperarousal, regardless of foster mother condition, compared to offspring whose parents were controls. In the MWM, there were no differences across groups during training. However, during the probe trial, offspring whose biological parents were stressed, regardless of foster mother condition, showed longer latencies to reach the platform location, crossed the platform location less often, and spent less time in the target quadrant than offspring whose biological parents were controls. The spatial memory deficit during the probe trial is consistent with our previous experiment assessing the effects of preconception stress on spatial learning and memory.

Lack of foster mother influence on offspring behavior is consistent with our data showing that maternal behavior was similar across stressed and control mothers (foster and biological). Furthermore, our results support previous studies (Dias and Ressler, 2014; Kikusui et al., 2011) that behavioural alterations are inherited and not socially transmitted from the F0 generation (Faraji et al., 2018). Hence, we suggest that experience of the biological parent, and not maternal social environment, drives the offspring behavioral phenotype. In the future, *in-vitro* fertilization (IVF) can be used as an alternative method for this research. In mammals fertilization occurs in the maternal oviduct, where there are unique conditions for guaranteeing the encounter of the gametes and the first stages of development of the embryo and thus its future. During this period a major epigenetic reprogramming takes place that is crucial for the normal fate of the embryo. This epigenetic reprogramming is vulnerable to changes in environmental conditions such as nutrition, light, temperature, oxygen tension, embryo-maternal signaling, and the general absence of protection against foreign elements that could affect the stability of this process. In women the ovary normally produces one unique egg in each cycle. To count on more than one egg to perform IVF, the superovulation helps make the process more productive. Superovulation is the drug-

induced process of inducing a woman to release more than one egg in a month. Evidence suggests that the hormone dose used for this procedure seems to be important in the degree of methylation(Duffy et al., 2010; Schwarz et al., 2010; Uyar and Seli, 2014). To improve breeding, reproductive and productive efficiency in animal model, IVF will be an ideal alternative method for the transgenerational study. A substantial amount of research available in animals shows convincingly that IVF, produces significant epigenetic modifications, altering the expression of different genes, particularly of imprinted genes that have a major role in normal animal development.

4.1.5 Preconception predator stress produces transgenerational behavioral effects (F2 generation)

As discussed above, there is growing evidence in both humans and animals that stress effects can be transferred to the next generation. Whether these changes can be transferred to additional generations (i.e. F2, F3) is less well documented. There are a few studies in humans that report that offspring of Israeli Holocaust survivors have a higher incidence of PTSD, mood disorders, anxiety disorders, and substance abuse disorders three generations downstream (Barocas and Barocas, 1979; Dasberg, 1987; Dasberg and Sheffler, 1987; Scharf, 2007; Yehuda et al., 2008, 2015). This is consistent with the animal literature showing that the stress experienced by the F0 can affect the F2 offspring (Dias and Ressler, 2014; He et al., 2016; Schöpper et al., 2011). For example, prenatal maternal stress increased anxiety-like behavior in adult F2 male offspring (He et al., 2016; Schöpper et al., 2011). Similarly, preconception exposure to footshock altered behavior in the F2 generation (Dias and Ressler, 2014). To our knowledge, the effects of preconception predator stress, however, has not been assessed in the F2 generation. Thus, we

examined the effects of preconception predator stress on anxiety-, and depressive-like behaviors, social interaction, hyperarousal, and spatial learning and memory in the F2 generation. In this experiment, male offspring from stressed parents were bred to female offspring from stressed parents, male offspring from stressed parents were bred to female offspring from control parents, male offspring from control parents were bred to female offspring from stressed parents, male offspring from control parents were bred to female offspring from control parents which resulted in four groups of F2 mice. First, we compared offspring who had at least one set of grandparents that were predator stressed (three groups of offspring) to offspring whose both set of grandparents were controls (one group). We show that adolescent F2 offspring who had at least one set of grandparents that were predator stressed showed increased anxiety- (EPM, OF, LDB) and depressive-like behaviours (FST), decreased social interaction (SI ratio), and enhanced hyperarousal (acoustic startle) compared to offspring whose both sets of grandparents were controls. As adults, F2 offspring with at least one set of grandparents who were predator stressed showed increased freezing frequency and duration during the 2 min RET (mild stressor). Following the mild stressor, offspring with at least one set of grandparents who were predator stressed showed increased anxiety-like behavior (EPM, OF, LDB), decreased social interaction (SI ratio), and enhanced hyperarousal (acoustic startle) compared to offspring whose both sets of grandparents were controls. Despite a normal learning curve (duration and distance travelled to reach the platform) during MWM training, offspring who had at least one set of grandparents who were predator stressed showed spatial learning deficits in the probe trial compared to offspring whose both sets of grandparents were controls. Specifically, offspring with at least one set of grandparents who were predator stressed spent less time in the target quadrant, crossed the platform location less often and took longer to reach the target platform then offspring whose

bother sets of grandparents were controls. Next, we compared offspring whose paternal grandparents were predator stressed and maternal grandparents were controls to offspring whose paternal grandparents were controls and maternal grandparents were predator stressed. We show that during adolescence, offspring whose paternal grandparents were predator stressed and maternal grandparents were controls show increased anxiety-like behaviour (in the light/dark box and open field) and decreased social behavior (in the social interaction test) compared to offspring whose maternal grandparents were predator stressed and paternal grandparents were controls. Following the mild stressor, offspring whose paternal grandparents were predator stressed and maternal grandparents were controls show increased anxiety-like behaviour (in the elevated plus maze, light/dark box, and open field) and decreased social behavior (in the social interaction test) compared to offspring whose maternal grandparents were predator stressed and paternal grandparents were controls.

Our study supports previous studies where preconception (Dias and Ressler, 2014) or prenatal (Grundwald et al., 2016; McGowan and Matthews, 2018) parental traumatic experience influences behavioral phenotypes in F2 offspring. We are the first, however, to show that preconception predator stress alters behavior two generations downstream. Furthermore, our data suggest that the experience of the paternal grandparents play a larger role in grandchildren behavior than the experience of the maternal grandparents. Future experiments will cross predator stressed males with control females (and vice versa) to fully elucidate the contribution of the mother and father on offspring behavior.

4.1.6 Predator stress increases neuronal activity in stress-related brain areas

Next we examined neuronal activity following predator stress in the F0 and F1 generations. We show that a single, five minute exposure to a rat increases neuronal activity in the hippocampus of a mouse. Specifically, rat-exposed mice show increased c-FOS expression in the dentate (dorsal: right and left hemisphere; ventral: left hemisphere) and CA1 (ventral: right and left hemisphere; dorsal: right hemisphere) regions of the hippocampus compared to control mice. Our findings are consistent with several published studies which report increased c-FOS expression in the hippocampus in response to predator stress (Baisley et al., 2011; Lipski et al., 2017; VanElzakker et al., 2011). Surprisingly, we did not see increased c-FOS expression in the ventral dentate (right hemisphere) or dorsal CA1 (left hemisphere) in rat-exposed mice. Differential responses of the ventral and the dorsal hippocampus have been reported in the literature. For example, Pentkowski and colleagues showed that rats with ventral, but not dorsal, hippocampal lesions exhibited deficits in freezing and crouching when exposed to cat odor (Pentkowski et al., 2006). Moreover, ventral hippocampal CA1 lesioned mice exposed to coyote urine showed impaired avoidance and risk assessment behaviors (Wang et al., 2013). In contrast, coyote urine activates place cells in the dorsal CA1 (Wang et al., 2012). While our data, and others, shows a differential response of the dorsal versus the ventral hippocampus, future studies are necessary to fully understand whether the areas have overlapping or distinct roles in modulating predator stress-induced behavior. In addition to dorsal versus ventral, we also see differential c-FOS expression response across hemisphere. Future studies should include pharmacological inhibition or specific hemispheric lesion to fully understand predator stress- induced neural action.

Unlike the hippocampus, we show that a single, 5 minute exposure to a rat does not alter c-FOS expression in the amygdala (basolateral or central nuclei). This is consistent with Dielenberg and colleagues who reported that exposure to cat odor did not alter c-FOS expression

in the basolateral amygdala (Dielenberg et al., 2001; Dielenberg and McGregor, 2001). This is in contrast, however, to other studies that have demonstrated increased c-FOS expression or c-FOS mRNA levels in response to predator odours (Funk and Amir, 2000; Masini et al., 2005). Inconsistencies are also seen in lesioning studies. For example, while Wallace and Rosen (2001) showed that lesioning of the basolateral amygdala did not alter predator stress-induced behavior (Wallace and Rosen, 2001), others studies report altered freezing in response to predator odors as a result of lesioning or inactivating the basolateral amygdala (Müller and Fendt, 2006; Takahashi, 1995; Takahashi et al., 2005; Vazdarjanova et al., 2001). Similar to the basolateral amygdala, there are inconsistencies in the results of studies examining the role of the central amygdala in predator stress. Some studies suggest that the central amygdala is not involved in fear responses as indicated by a lack of an effect of central amygdala lesions on rat freezing behavior elicited by cat or fox odor (Fendt et al., 2005; Li et al., 2004; Rosen, 2004). In terms of c-FOS expression, studies have demonstrated no increase in c-FOS expression in the central amygdala following exposure to cat odor (Dielenberg et al., 2001; Dielenberg and McGregor, 2001) or ferret odor (Masini et al., 2005); whereas, another study reported a significant increase in the number of FOS-positive cells in the central amygdala in rats following fox odor exposure (Day et al., 2004). Clearly additional studies like pharmacological inhibition or specific brain area lesion are needed to fully understand the role of the amygdala in predator stress-induced behaviors.

While we did not examine the dorsal pre-mammillary nucleus, several studies have reported increased c-FOS expression in this area following exposure to a live cat or cat odor (Canteras et al., 1997; Dielenberg et al., 2001; Dielenberg and McGregor, 2001; McGregor et al., 2004). In addition, rats with dorsal pre-mammillary nucleus lesions showed a significant reduction in freezing to cat odor or to the presence of a cat (Blanchard et al., 2003a, 2003b; Canteras et al.,

1997; Markham et al., 2004, 2010). Hence, future studies should assess c-FOS in the pre-mammillary nucleus following the RET.

4.1.7 Neuronal activity following a mild stressor in preconception stressed offspring

As described above, preconception predator stress alters behavior in F1 offspring following a mild stressor. As a first step in identifying the neural mechanisms underlying this behavioral phenotype, we assessed c-FOS expression in the hippocampus and amygdala following a mild stressor in our F1 generation. We show that offspring from preconception predator stressed mice show increased c-FOS expression in the dentate and CA1 of the hippocampus following a mild stressor. In contrast, there were no differences in the amygdala (basolateral or central amygdala) across groups. In this study, similar brain area activation was observed in F0 and F1 generation mice. It is not surprising that that we see changes in neural activation in neurons known to be involved in the stress response in the F0 generation; these mice were exposed to a stressor that was sufficient to produce lasting changes in anxiety-like behavior. In the F1, we exposed the mice to a 2 min RET; a stressor that does not alter behavior in naïve mice. Nevertheless, in response to the mild stressor, offspring from preconception stress mice show robust alterations in c-FOS expression in stress-related brain areas. Our data suggest that the experience of the parents not only alters behavior of the offspring, but also neural activation. To our knowledge, this is the first demonstration that a mild stressor induces c-FOS expression in the hippocampus in offspring from preconception predator stressed parents. The data suggest that alterations in hippocampal neurons (notably in dorsal CA1 area; Franklin et. al., 2019), at least initially, may be important in the transmission of stress across generations.

4.1.8 Predator stress alters glucocorticoid receptors and FKBP5 expression

The glucocorticoid receptor (GR) is expressed in almost every cell in the body and regulates genes controlling the development, metabolism, and immune response. GR, also known as Nr3c1 (nuclear receptor subfamily 3, group C, member 1), is the receptor to which cortisol and other glucocorticoids bind. In the absence of cortisol or corticosterone hormone, GR resides in the cytosol complexed with a variety of proteins including heat shock protein 70 and 90 (hsp70 and 90), and the protein chaperone FK506-binding protein (FKBP5). Given that predator stress increases stress-related behaviors and plasma corticosterone, we assessed GR and FKBP5 expression two days after a single, 5 minute exposure to a rat or control condition in brain areas known to be important in the stress response (e.g. hippocampus, amygdala, hypothalamus, and medial prefrontal cortex). Rat exposure significantly increased GR expression in the dentate gyrus, CA1, and hypothalamus. In contrast, rat exposure suppressed GR expression in CA2, CA3, the thalamus and the basolateral amygdala (BLA). There were no differences between groups in the medial prefrontal cortex.

After stress, the HPA axis mediates the secretion of glucocorticoids (cortisol in humans and corticosterone in rodents) that bind to glucocorticoid (GR) receptors throughout the brain. Activation of these receptors serves as a negative feedback loop controlling or terminating the cellular stress response (Gjerstad et al., 2018). Imbalance in this feedback loop at any stage can result in short- and long-term detrimental effects in the brain, inducing neuronal death, slowed neurogenesis, weakened synaptic connections, and as well as impaired learning and memory processes (Shors et al., 2012). One key regulator for reducing the affinity of GR for glucocorticoids is the 51 kDa FK506-binding protein, also known as FKBP5/FKBP51 (Fries et al., 2017; Hähle et al., 2019; Pratt and Toft, 1997). Increased FKBP5 protein expression and common polymorphisms in the FKBP5 gene are significantly associated with generalized or tissue-specific GR resistance

or insensitivity to glucocorticoids (Charmandari et al., 2004). In addition, the interaction of early life stressors (e.g., childhood abuse) with several common FKBP5 allelic variations has been shown to increase susceptibility of many mental health disorders (Binder, 2009; Binder et al., 2008; Criado-Marrero et al., 2019), indicating that dysregulation of FKBP5 may contribute to a maladaptive stress response. Hence, we measured FKBP5 expression two days after the 5 min rat exposure or control condition. Predator stress increased FKBP5 expression in the dentate gyrus (right hemisphere), CA2 (right hemisphere), CA3 (left hemisphere), and basolateral amygdala. These data are consistent with animals exposed to chronic stress showed increased expression of FKBP5 as well as enhanced GR expression in hippocampus (Guidotti et al., 2013). This current study also revealed a hemispheric laterization effect in GR and FKBP brain expression. To further examine the hemispheric laterization in RET model, we should apply nostril occlusion (Galliano et al., 2020) during RET. Unilateral nostril occlusion (left or right) may help to clarify the hemispheric laterization effect of GR and FKBP expression in predator stressed mice.

We measured GR and FKBP5 expression at one time point: 2 days after predator stress exposure. We did this because our behavioural data showed changes 2 days after exposure. However, to our knowledge no studies have been conducted on GR expression changes over time. Therefore, future studies should include different time intervals (immdiate, 20 days or 2 month after predator exposure) for better understaining the effectiveness of RET among next generations, would be very informative.

4.1.9 GR and FKBP5 expression following a mild stressor in preconception stressed offspring

We next assessed GR and FKBP5 expression two days following the mild stressor (2 min RET) in offspring from preconception predator stressed and control parents. The pattern of GR and FKBP5 expression across brain areas in the F1 mice in response to the mild stressor is different than the pattern seen following exposure to the 5 min RET in naïve (F0) mice. Despite a robust anxiety-like behavioral response, GR expression is *decreased* in offspring from preconception stressed mice compared to offspring from control mice following the mild stressor in the dentate and CA1 regions of the hippocampus. In contrast, GR expression was significantly increased in CA3 (left hemisphere), the thalamus, and hypothalamus of offspring from preconception stressed parents compared to offspring from control parents. Furthermore, there were no differences in GR expression across F1 groups in the CA2, CA3 (right hemisphere), BLA and mPFC in response to the mild stressor. In addition, offspring from preconception stressed parents show increased FKBP5 expression in the dentate gyrus, CA1, thalamus, and BLA but decreased expression in CA2 and CA3 compared to offspring from control parents. There was no significant differences in hypothalamus and mPFC area between across groups.

Interestingly, following the mild stressor (2 min RET), offspring from preconception stressed mice show increased FKBP5 expression in the dentate and CA1 of the hippocampus, yet decreased GR expression in these areas, compared to offspring from control parents. Decreased GR expression may be due to impaired ability of the receptor to translocate to the nucleus and activate GR-dependent transcriptional mechanisms. It may be that FKBP5 acts as a negative feedback regulator of the GR, so high levels of FKBP5 reduce available GRs and promote glucocorticoid resistance, which refers to a decrease in the sensitivity of target tissues to cortisol. After 2 min RET we showed that increased FKBP5 expression in in F1 stressed mice is associated with decreased GR levels in the hippocampus, which may be suggestive of an impaired ability of

the receptor to translocate to the nucleus and activate GR-dependent transcriptional mechanisms. Though our results suggest a relationship between acute RET and brain region specific glucocorticoid receptor or FKBP expression, the molecular mechanisms underlying this mechanism remain largely unknown. I predict that FKBP5 is acting as a negative-feedback regulator of the GR, so high levels of FKBP5 reduce available GRs and promote glucocorticoid resistance (or a decrease in the sensitivity of target tissues to cortisol). Studies suggest that the stress response is finely tuned through a series of mechanisms that control the trafficking of glucocorticoid receptors (GRs) from cytoplasm to the nucleus, including binding to the chaperone protein FKBP5 and receptor phosphorylation, suggesting that these elements may also be affected under pathologic conditions (Guidotti et al., 2013). On these bases, future studies should include investigation of FKBP5 and GR translocation and trafficking mechanisms from cytoplasm to nucleus over time in the different brain regions of rodents and their offspring exposed to predator stress.

4.1.10 Sex differences following stress

PTSD consistently emerges as one of the most sex differentiated psychiatric disorders. PTSD animal models are based on exposure to an acute stressor shows sex differences in acute stress responses, as well as in associative learning and extinction of aversive conditioned responses (Cohen and Yehuda, 2011; Cohen and Zohar, 2004; Dalla and Shors, 2009; Olff et al., 2007). Prior to 2006, almost all of the experiments on the effects of prenatal stress in rodents were performed only on male offspring (Weinstock, 2007). Since then, only a few have assessed the effect of prenatal stress in both sexes. In adult female rats, no effect was found on the rate of

learning (Weinstock, 2011; Zuena et al., 2008), but it was slower than in controls in pre-pubertal females (Weinstock, 2011). Others showed a deficit in memory consolidation in the passive avoidance test in rats of both sexes (Palacios-García et al., 2015). In adult males of the SD and Wistar strains, prenatal stress slowed the rate of acquisition of spatial learning and memory retention, irrespective of the nature of the maternal stressor (Barzegar et al., 2015; Liu et al., 2012; Markham et al., 2004, 2010; Modir et al., 2014; Ratajczak et al., 2015; Schulz et al., 2011). In contrast to the findings in the majority of studies, thrice-daily restraint improved learning in the MWM test in adult SD male offspring (Zuena et al., 2008). Prenatal stress also reduced spatial learning but not memory retention in pre-pubertal and adult male C57/BL mice (Zhao et al., 2013), or in adults of both sexes (Benoit et al., 2015a, 2015b). In the fear-conditioning paradigm, animals are trained to associate a conditioned stimulus (CS), such as a cue (e.g. tone) or a context with an unconditioned stimulus (US) of aversive nature, such as a footshock showed sex differences. In contextual fear conditioning, female rats show less learned freezing behaviour than male rats (Maren et al., 1997; Pryce et al., 1999). Whereas in cue fear conditioning, male rats again exhibit more conditioned fear than female rats, either when freezing or when ultrasonic vocalizations are used as a CR (Kosten et al., 2005; Maren et al., 1997; Pryce et al., 1999). In the fear-potentiated startle paradigm, the rats learn that the presence of a cue, such as a light is paired with a footshock. Therefore, when they are later exposed to the light without the footshock, they show enhanced startle reflex in response to a sudden noise (Davis, 2006). In this paradigm intact females show a greater potentiation of startle than males (De Jongh et al., 2005). Sex differences have also been found in adult rodents after receiving chronic stressor. Maternal deprivation and chronic unpredictable variable stress caused decreased anxiety-related behaviour in females and increased anxiety behaviour in male rats (Renard et al., 2005). Male and female rodents received

21 days of daily 6h restraint stress significantly impairs male and enhances female radial arm maze performance (Luine et al., 2017). In an object placement task, stressed males could not discriminate the old from new location which suggests that stress impairs male and enhances female spatial memory (Luine et al., 2017). Moreover, acute or chronic foot shock stress exposure decreases pCREB in dentate gyrus (DG) and prelimbic area (PL) of prefrontal cortex (PFC) in male rats whereas not in female rats. However, open-field tests showed no behavioural difference between male and female rats following stress (Lin et al., 2008). Though data suggesting that acute or chronic stress does not elicit identical behavioural responses in males and females, in recent study Shaw et al. showed that chronic repeated predatory stress increased anxiety-like behaviours in the open field in both male and female mice as compared to control mice (Shaw et al., 2020). Overall, it seems that sex differences in the various PTSD animal models depend on the testing paradigm, testing condition, and the behavioural responses that are assessed. Interestingly, the prevalence rates of severely affected male and female rodents are the same. However, males and females respond in a different way, probably because baseline stress levels are higher in females and the magnitude of response is lower (Cohen et al., 2014; Cohen and Yehuda, 2011; Mazor et al., 2009). In the current study, both in the F0 and F1 stressed mice, there were almost no sex differences in behavioral responses suggesting that male and female mice react similarly to the RET. It may be that other behavioral measures, not assessed in the current study would show sex differences but given the variety of behavioral measures assessed, this is unlikely. In light of the lack of sex differences across behavioral tests, brain changes were only assessed in male mice (F0 and F1 generations). Nevertheless, future studies should assess changes in brain activation and function in both males and females.

4.2 Epigenetics as future research

4.2.1 Epigenetics and PTSD

Given our cross-fostering data which indicates that the maternal social environment plays little part in the offspring phenotype, it is likely that the offspring phenotype is due to epigenetic mechanisms. Indeed, epigenetic modifications have been espoused to help explain the transmission of experience to future generations. Epigenetic modifications are changes in the transcriptional potential of a cell by the environment, which occurs independent of alterations in the gene sequence. Epigenetic modifications provide a mechanism that links genes and environment, while playing an important role in the modulation of behavioral responses to stress (Day and Sweatt, 2011). Although the mechanism by which epigenetic modifications lead to the transmission of environmental influences to subsequent generations is not known, one possibility involves the transmission of DNA methylation (Day and Sweatt, 2011; Dias and Ressler, 2014; Meaney, 2001).

DNA methylation is one of the most broadly studied and well-characterized epigenetic modifications. DNA methylation plays a significant role in the maintenance of cellular identity and heritable changes in gene expression throughout the cell cycle, typically by prohibiting DNA transcription by the addition of a methyl group to a gene promoter region. Indeed, DNA methylation may be important in fundamental mechanisms for the induction and stabilization of PTSD (Zovkic et al., 2013).

Several studies have identified alterations in methylation states of specific genes in people with PTSD. Of the gene methylation studies in people with PTSD, the most commonly studied target is the glucocorticoid receptor (GR). Labonté and colleagues found that individuals with

lifetime PTSD had lower morning cortisol release, higher mRNA expression of the human GR (hGRtotal, 1B, and 1C) and lower overall methylation levels in hGR 1B and 1C promoter regions (Labonté et al., 2014). Similarly, lower *Nr3c1* 1F promoter methylation was observed in combat veterans with PTSD compared with combat veterans without PTSD (Yehuda et al., 2014). Interestingly, DNA methylation of the Nr3c1 promotor appears to be sex-specific as methylation at the *Nr3c1* 1F promoter was linked to traumatic memories and PTSD risk in male, but not in female genocide survivors in Rwanda (Vukojevic et al., 2014). However, much more research is necessary to fully assess the role of sex on DNA methylation of specific targets in people with PTSD.

As described by Klengel and colleagues, it was once thought that DNA methylation was completely erased during development of primordial germ cells and during fertilization (Klengel et al., 2016). However, this erasure, or reprogramming, appears not to be complete. Evidence for certain loci that escape reprogramming is growing with examples including imprinted genes and repetitive elements (Kobayashi et al., 2013; Radford et al., 2014; Tang et al., 2015). Hence, altered GR promotor methylation may be one mechanism by which parental stress is translated into changes in gene expression and physiology, ultimately resulting in psychologically vulnerable offspring phenotypes. Perroud and colleagues examined the impact of the Tutsi genocide on the children of women who were pregnant while genocide was ongoing in Rwanda (Perroud et al., 2014). In 2011, more than 20% of the Rwandan population met the criteria for PTSD. Peripheral blood leukocytes were obtained and methylation levels of the promoter regions of the glucocorticoid receptor *NR3C1* was examined in trauma exposed woman and their children. As expected, both mothers exposed to genocide and their children had significantly higher levels of PTSD than the control group. They also showed higher methylation levels at exon 1F promoter of

Nr3c1, at CpG3-CpG9. Furthermore, there was a negative correlation between *Nr3c1* methylation and glucocorticoid levels in plasma (Perroud et al., 2011, 2014). Changes in methylation of the GR receptor appears to be dependent on parental status of PTSD. Offspring with just paternal PTSD showed higher GR-1F promoter methylation, whereas offspring with both maternal and paternal PTSD showed lower methylation (Yehuda et al., 2014). Interestingly, in comparison to demographical controls, Holocaust survivors showed increased methylation of the promotor region for FK506 binding protein 5 (FKBP5), a protein that lowers the affinity of cortisol when it is bound to GR; thereby potentially hindering the negative HPA axis feedback loop (Binder, 2009), while methylation at this site was lower in Holocaust survivor offspring (Yehuda et al., 2016). Although gender-specific effects cannot be disentangled in this study, Yehuda et al., suggest that FKBP5 hypermethylation, leading to decreased FKBP expression and increased GR sensitivity in the F0 mothers, may result in lowered circulating glucocorticoid levels during pregnancy, promoting demethylation in the fetus to optimize or increase glucocorticoid levels (Yehuda et al., 2016). Alternatively, preconception or postnatal social influences may also influence offspring cortisol levels, and hence regulate FKBP methylation levels. Currently, whether changes in glucocorticoids in offspring reflect intergenerational consequences of parental exposure or offspring recalibration of glucocorticoid regulation is not known.

While epigenetic studies investigating the intergenerational effects of stress have primarily looked at changes in the methylation state of GR, recent reports suggest that other targets may play a role in this transmission (see Table 7). While methylation of the *Bdnf* gene promoter region has been associated with the development of PTSD (Kim et al., 2017; Voisey et al., 2019), a recent report suggests that maternal trauma exposure may be linked to high *Bdnf* methylation levels in offspring (Kertes et al., 2017). Among 24 mothers and newborns in the eastern Democratic

Republic of Congo, a region with extreme conflict and violence to women, maternal experiences of war trauma and chronic stress were associated with higher *Bdnf* methylation in umbilical cord blood, placental tissue, and lower methylation in maternal venous blood. While the studies described above examined parental and first-generation methylation status of specific genes, Serpeloni and colleagues examined the grandchildren of grandmothers exposed to psychosocial stress during pregnancy (Serpeloni et al., 2017). Grand maternal exposure to community and domestic violence during pregnancy was significantly associated with decreased methylation of the *CORIN* (corin, serine peptidase), *SMYD3 (*SET and MYND domain-containing protein 3, is a histone methyltransferase*),* and *BARX1* (BARX Homeobox 1 is a protein coding gene) genes, as well as increased methylation of the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene in the grandchildren. *CFTR* and *CORIN* genes are involved in circulatory system processes and congenital abnormalities and dysregulation of these genes impact the release of vitamin D, blood pressure regulation, heart failure and hypertension. *SMYD3* and *BARX1* genes are involved in embryonic development, craniofacial development, odontogenesis, and stomach organogenesis. The exact role methylation of these genes plays in the intergenerational effects of stress, however, has not be elucidated.

In all, there appears to be strong support for epigenetic regulation, specifically DNA methylation, in human PTSD (see Table 7). Furthermore, evidence suggests that these epigenetic changes may be passed on to future generations and lead to a vulnerability to psychiatric illnesses in the offspring. Although these findings are exciting, several limitations should be noted. For instances, low sample size, age variability, a lack of replication and sufficient controls, and a paucity of information on sex effects/differences and ancestry information (Lacal and Ventura, 2018; Rady, 2010; Zannas et al., 2016) plague these studies. Furthermore, maternal and paternal

lineages may have differing effects on the epigenetic transmission of stress. Moreover, the exact nature of the transmission is dependent on which parent underwent the stressor (and when) as the maternal lineage can be observed in the F0, F1 and F2 generations, and the transgenerational phenotype in F3, whereas the paternal lineage can be seen in the F0 and F1 generations, and the transgenerational phenotype in F2 (Bale, 2011; Gabory et al., 2009). Hence, more research is necessary to fully understand the role DNA methylation plays in the development of, or vulnerability for, PTSD. In addition to these issues, there are several confounds that must be considered in the human research that question the role of epigenetics in the transmission of stress effects across generations. For instance, it may be that the children of survivors of trauma adopt ineffective coping techniques like their parents. Alternatively, the children may have shared trauma with their parents, and/or been exposed to parental PTSD symptoms, or experienced parental emotional abuse or neglect. These, and others not listed, may contribute to the increased likelihood of a child developing a stress-induced psychopathology (Lehrner et al., 2014; Sturge-Apple et al., 2012; Yehuda et al., 2005). Both ethical limitations and the logistical constraints associated with human research limit full understanding of the mechanisms underlying these transgenerational effects. For this reason, recent efforts have been made to assess epigenetic effects of stress in rodents.

4.2.2 Epigenetic modifications: DNA methylation and predator stress

As can be seen in Table 8, several studies have identified alterations in methylation states of specific genes following predator stress. For instance, *Dlgap2*, a gene that encodes a postsynaptic density protein, is more likely to be unmethylated in animals that display an anxious phenotype following the predator stress paradigm (Chertkow-Deutsher et al., 2010). Similarly,

predator stress induces phenotypic variability in stress coping responses that can be linked to the degree of methylation of the hormone vasopressin (*Avp*) in the amygdala (Bowen et al., 2014). However, it is currently unknown whether changes in Dlgap2 and Avp DNA methylation persist into future generations.

Similar to human literature described above, methylation of the GR and Fkbp5 appear to play a role in the transmission of predator stress effects to future generations (see Table 8). Female offspring from prenatal predator odor-exposed dams showed increased transcript abundance of both the glucocorticoid receptor gene (*Nr3c1*; on the day of birth) and *Fkbp5* (in adulthood) in the amygdala (St-Cyr et al., 2017). Moreover, increased *Fkbp5* expression was inversely correlated with decreased DNA methylation for this product's gene (St-Cyr et al., 2017), a finding consistent with the human literature (Yehuda et al., 2016). In a related study, female offspring of mice exposed to predator odor during pregnancy had decreased *Bdnf* transcript abundance which was positively correlated with a concomitant decrease in DNA methylation of *Bdnf* exon IV in the hippocampus (St-Cyr and McGowan, 2015). Epigenetic alterations of the *Bdnf* gene have been linked to impaired brain functioning, memory, stress, and neuropsychiatric disorders (Andero et al., 2013, 2014; Fuchikami et al., 2010; Ikegame et al., 2013). These results are consistent with other work in which predator scent stress induced a significant down-regulation of *Bdnf* mRNA in the CA1 region of the hippocampus (Kozlovsky et al., 2007). Hypermethylation of hippocampal *Bdnf* DNA may be a cellular mechanism underlying the persistent hippocampus-specific cognitive deficits which are prominent features of the pathophysiology of PTSD. Indeed, selective hypermethylation of the *Bdnf* gene in the dorsal hippocampus appears to be an important component of local synaptic structure, plasticity, and maintenance of intrusive memories of the trauma in an active state following exposure to a life-threatening event (Zoladz et al., 2012).

Despite these findings, more research is necessary to fully assess the role of DNA methylation in the transmission of stress effects across generations (Blouin et al., 2016).

The current study reveals changes of GR and FKBP5 expression in different brain regions in response to traumatic stress, and these changes appear to be transferred to their offspring. Hence, our current animal model will open new avenue to fully understand the role of GR and FKBP5 methylation in the development and vulnerability to mental illness. In light of the literature described above, future studies assessing BDNF expression in the F0 and F1 generation are also warranted.

4.3 Conclusion

It is well known that traumatic stress can have deleterious effects on an individual. Recent data suggests that these harmful effects can propagate into future generations, making offspring more prone to mental illness (i.e. anxiety, depression and/or posttraumatic stress disorder). While recent data from the animal literature supports inter- and trans-generational effects of stress, little is known regarding the consequences of pre-conception stress on offspring. Furthermore, whether these changes persist into adulthood and make offspring more susceptible to future stressors is not known. Overall, our data suggest that traumatic stress not only affects an individual, but it can alter the brain and behavioural responses of future generations. Ultimately, identification of the mechanisms that promote anxiety in children, as well as increased stress-susceptibility in adulthood, will represent a major advance in the field, and may lead to novel treatments for such devastating, and often treatment-resistant disorders.

Figure Captions

Figure 1. **Breeding protocol followed by 5 min rat exposure test (RET).** F0: parent generation and F1: first filial generation. Male and female mice (F0) were exposed to a live rat (RET) in the exposure chamber while mice in the control group were exposed to an empty chamber (similar to habituation days). Full details of the habituation and rat exposure test (RET) can be found in section 2.4.1. Two days after the RET, all mice were tested on the elevated plus maze (EPM) to evaluate anxiety-like behaviour. A description of the EPM can be found in section 2.4.2. Ten days after the RET (or 8 days after the EPM), male control mice were bred to female control mice and male predator stressed mice were bred to female predator stressed mice. Approximately twenty days later, babies (F1) were born.

Figure 2. A schematic of the F1 behavioural procedure. Behavioural testing for F1 mice (F1 stressed mice: PS and F1 control mice) began at PND 24. All offspring underwent a behavioural battery for six days (one test/day) starting with the EPM, followed by the open field (OF), light/dark (LD) box, acoustic startle (AS), forced swim (FS), and the social interaction (SI). A detailed description of the behavioural battery can be found in section 2.4. Seventeen days later (PND 55), mice began habituation for the RET. After five days of habituation, all F1mice were exposed to a rat (PND 60) for two minutes (2 min RET exposure). Note that this was the first exposure to a rat by F1 mice. Two days later (PND 62), all offspring underwent a second behavioural battery identical to the first one.

Figure 3. **Experimental protocol to identify the role of the maternal social environment on offspring behaviour.** The exact same protocol was followed for the F0 and F1 mice as described in experiment 1 excepting the fostering procedure. Fostering was performed 3–4 h after parturition. All offspring were fostered to either a control mother or were fostered in four different groups (mentioned in section *2.3.3*). Fostered mice underwent a baseline behavioural battery. On PND 60, fostered mice were exposed to 2 min RET exposure. Two days later (PND 62), all offspring underwent a second behavioural battery including MWM.

Figure 4. **Experimental protocol to the effects of preconception predator stress on second filial generation offspring behavior.** Second filial generation (F2) animals are the offspring from the mating of F1 mice. F1 mice were generated as described in experiment 1. At weaning, however, F1 mice did not undergo the behavioral test battery; instead the mice were left undisturbed. At PND 60, mice were bred to produce four F2 groups (mentioned above section *2.3.7*). The F2 generation followed the exact same behavioural protocol as the F1 generation described in experiment 1.

Figure 5. **Rat Exposure Test (RET) in exposure chamber.**

Figure 6. **Protocol for molecular measures. 1)** To measure neural activation, mice were anesthetized and perfused 90 min (for c-FOS) after the 5 min RET or 2 min RET (F1 mice). **2)** To measure GR and FKBP5 activation, mice were anaesthetized and perfused two days after the RET or 2 min RET (F1 mice).

Figure 7. **Five min RET produces lasting changes in anxiety-like behaviour.**

Mean + SEM of freezing duration (A) and frequency (B) plotted over four groups: predator stressed males (PS-M), predator stressed females (PS-F), control males (C-M), control females (CF). Mice freeze longer (A) and more frequently (B) when exposed to a predator compared to mice exposed to an empty cage. Predator stressed mice spend less time (C) and enter (D) the open arms than controls. C: control, PS: predator stressed, M: male, F: female, F0: parent generation. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 8. **Preconception predator stress produces anxiety-like behaviour in adolescent offspring (F1 mice).** Mean + SEM are plotted over four groups: predator stressed adolescent males (PS-M adol), predator stressed adolescent females (PS-F adol), control adolescent males (C-M adol), control adolescent females (C-F adol). Offspring from predator stressed (PS) parents spend less time (panel A) in the open arms and enter (panel B) the open arms of the elevated plus maze less often than controls. Offspring from PS parents spend less time (panel C) and enter (panel D) the light side of the light/dark box than controls. Offspring from PS parents spend less time in the center (panel E) and travel less distance (panel F) in the open field than controls. Offspring from PS parents interact less with a novel conspecific than controls (panel G). Offspring from predator stressed parents show increased peak startle amplitude (panel H) compared to controls. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 9. **Preconception predator stressed F1 offspring show increased anxiety-like behaviour when exposed to a mild stressor in adulthood.** Mean + SEM are plotted over four groups: predator stressed adult males (PS-M adult), predator stressed adult females (PS-F adult), control adult males (C-M adult), control adult females (C-F adult). Offspring from predator stressed (PS) parents freeze longer (panel A) and more often (panel B) during a 2 min exposure to a rat (2 min RET) than controls. PS offspring spent less time in the open arms (panel C) and entered (panel D) the open arms of the elevated plus maze less often than controls after exposure to the 2 min RET. Offspring from PS parents entered less often (panel E) and spent less time (panel F) in the light side of the light/dark box than controls after exposure to the 2 min RET. Offspring from PS parents spend less time in the center (panel G) and travel less distance (panel H) in the open field than controls after exposure to the 2 min RET. Offspring from predator stressed parents show

increased peak startle amplitude (panel H) compared to controls after exposure to the 2 min RET.. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 10. **Two min predator exposure improved MWM performance across training trials and produced spatial memory deficits on the probe trial in the offspring of predator-stressed mice**. Mean + SEM are plotted over two groups. All F1-mice travelled significantly shorter distances (Panel A) and took less time (Panel B) to reach the platform over trials. During the probe trial, control-F1 mice spent significantly more time in the target quadrant (Panel C) than mice in the stressed conditions. Stressed-F1 mice had a slower platform latency (Panel D), whereas control-F1 mice crossed the platform location more often (Panel E) than mice in the stressed group. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 11. **Preconception predator stressed F1 mice show increased anxiety-like behaviour in the absence of mild stress exposure.** Mean + SEM are plotted over four groups: predator stressed adult males (PS-M adult), predator stressed adult females (PS-F adult), control adult males (C-M adult), control adult females (C-F adult). Offspring from predator stressed (PS) parents spend less time (panel A) in the open arms and enter (panel B) the open arms of the elevated plus maze less often than controls. Offspring from PS parents spend less time (panel C) in the light side of the light/dark box than controls. Offspring from PS parents spend less time in the center (panel D) and travel less distance (panel E) in the open field than controls. Offspring from predator stressed parents show increased peak startle amplitude (panel F) compared to controls. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$. Means marked with the same letter do not differ; means marked with different letters differ (for panel C).

Figure 12A. **Predator stress induced c-FOS expression after 5 min acute predator stress (PS) exposure.** c-FOS expression was identified in the hippocampus area within a) dentate gyrus: DENTRH, DENTLH; b) CA1 area: CA1RH, CA1LH; c) CA2 area: CA2RH, CA2LH and CA3 area: CA3RH, CA3LH area. c-FOS staining images were represented for control and stressed-F0 mice.

Figure 12B. **Predator stress induced c-FOS expression after 2 min acute predator stress (PS) exposure in F1 adult offspring.** c-FOS expression was identified in the hippocampus area within a) dentate gyrus: DENTRH, DENTLH; b) CA1 area: CA1RH, CA1LH; c) CA2 area: CA2RH, CA2LH and CA3 area: CA3RH, CA3LH area. c-FOS staining images were represented for control and stressed-F1 mice.

Figure 13: **predator stress induced c-FOS activation after 5 min acute predator stress exposure and 2min mild stress exposure on their offspring.** Mean + SEM are plotted over different brain regions: Panel A represents significantly elevated c-FOS activation after 5min exposure to a predator in different regions within dentate gyrus: DENTRH, DENTLH, VDLH and b) CA1 area: CA1RH, VCA1RH, and VCA1LH. But with no significant difference were identified in the dentate gyrus: VDRH, CA1 area: CA1LH, amygdala: BLA and CeA between the stressed-

F0 and control-F0 groups. Panel B shows preconception predator stressed F1 offspring show increased c-FOS activation when exposed to a mild stressor in adulthood. a) dentate gyrus: DENTRH, DENTLH, VDLH, VDRH and b) CA1 area: CA1RH, CA1LH, VCA1RH, VCA1LH. There was no significant difference was observed in the BLA and CeA between stressed-F1 and control-F1 groups. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 14. **Corticosterone assay.** Mean + SEM are plotted over two groups: Panel A represents corticosterone assay after 5min exposure to a predator. Five min exposure to a predator increase plasma corticosterone. Panel B shows preconception predator stressed F1 offspring show increased serum corticosterone when exposed to a mild stressor in adulthood. In the absence of mild stressor, there was no significant increase of corticosterone observed in preconception predator stressed adult offspring compared with control (Panel C). Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 15A. **predator stress induced GR expression after 5 min acute predator stress exposure.** GR expression was identified in the hippocampus area within a) dentate gyrus: DENTRH, DENTLH; b) CA1 area: CA1RH, CA1LH; c) CA2 area: CA2RH, CA2LH and CA3 area: CA3RH, CA3LH area. Dapi and GR staining images were represented for control (**i-viii**) and stressed-F0 mice (**ix-xvi**).

Figure 15B. **predator stress induced GR expression after 5 min acute predator stress exposure.** GR expression was identified in the hippocampus area within a) thalamus; b) hypothalamus; c) basolateral amygdala (BLA) and d) medial prefrontal cortex (mPFC). Dapi and GR staining images were represented for control (**xvii-xxiv**) and stressed-F0 mice (**xxv-xxxii**).

Figure 15C. **predator stress induced GR expression after 2 min acute predator stress exposure in F1 adult offspring.** GR expression was identified in the hippocampus area within a) dentate gyrus: DENTRH, DENTLH; b) CA1 area: CA1RH, CA1LH; c) CA2 area: CA2RH, CA2LH and CA3 area: CA3RH, CA3LH area. Dapi and GR staining images were represented for control (**i-viii**) and stressed-F1 (**ix-xvi**) mice.
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Figure 15D. **predator stress induced GR expression after 2 min acute predator stress exposure in F1 adult offspring.** GR expression was identified in the hippocampus area within a) thalamus; b) hypothalamus; c) basolateral amygdala (BLA) and d) medial prefrontal cortex (mPFC). Dapi and GR staining images were represented for control (**xvii-xxiv**) and stressed-F1 mice (**xxv-xxxii**).

Figure 16: **predator stress induced GR expression after 5 min acute predator stress exposure and 2min mild stress exposure on their offspring.** Mean + SEM are plotted over different brain

regions: Panel A represents significant increase of GR expression was identified in different locations within a) dentate gyrus: DENTRH, DENTLH and b) CA1 area: CA1RH, CA1LH predator stressed-F0 groups compared with control. Significantly elevated GR expression also observed in hypothalamus (Hypotha) brain area in stressed-F0 group. Surprisingly, in the control (no stress) mice, elevated GR expression was observed in CA2 area: CA2RH, CA2LH and CA3 area: CA3RH, CA3LH, thalamus (Thla) and BLA compared with stressed-F0 group. But no significant difference was identified in the medial prefrontal cortex (mPFC) between stressed-F0 and control-F0 groups. Panel B shows GR expression were significantly elevated within dentate gyrus and CA1 area of hippocampus in stressed-F0 group, whereas 2 min RET significantly increased GR expression in the similar area of control-F1 offspring. There were no significant differences between stressed-F1 and control-F1 groups in CA2 area: CA2RH, CA2LH and CA3 area: CA3RH, BLA and mPFC. GR expression only significantly increased in CA3LH area of stressed-F1 offspring. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 17A. **predator stress induced FKBP expression after 5 min acute predator stress exposure.** FKBP expression was identified in the hippocampus area within a) dentate gyrus: DENTRH, DENTLH; b) CA1 area: CA1RH, CA1LH; c) CA2 area: CA2RH, CA2LH and CA3 area: CA3RH, CA3LH area. Dapi and FKBP staining images were represented for control (i-viii) and stressed-F0 mice (ix-xvi).

Figure 17B. **predator stress induced FKBP expression after 5 min acute predator stress exposure.** FKBP expression was identified in the hippocampus area within a) thalamus; b) hypothalamus; c) basolateral amygdala (BLA) and d) medial prefrontal cortex (mPFC). Dapi and FKBP staining images were represented for control (xvii-xxiv) and stressed-F0 mice (xxv-xxxii).

Figure 17C. **predator stress induced FKBP expression after 2 min acute predator stress exposure in F1 adult offspring.** FKBP expression was identified in the hippocampus area within a) dentate gyrus: DENTRH, DENTLH; b) CA1 area: CA1RH, CA1LH; c) CA2 area: CA2RH, CA2LH and CA3 area: CA3RH, CA3LH area. Dapi and FKBP staining images were represented for control (i-viii) and stressed-F1 (ix-xvi) mice.

Figure 17D. **predator stress induced FKBP expression after 2 min acute predator stress exposure in F1 adult offspring.** FKBP expression was identified in the hippocampus area within a) thalamus; b) hypothalamus; c) basolateral amygdala (BLA) and d) medial prefrontal cortex (mPFC). Dapi and FKBP staining images were represented for control (xvii-xxiv) and stressed-F1 mice (xxv-xxxii).

Figure 18: **predator stress induced FKBP5 expression after 5 min acute predator stress exposure and 2min mild stress exposure on their offspring.** Mean + SEM are plotted over different brain regions: Significantly elevated FKBP5 expression was observed in four different areas, DENTRH, CA2RH, CA3LH and BLA after 5 min predator stress exposure stressed-F0 group (Panel A). Whereas, predator stressed-F1 offspring shows increase FKBP5 expression in a) dentate gyrus: DENTRH, DENTLH and b) CA1 area: CA1RH, CA1LH, thalamus and BLA. Interestingly, in control-F1 offspring shows elevated FKBP5 in CA2 area: CA2RH, CA2LH and

CA3 area: CA3RH, CA3LH compared with stressed-F1 offspring. There was no significantly changes observed in hypothalamus and mPFC area between stressed-F1 and control-F1 offspring after 2 min RET (Panel B). Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 19. **Biological parent stress experience, and not social environment, determines anxiety-like behaviour and hyperarousal in the adolescent F1 mice.** Mean + SEM are plotted over four groups: offspring from PS biological parents fostered to a PS mother (BS-FS), offspring from PS biological parents fostered to a C mother (BS-FC), offspring from control biological parents fostered to a PS mother (BC-FS), offspring from control biological parents fostered to a control mother (BC-FC) for panels A-F. Panel A-B represents EPM ratio time and frequency data. The data shown on panel C-D represents distance traveled and center time in the OF. Panel E shows the ratio time of SI and panel F shows mean peak amplitude startle response between groups. Offspring from PS biological parents fostered to a PS mother (BS-FS) or a C mother (BS-FC) spend less time (panel A) in the open arms and enter (panel B) the open arms of the elevated plus maze less often than controls. Offspring from PS parents spend less time (panel C) and enter (panel D) the light side of the light/dark box than controls. Offspring from PS parents spend less time in the center (panel E) and travel less distance (panel F) in the open field than controls. Offspring from PS parents interact less with a novel conspecific than controls (panel G). Offspring from predator stressed parents show increased peak startle amplitude (panel H) compared to controls. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 20: **Biological parental stress, and not behavioural transmission, determines anxiety-like behaviour and hyperarousal response in adult F1 mice exposed to a mild stressor.** Mean + SEM are plotted over four groups: offspring from PS biological parents fostered to a PS mother (BS-FS), offspring from PS biological parents fostered to a C mother (BS-FC), offspring from control biological parents fostered to a PS mother (BC-FS), offspring from control biological parents fostered to a control mother (BC-FC) for panels A-I. Offspring from PS biological parents fostered to a PS mother (BS-FS) or a C mother (BS-FC) show less freezing duration and frequency (Panel A-B), spend less time (panel C) in the open arms and enter (panel D) the open arms of the elevated plus maze less often than controls. Offspring from PS biological parents fostered to a PS mother (BS-FS) or a C mother (BS-FC) spend less time in the center (panel E) in the open field than controls. BS-FS and BS-FC enter less number (panel F) and spend less time (panel G) and the light side of the light/dark box than controls. In SI test, BS-FS and BS-FC interact less with a novel mouse than controls (panel H). Offspring from predator stressed grandparents fostered to control or stressed group show increased peak startle amplitude (panel I) compared to controls. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 21. **Biological mother's stress experience shows spatial memory deficits in adult F1-crossfostered mice to a mild stressor.** Mean + SEM are plotted over four groups: offspring from PS biological parents fostered to a PS mother (BS-FS), offspring from PS biological parents fostered to a C mother (BS-FC), offspring from control biological parents fostered to a PS mother (BC-FS), offspring from control biological parents fostered to a control mother (BC-FC) for panels A-E. Panel A-B represents distance travelled and time taken for twelve days training sessions.

During probe trial, offspring from PS biological parents fostered to a PS mother (BS-FS) and a control mother (BS-FC) spent significantly more time in the target quadrant (Panel C) compared with control. BS-FS and BS-FC show significant main effect for latency to reach the platform location (Panel D) and the number of platform location crosses (Panel E) compared than control. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 22. **Preconception predator stress increased anxiety-like behaviour in second filial (F2) adolescent mice.** Mean + SEM are plotted over four groups: male F1 mice (from control F0 parents) were bred with female F1 mice (from control F0 parents) to generate F2 mice labelled FC-MC (father control-mother control); male F1 mice (from predator stressed F0 parents) were bred with F1 females (from control F0 parents) to generate F2 mice labelled FS-MC (father stressed-mother control); male F1 mice (from control F0 parents) were bred with female F1 offspring (from predator stressed F0 parents) to produce F2 mice labelled FC-MS (father control-mother stressed); male F1 mice (from predator stressed F0 parents) were bred with F1 females (from predator stressed F0 parents) to generated F2 mice labelled FS-MS (father stressed-mother stressed) for panels A-H. Panel A-B represents EPM ratio time and frequency data. The data shown on panel C-D represents distance traveled and center time in the OF. Panel E shows the ratio time of SI and panel F shows mean peak amplitude startle response between groups. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 23. **Preconception predator stressed F2 mice show increased anxiety-like behaviour in the adulthood.** Mean + SEM are plotted over four groups: F2 mice labelled FC-MC (father control-mother control); F2 mice labelled FS-MC (father stressed-mother control); F2 mice labelled FC-MS (father control-mother stressed); F2 mice labelled FS-MS (father stressed-mother stressed) for panels A-J. F2 stressed offspring labelled FS-MS show less freezing time (panel A) and frequency (panel B) than control. F2 control mice labelled FC-MC spend more time in the open arms (panel C) and entered (panel D) the open arms of the elevated plus maze more often than other three stressed groups (FS-MC, FC-MS, FS-MS). F2 control mice labelled FC-MC spend more time in the center (panel E) and travel more distance (panel F) in the open field than stressed groups. Panel G: F2 control mice labelled FC-MC spent more time in light side than three stressed groups. In panel H-I: Offspring from FC-MC show increased social interaction ratio and immobile time compared to stress groups. In Panel J: Offspring from FC-MC show decreased peak startle amplitude compared to stress groups. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, $*p < 0.05$, $***p < 0.001$.

Figure 24. **Preconception predator stressed F2 mice show cognitive deficits in the adulthood.** Mean + SEM are plotted over four groups: F2 mice labelled FC-MC (father control-mother control); F2 mice labelled FS-MC (father stressed-mother control); F2 mice labelled FC-MS (father control-mother stressed); F2 mice labelled FS-MS (father stressed-mother stressed) for panels A-E. A linear trend analysis revealed that all F2 mice travelled significantly shorter distances (panel A) and took less time (panel B) to reach the hidden platform over trials. During probe trial, offspring labelled FC-MC spent significantly more time in the target quadrant (Panel C) which had contained the hidden platform than three stressed conditions. Number of platform location cross (Panel D) of FC-MC were significantly higher than stressed groups. Platform

latency (Panel E) of FC-MC were significantly lower than stressed groups. Graph shows use of the "asterisk bar" object for indicating significant differences between samples. In here, *$p < 0.05$, ***$p < 0.001$.

Figures

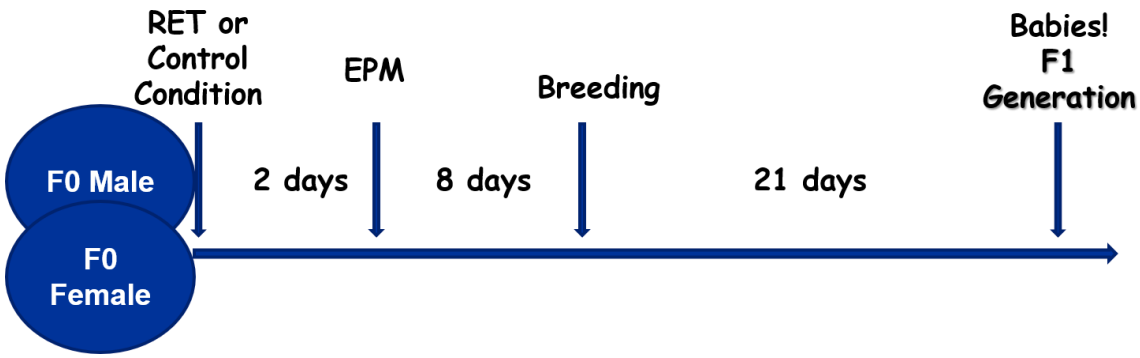


Figure 1. **Breeding protocol followed by 5 min rat exposure test (RET).**

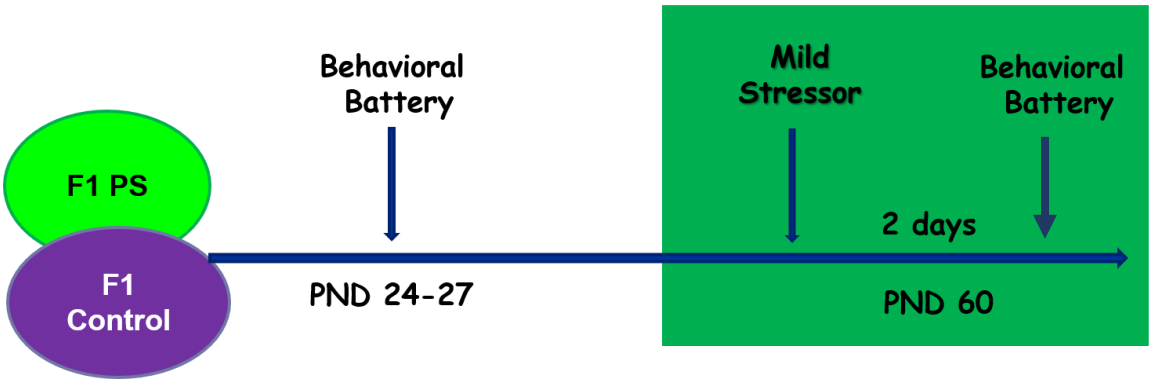
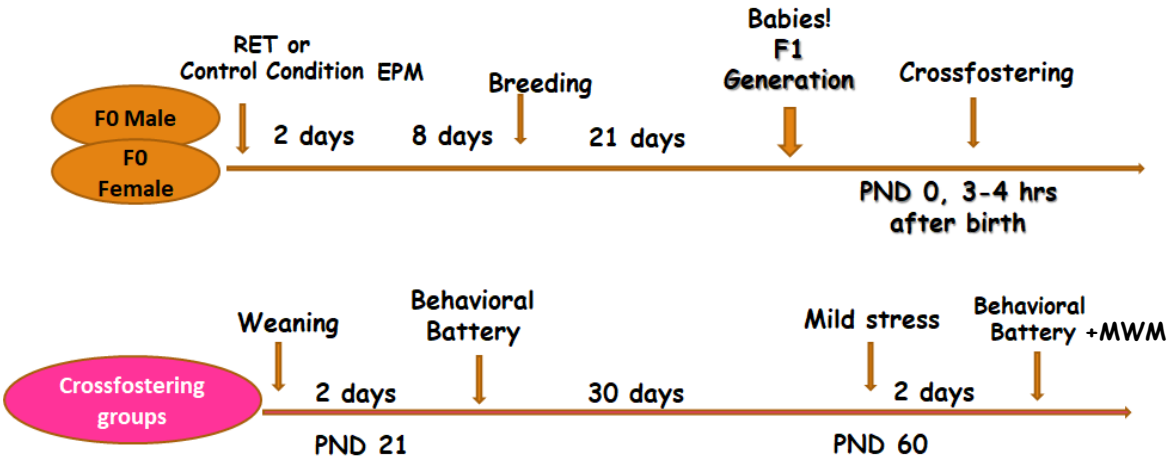


Figure 2. A schematic of the F1 behavioural procedure.



Control Mom's babies crossfosterd to control mom	Control Mom's babies crossfosterd to PS mom
PS Mom's babies crossfosterd to control mom	PS Mom's babies crossfosterd to PS mom

Figure 3. **Experimental protocol to identify the role of the maternal social environment on offspring behaviour.**

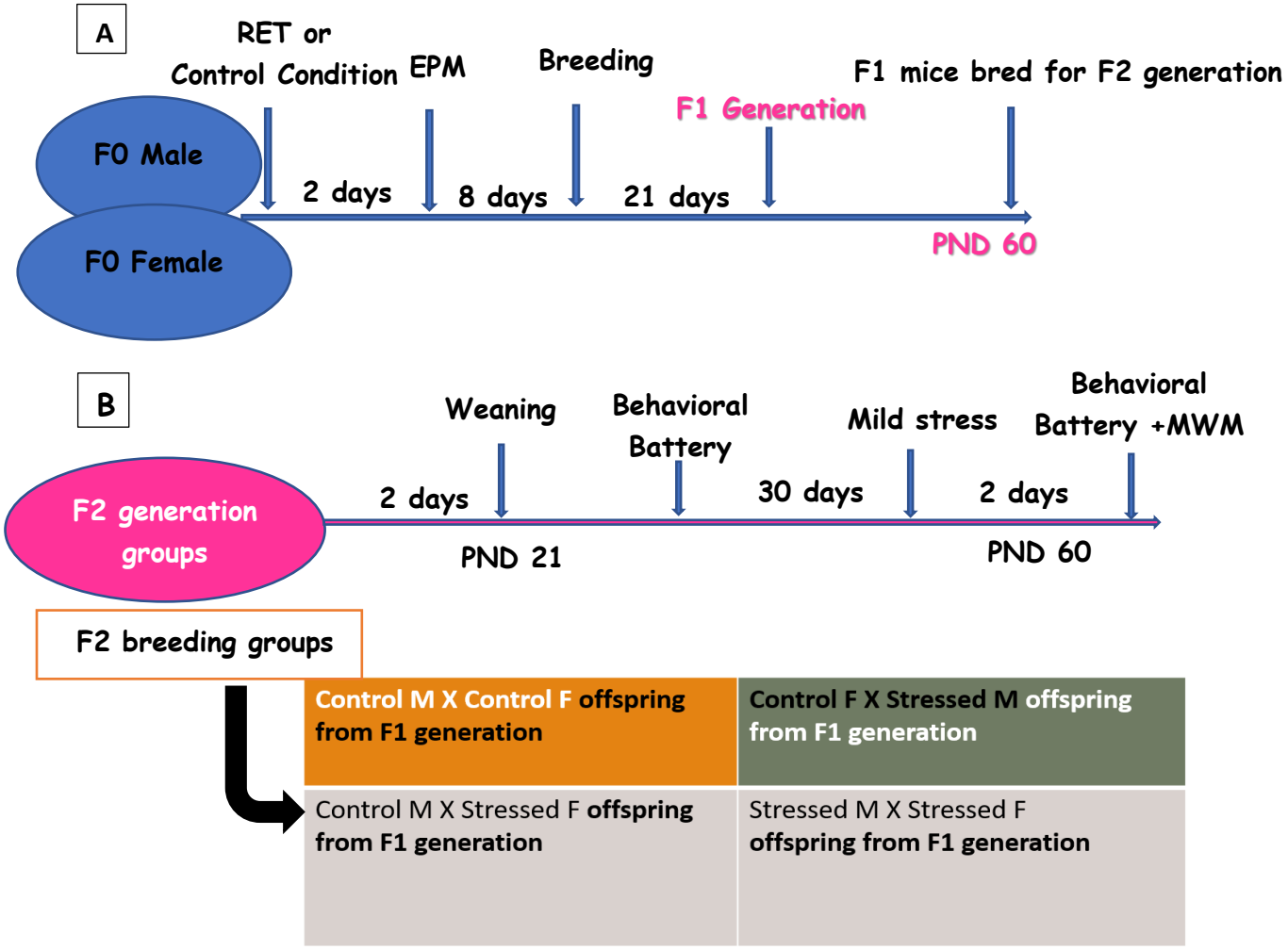


Figure 4. **Experimental protocol to the effects of preconception predator stress on second filial generation offspring behavior**

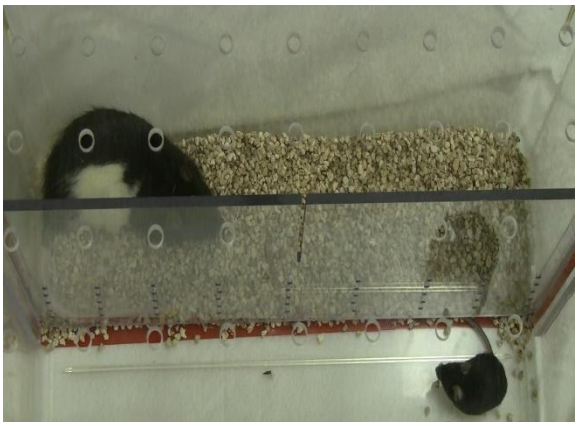


Figure 5. **Rat Exposure Test (RET) in exposure chamber.**

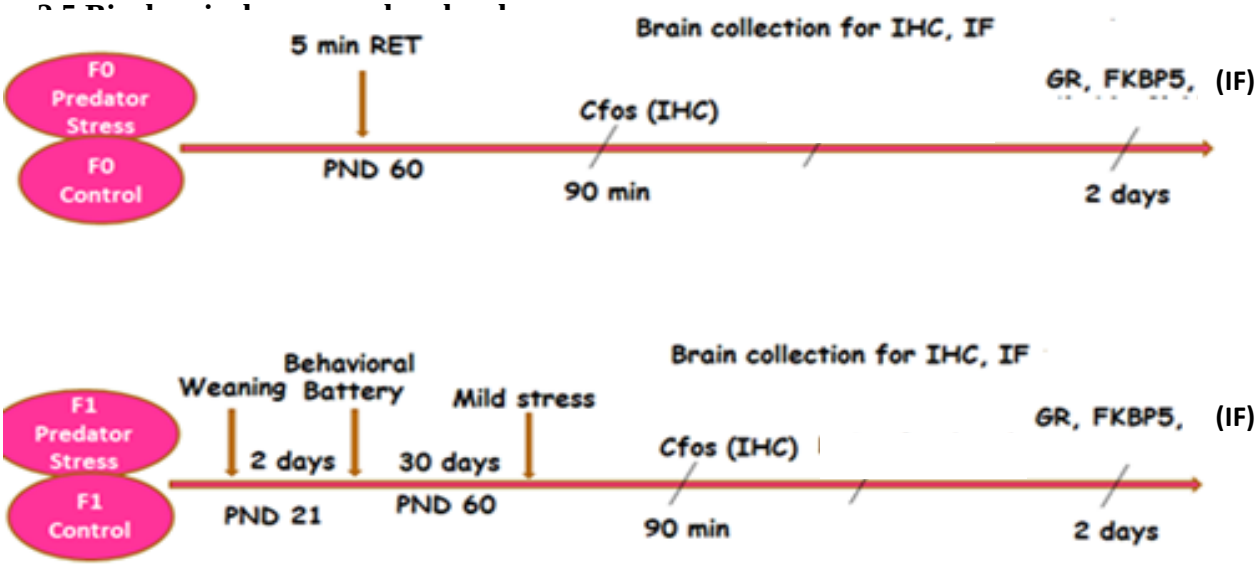


Figure 6. **Protocol for molecular measures.**

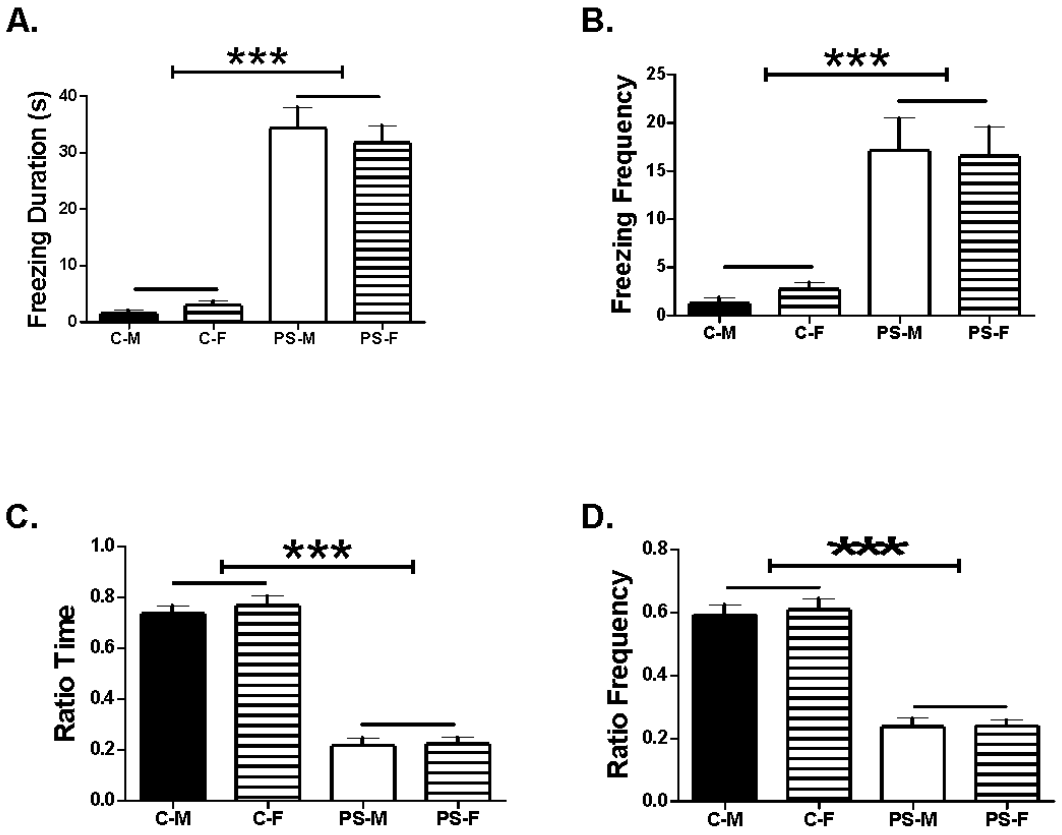


Figure 7. **Five min RET produces lasting changes in anxiety-like behaviour.**

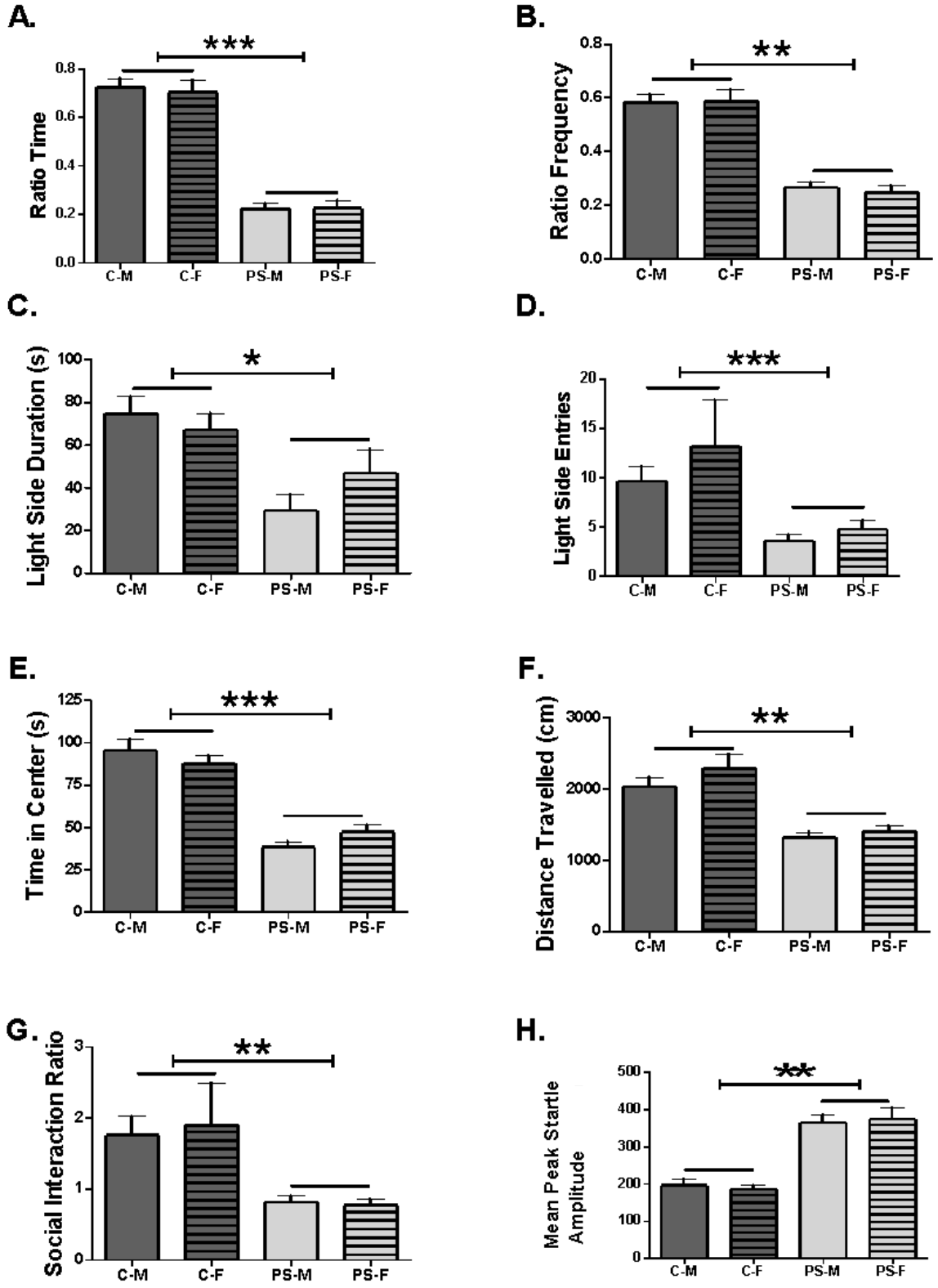


Figure 8. **Preconception predator stress produces anxiety-like behaviour in adolescent offspring (F1 mice).**

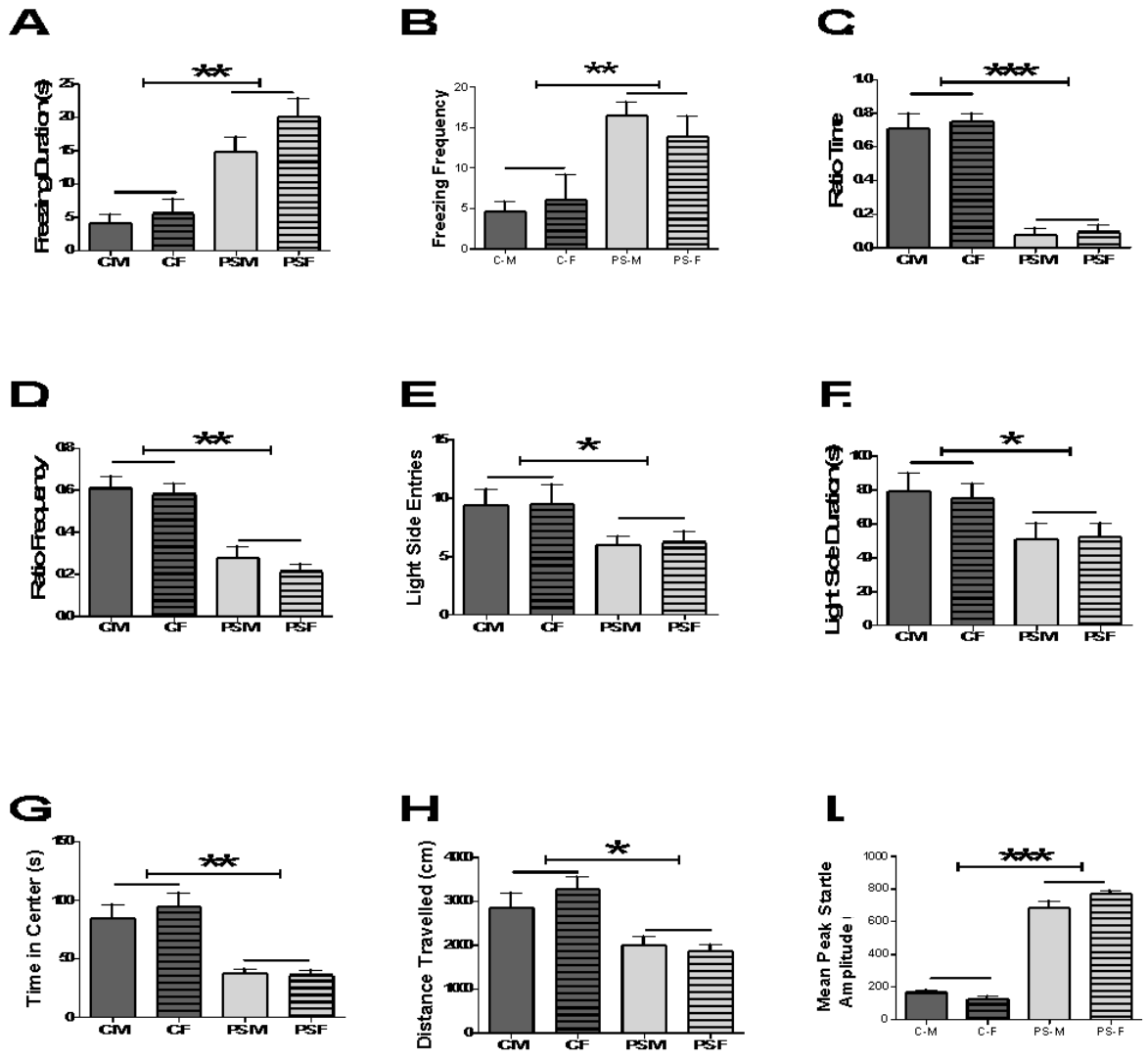


Figure 9. **Preconception predator stressed F1 offspring show increased anxiety-like behaviour when exposed to a mild stressor in adulthood.**

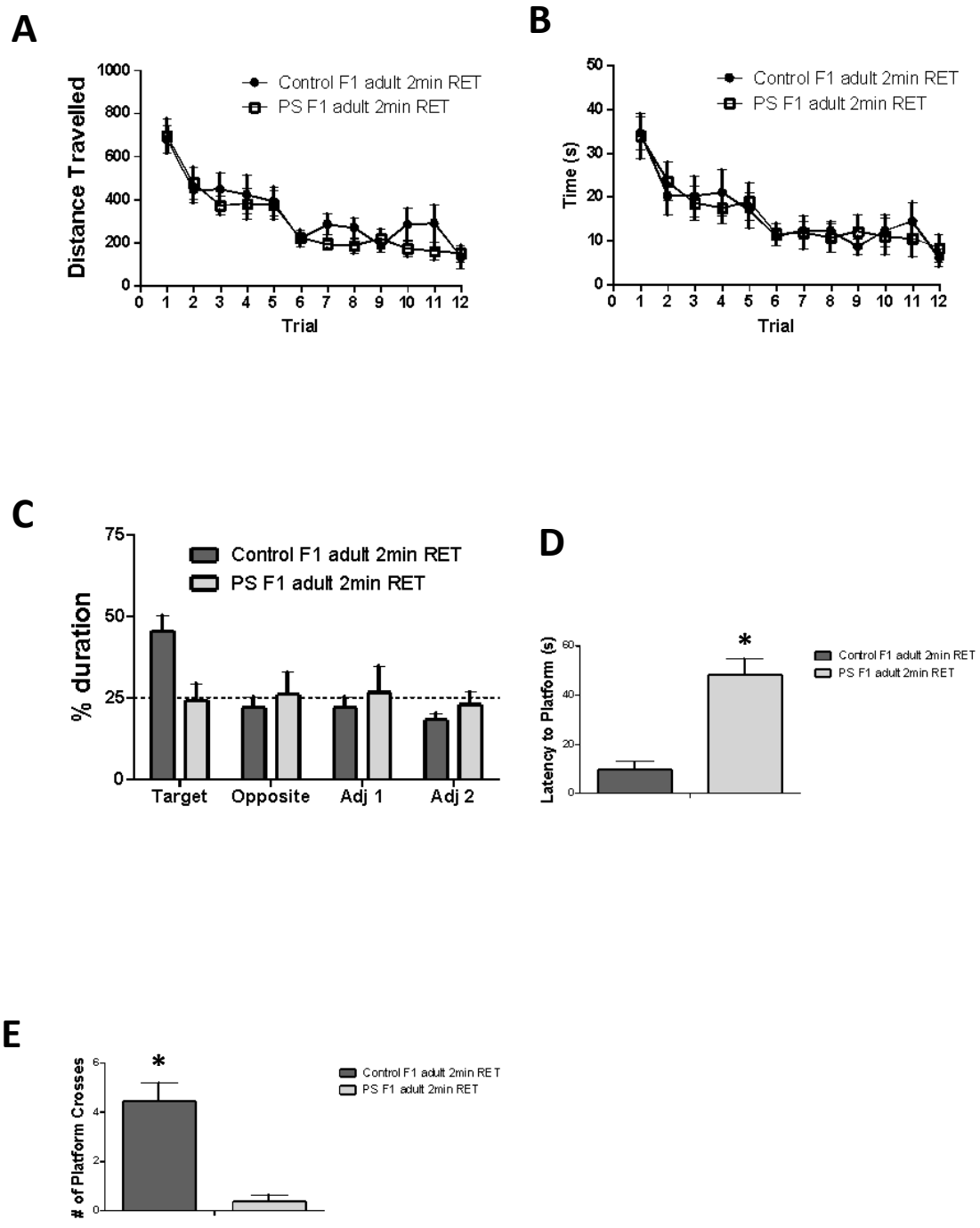


Figure 10. **Two min predator exposure improved MWM performance across training trials and produced spatial memory deficits on the probe trial in the offspring of predator-stressed mice**.

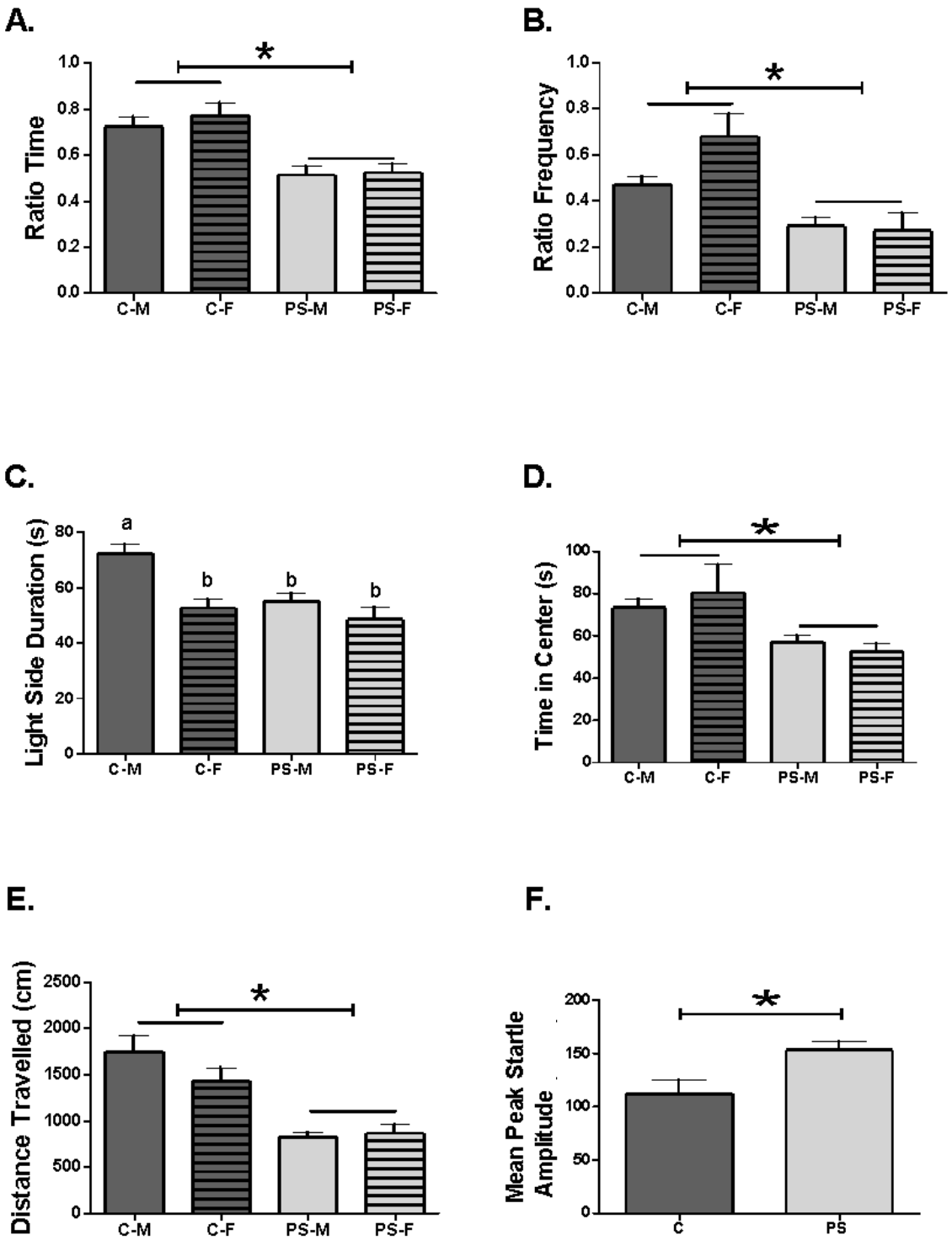


Figure 11. **Preconception predator stressed F1 mice show increased anxiety-like behaviour in the absence of mild stress exposure.**

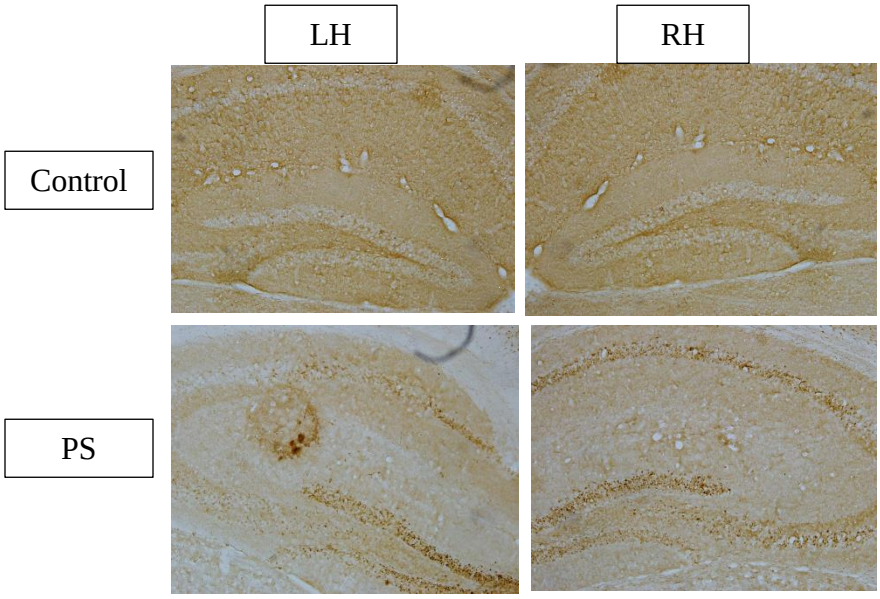


Figure 12A. **Predator stress induced c-FOS expression after 5 min acute predator stress (PS) exposure.**

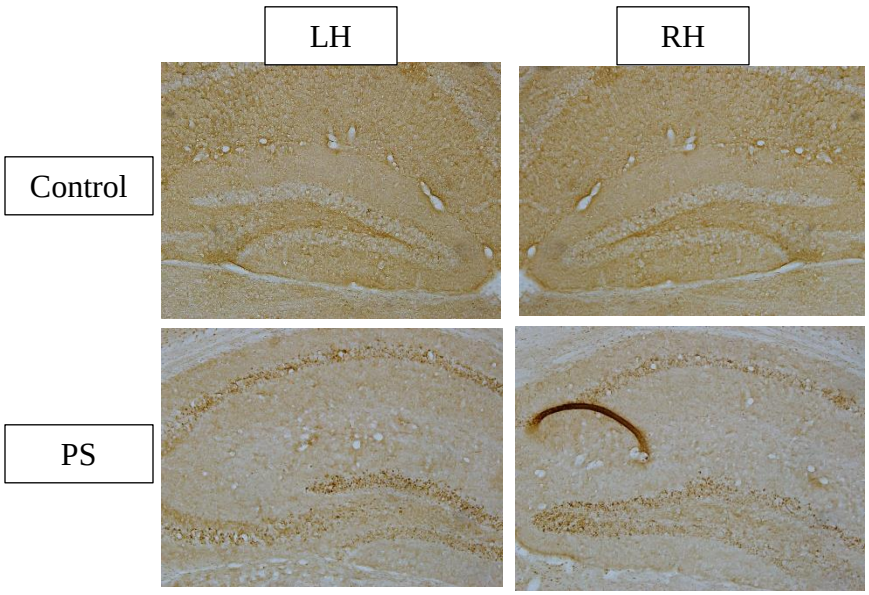


Figure 12B. **Predator stress induced c-FOS expression after 2 min acute predator stress (PS) exposure in F1 adult offspring.**

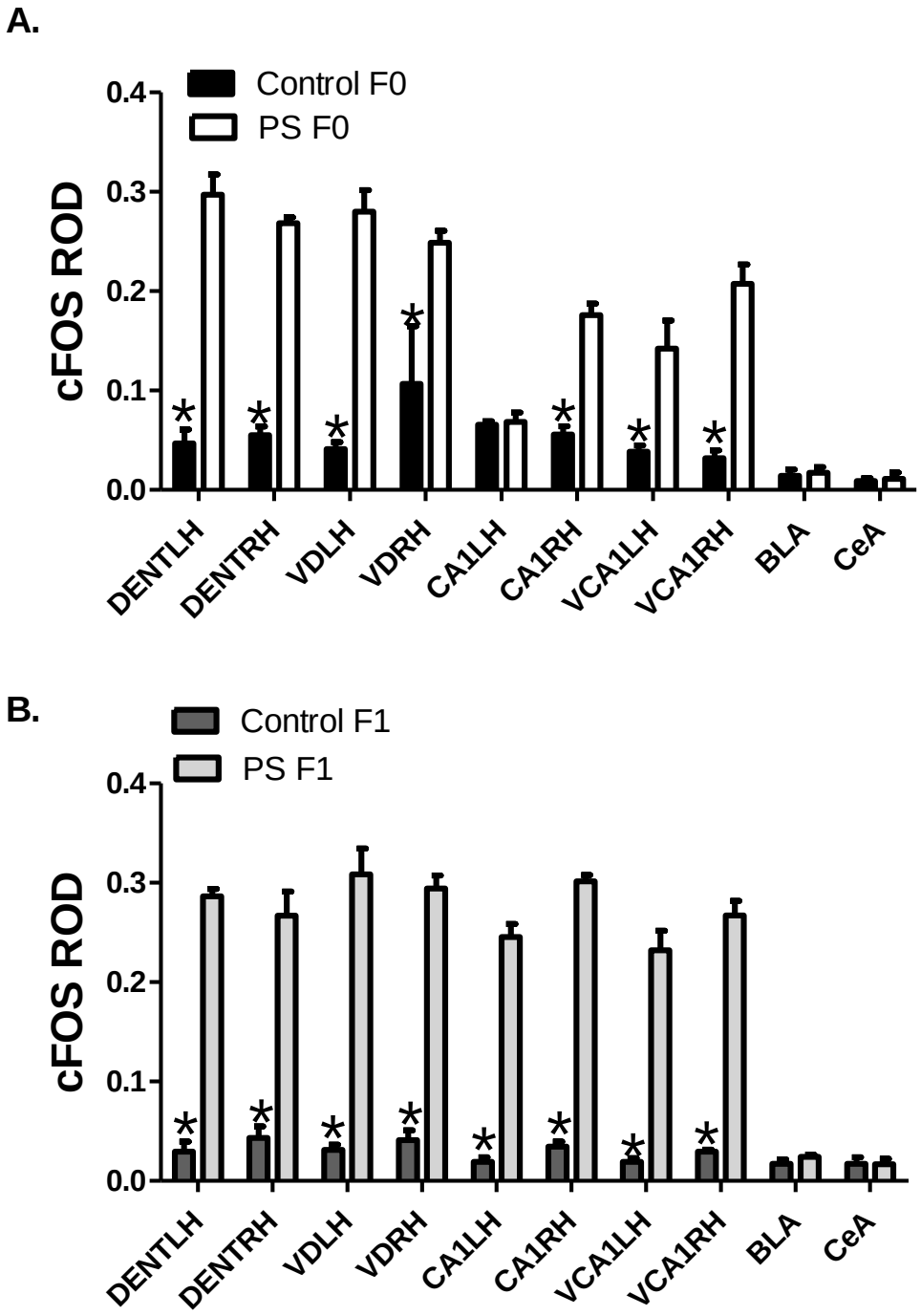


Figure 13: **predator stress induced c-FOS activation after 5 min acute predator stress exposure and 2min mild stress exposure on their offspring.**

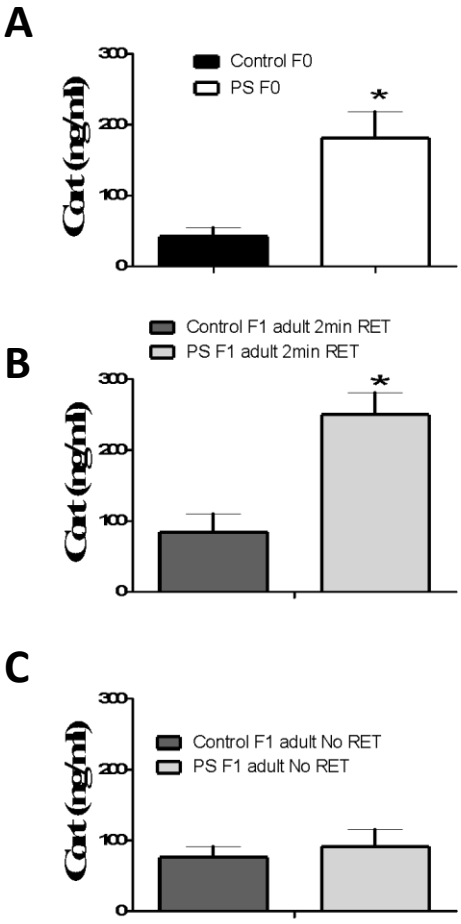


Figure 14. **Corticosterone assay.**

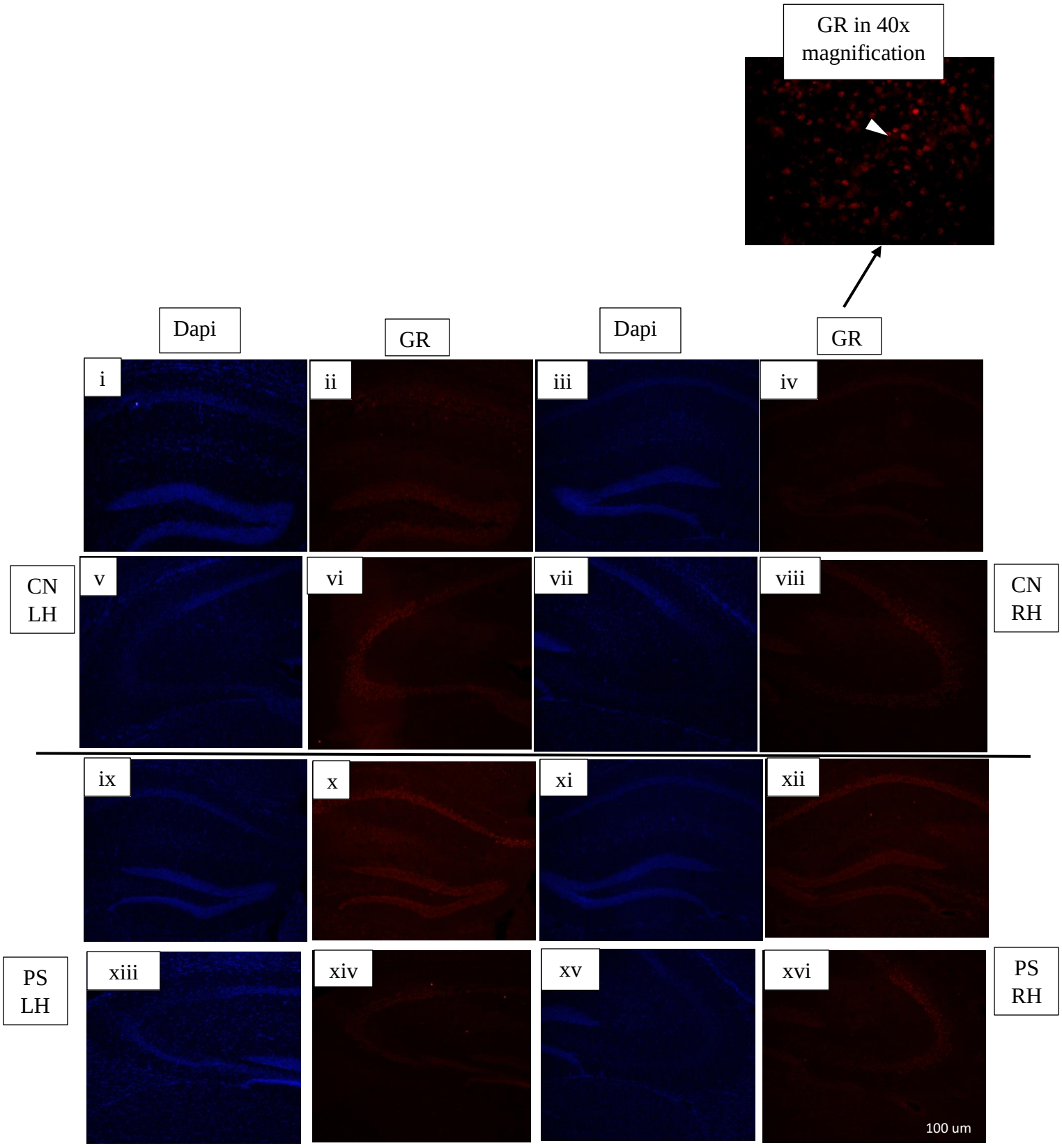


Figure 15A. **predator stress induced GR expression after 5 min acute predator stress exposure.**

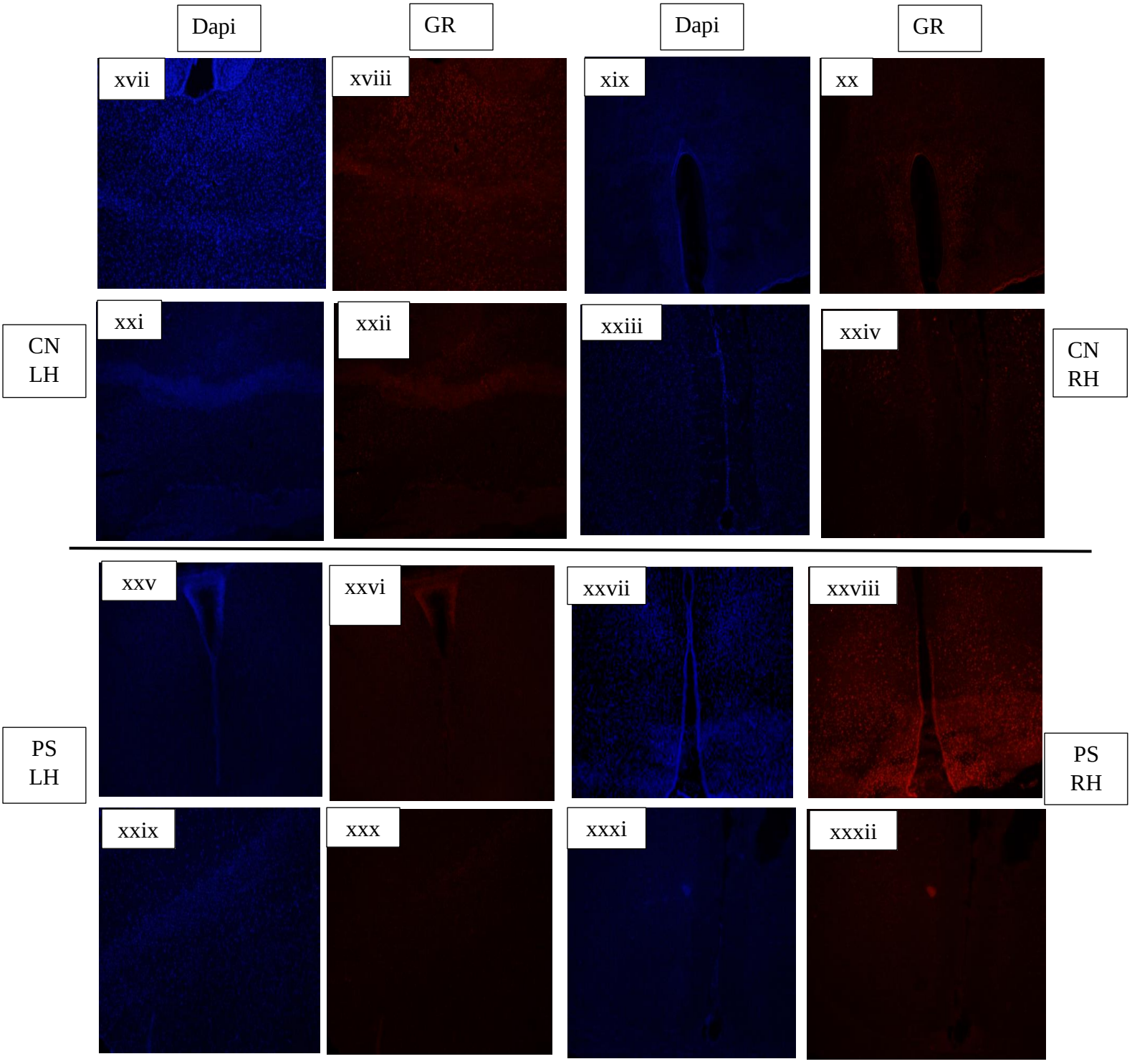


Figure 15B. **predator stress induced GR expression after 5 min acute predator stress exposure.**

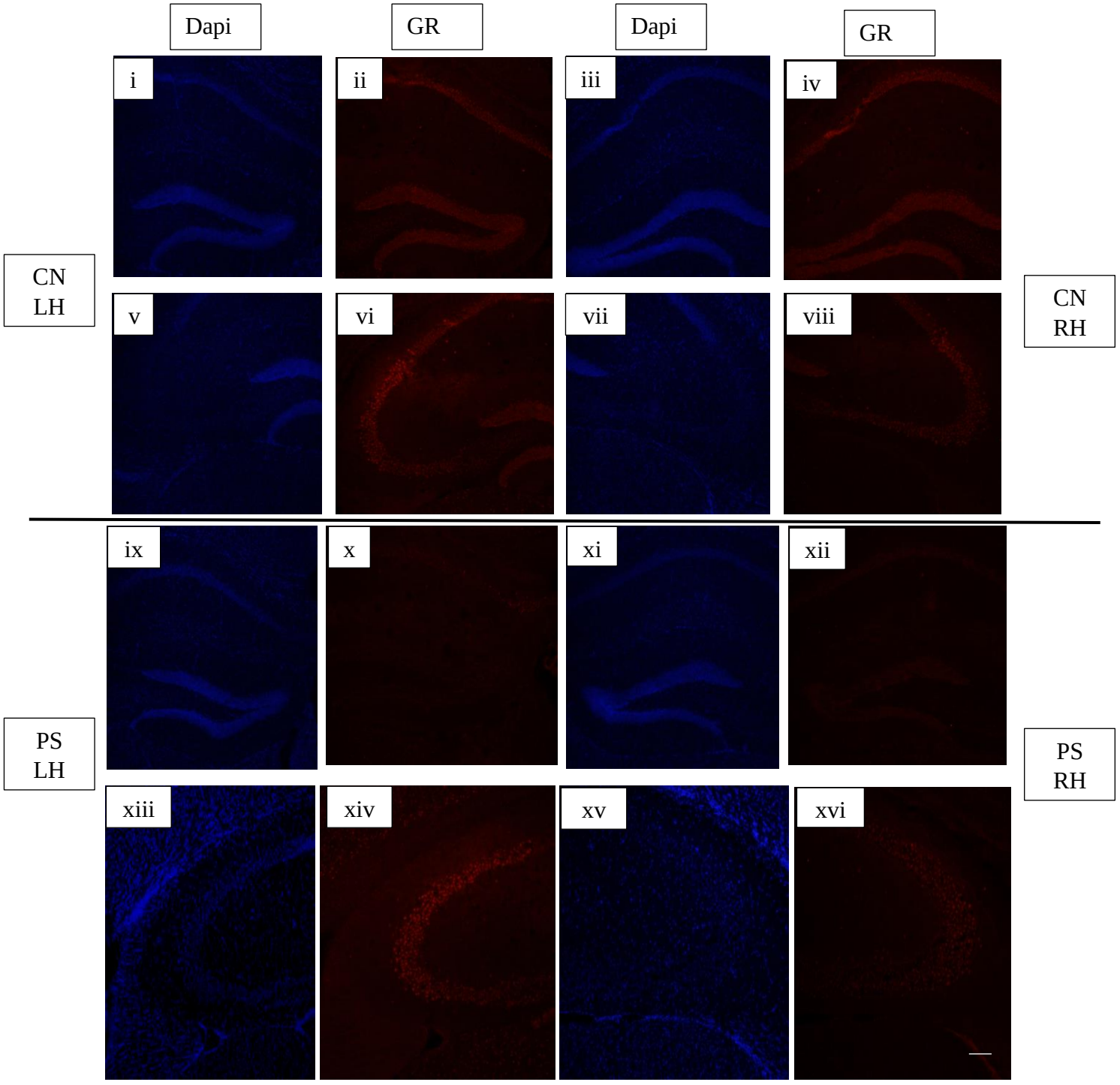


Figure 15C. **predator stress induced GR expression after 2 min acute predator stress exposure in F1 adult offspring.**

.

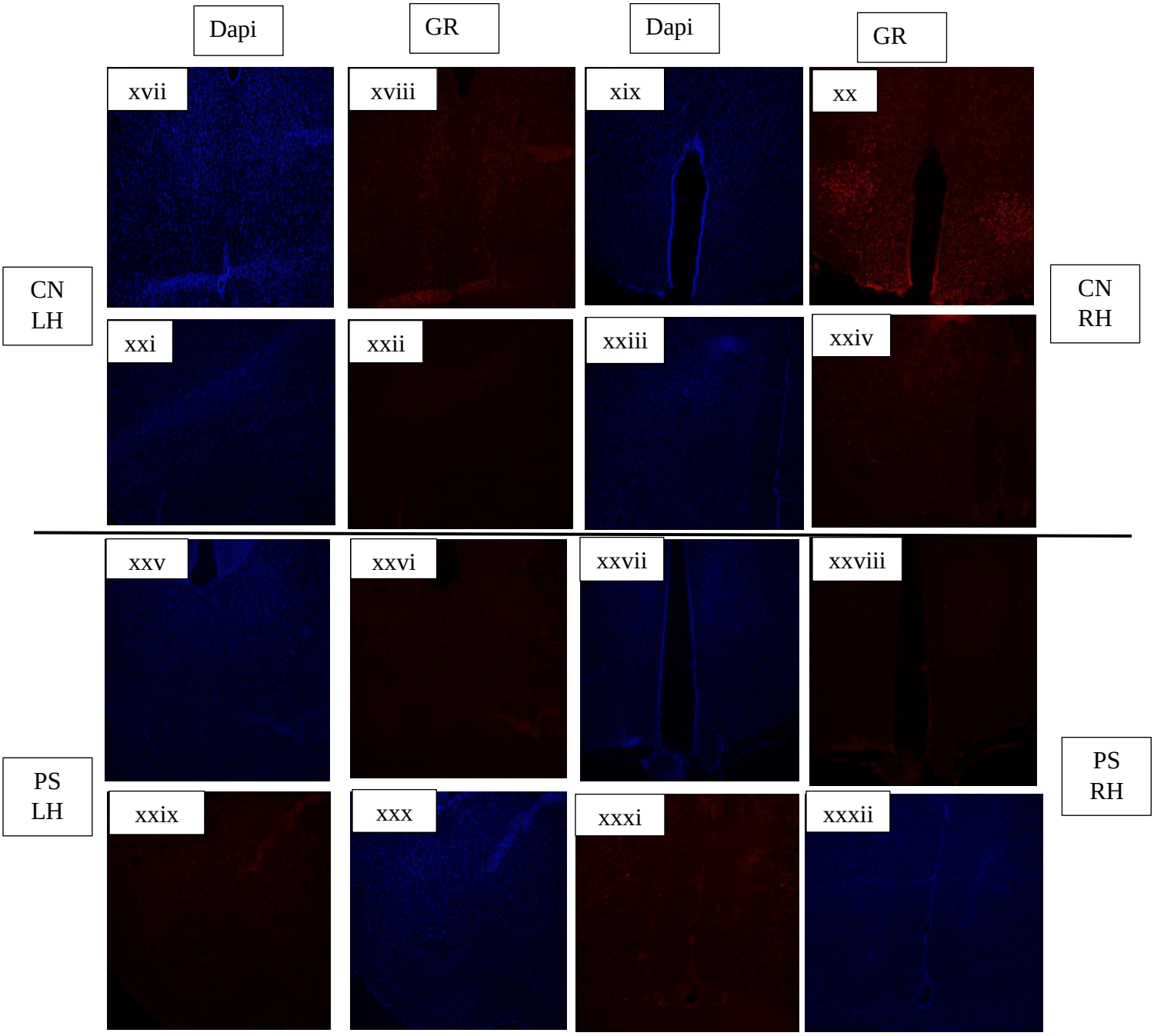


Figure 15D. **predator stress induced GR expression after 2 min acute predator stress exposure in F1 adult offspring**

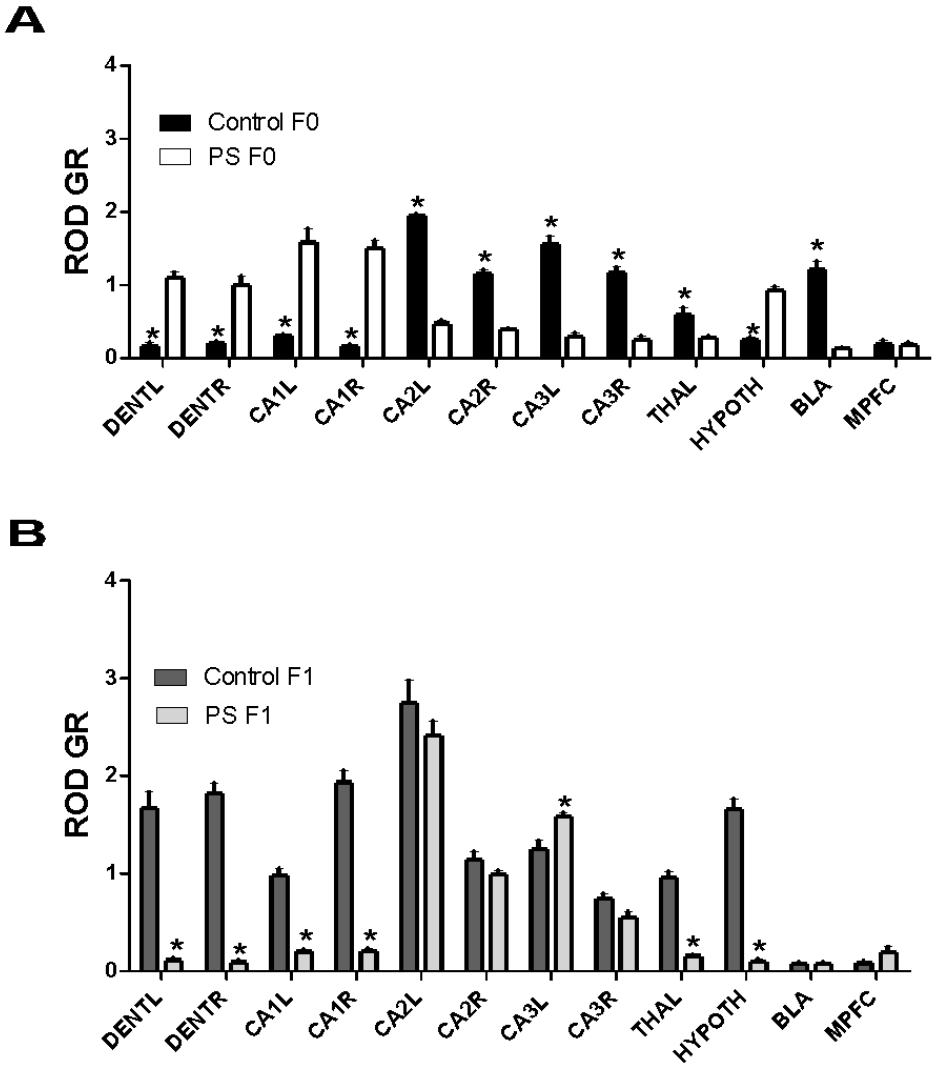


Figure 16: **predator stress induced GR expression after 5 min acute predator stress exposure and 2min mild stress exposure on their offspring.**

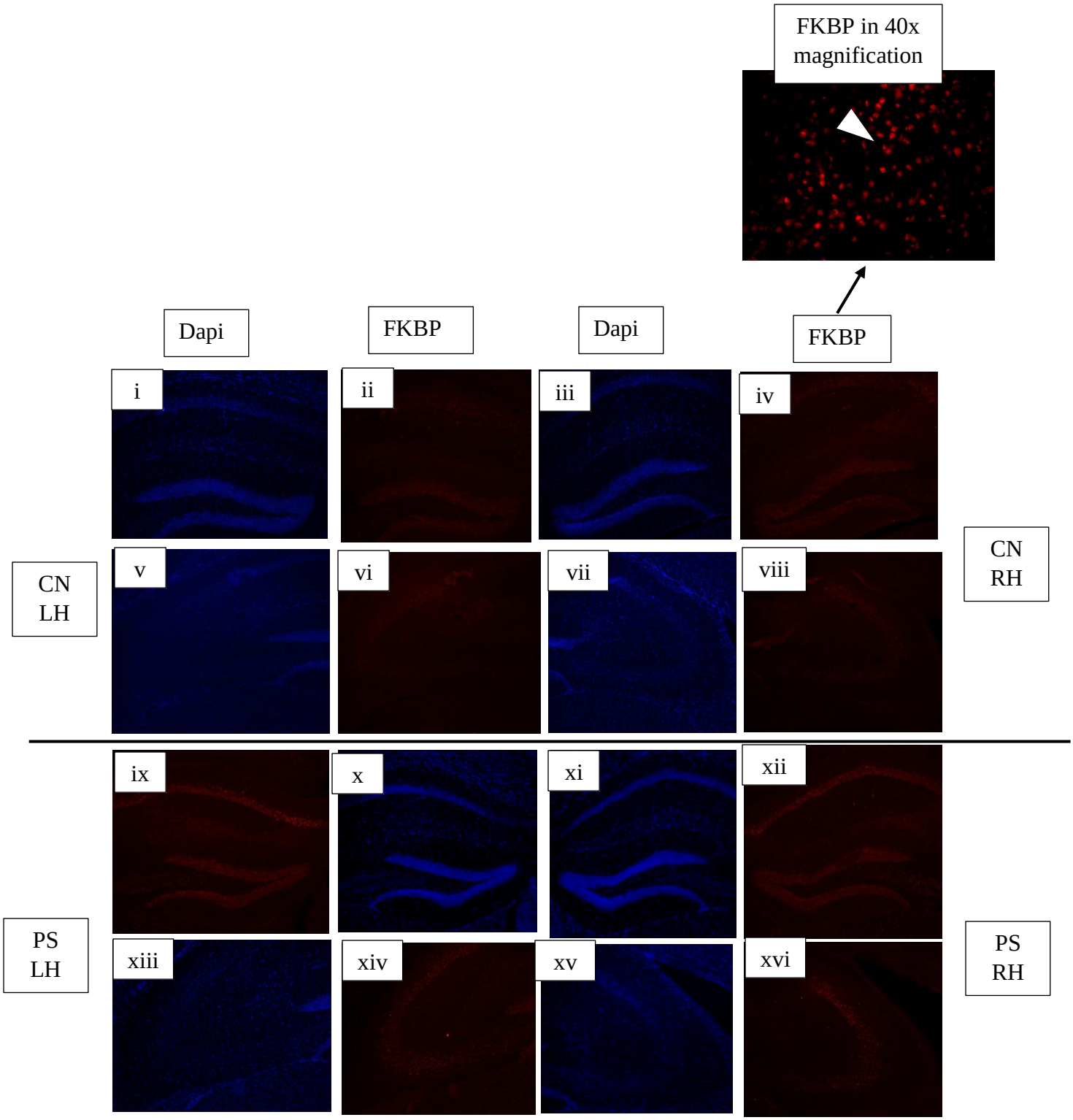


Figure 17A. **predator stress induced FKBP expression after 5 min acute predator stress exposure.**

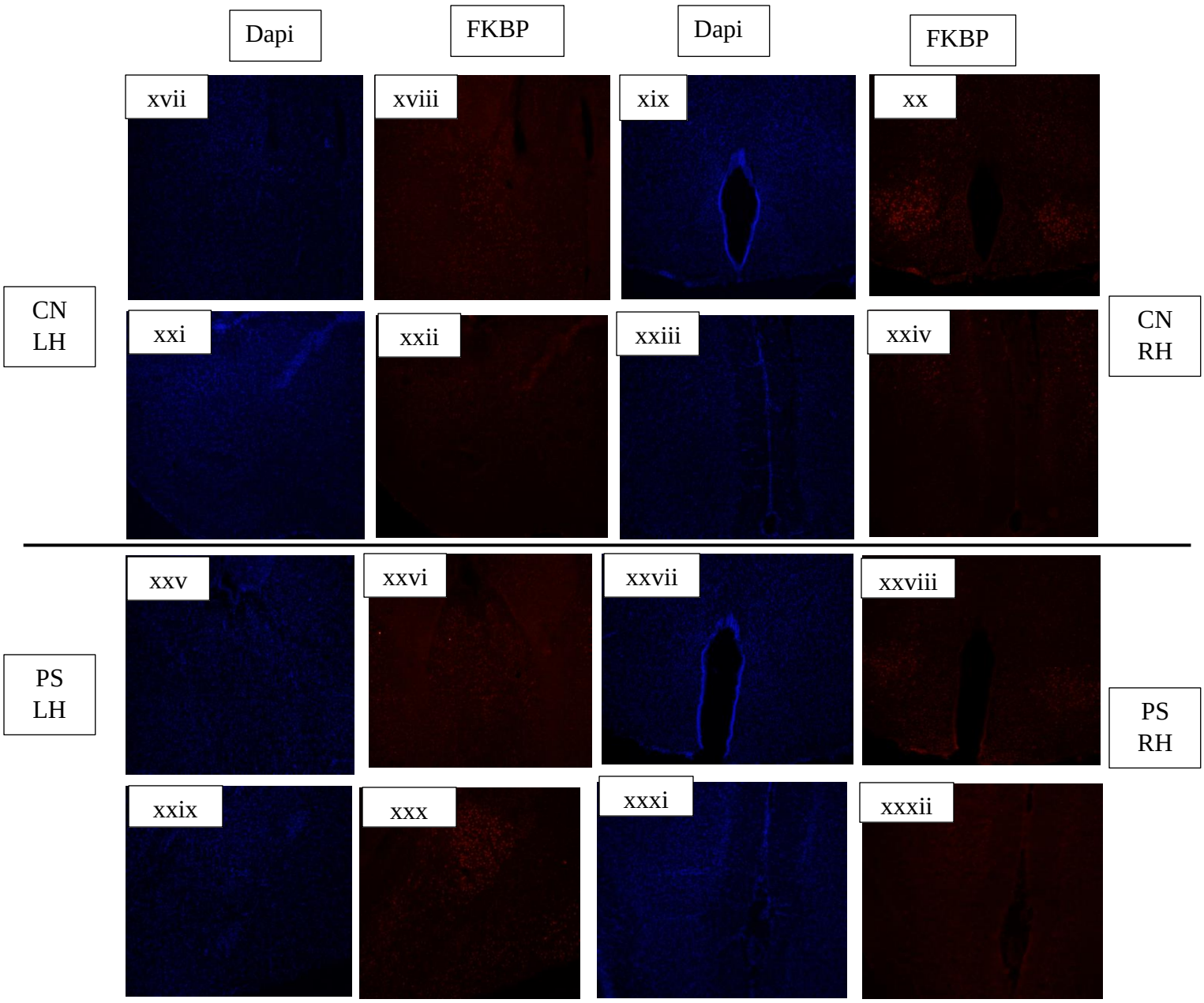


Figure 17B. **predator stress induced FKBP expression after 5 min acute predator stress exposure.**

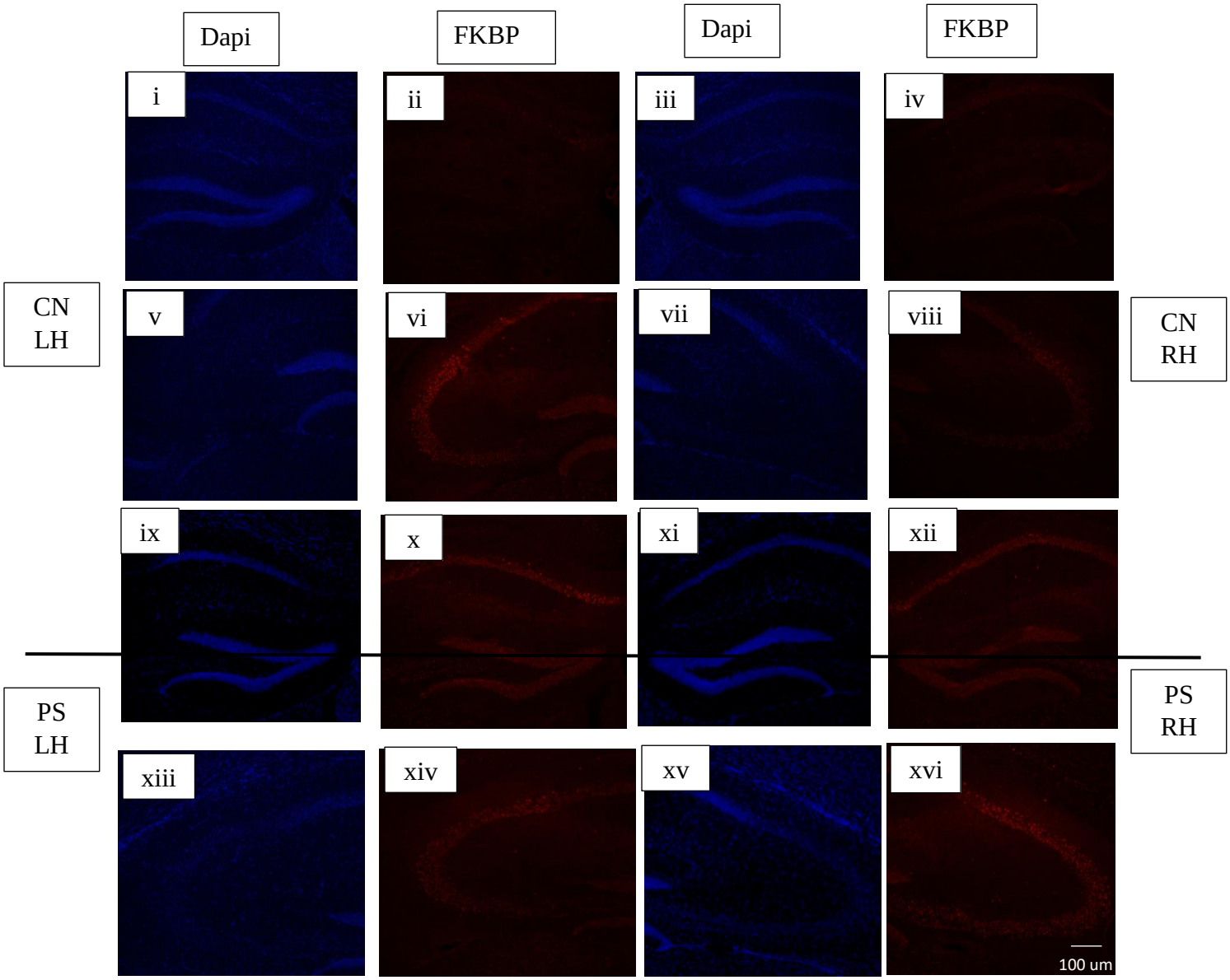


Figure 17C. **predator stress induced FKBP expression after 2 min acute predator stress exposure in F1 adult offspring.**

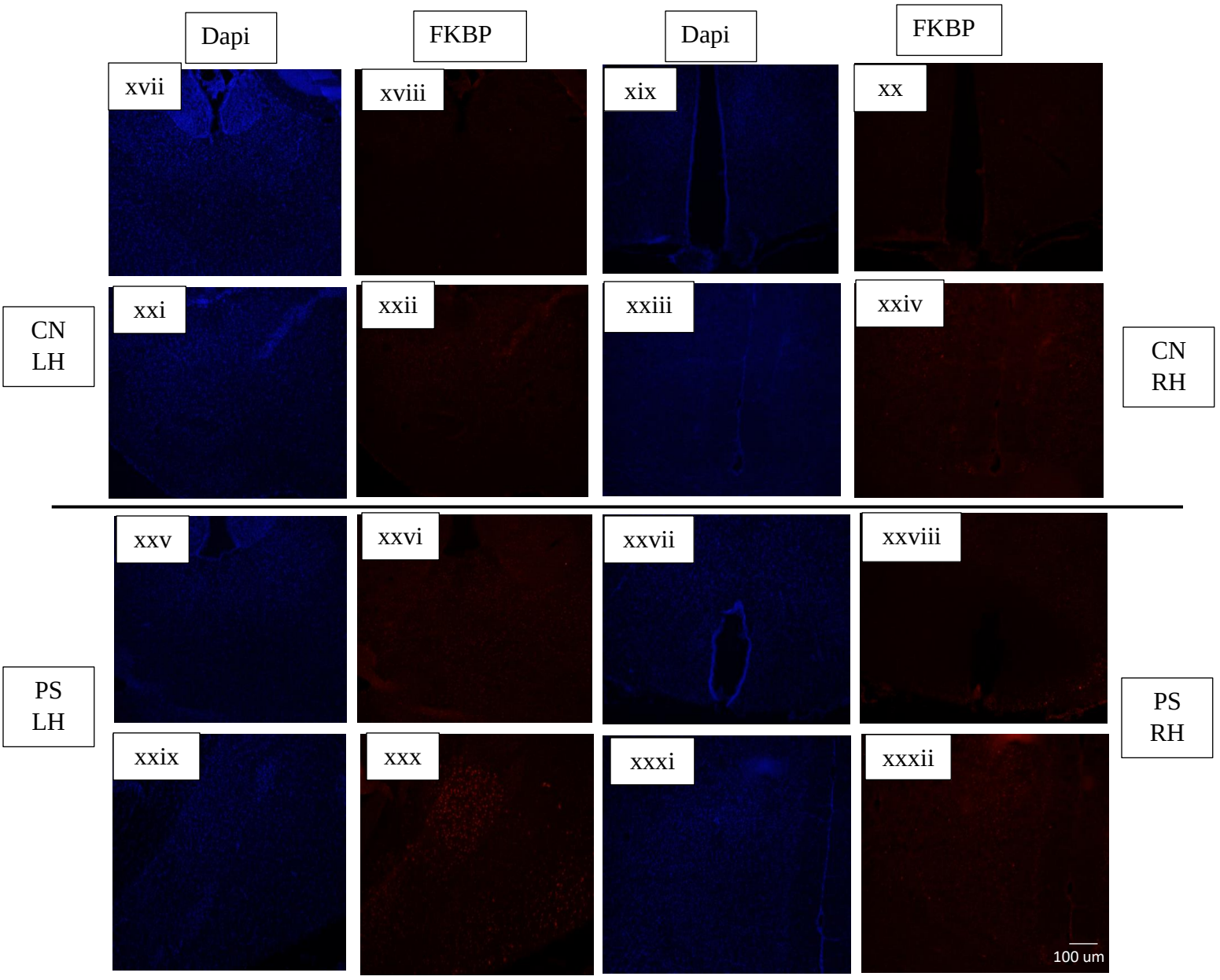


Figure 17D. **predator stress induced FKBP expression after 2 min acute predator stress exposure in F1 adult offspring.**

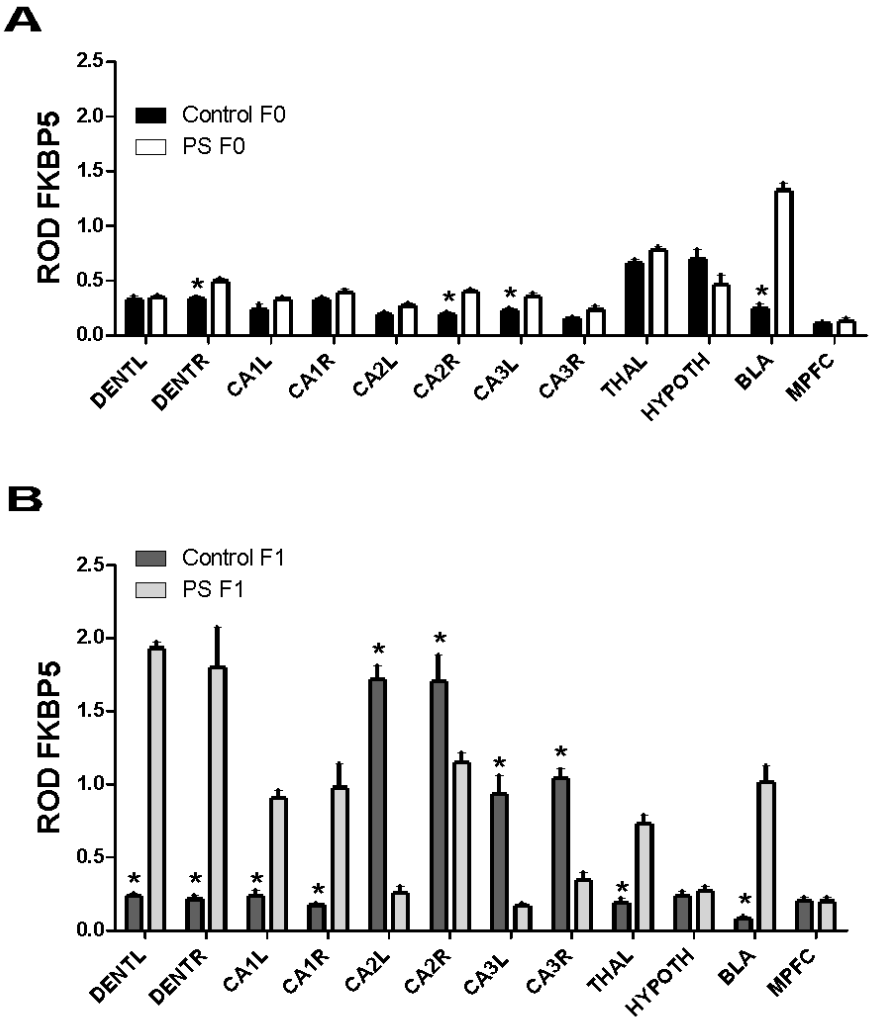


Figure 18: **predator stress induced FKBP5 expression after 5 min acute predator stress exposure and 2min mild stress exposure on their offspring.**

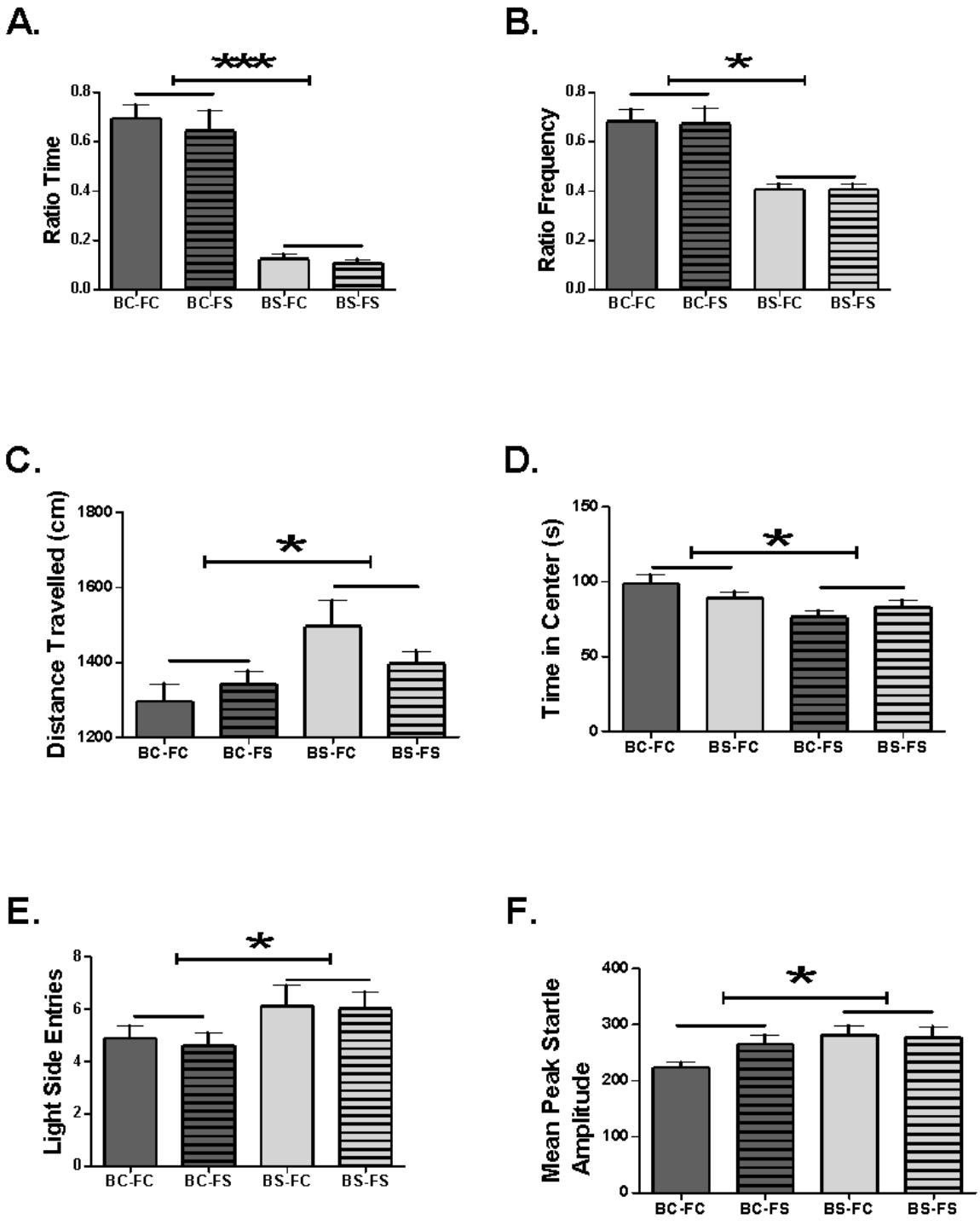


Figure 19. **Biological parent stress experience, and not social environment, determines anxiety-like behaviour and hyperarousal in the adolescent F1 mice.**

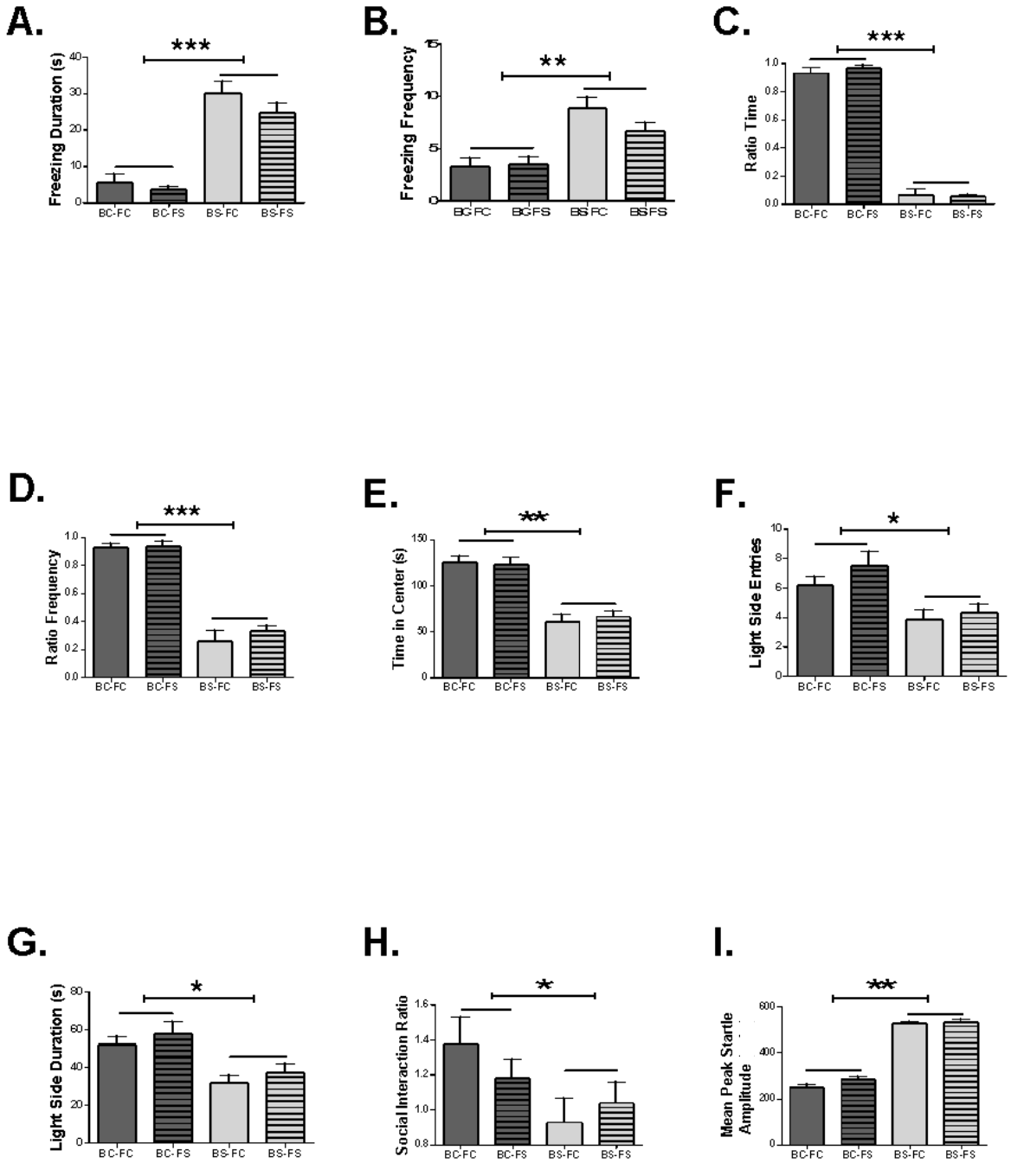


Figure 20: **Biological parental stress, and not behavioural transmission, determines anxiety-like behaviour and hyperarousal response in adult F1 mice exposed to a mild stressor.**

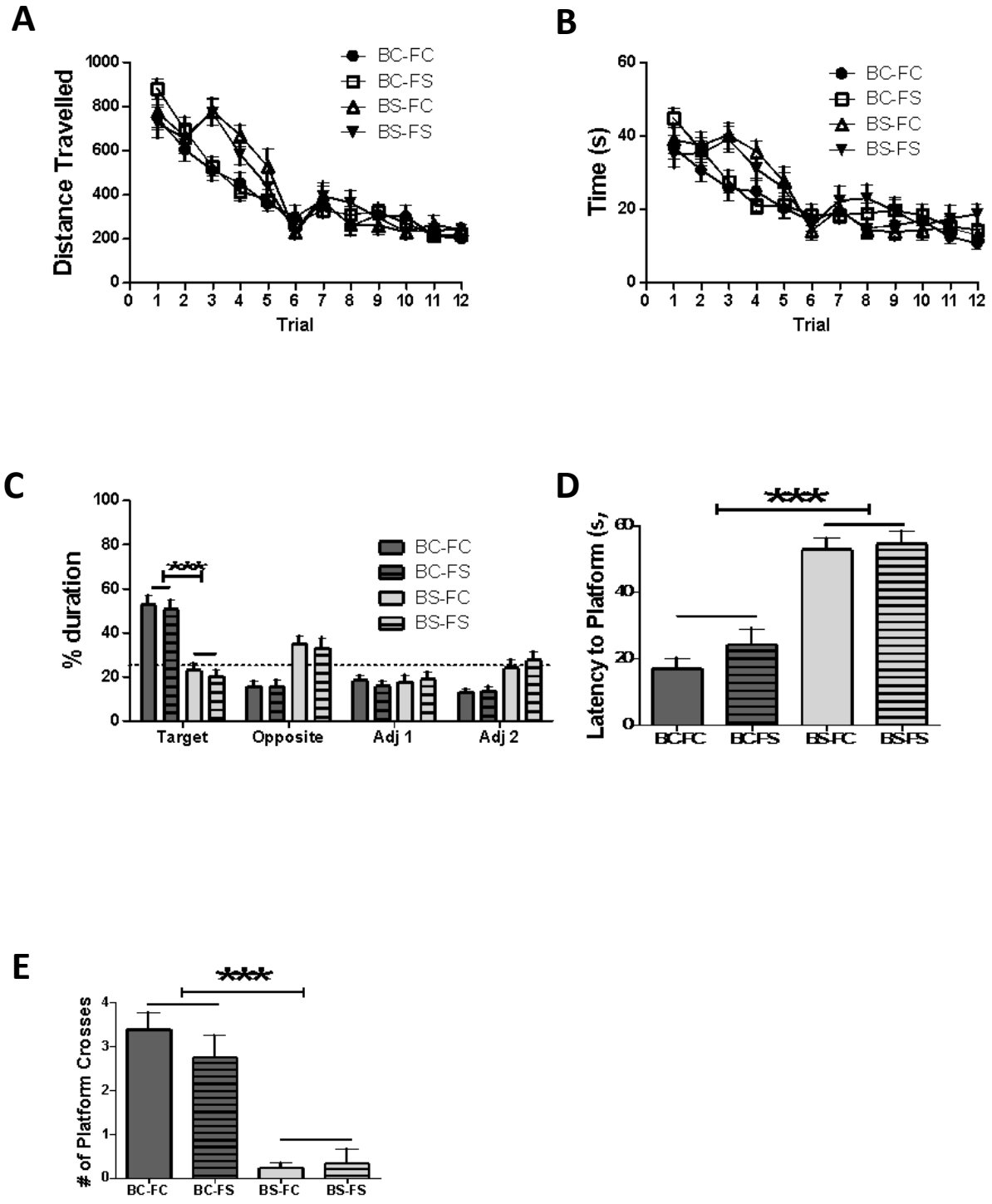


Figure 21. **Biological mother's stress experience shows spatial memory deficits in adult F1-crossfostered mice to a mild stressor.**

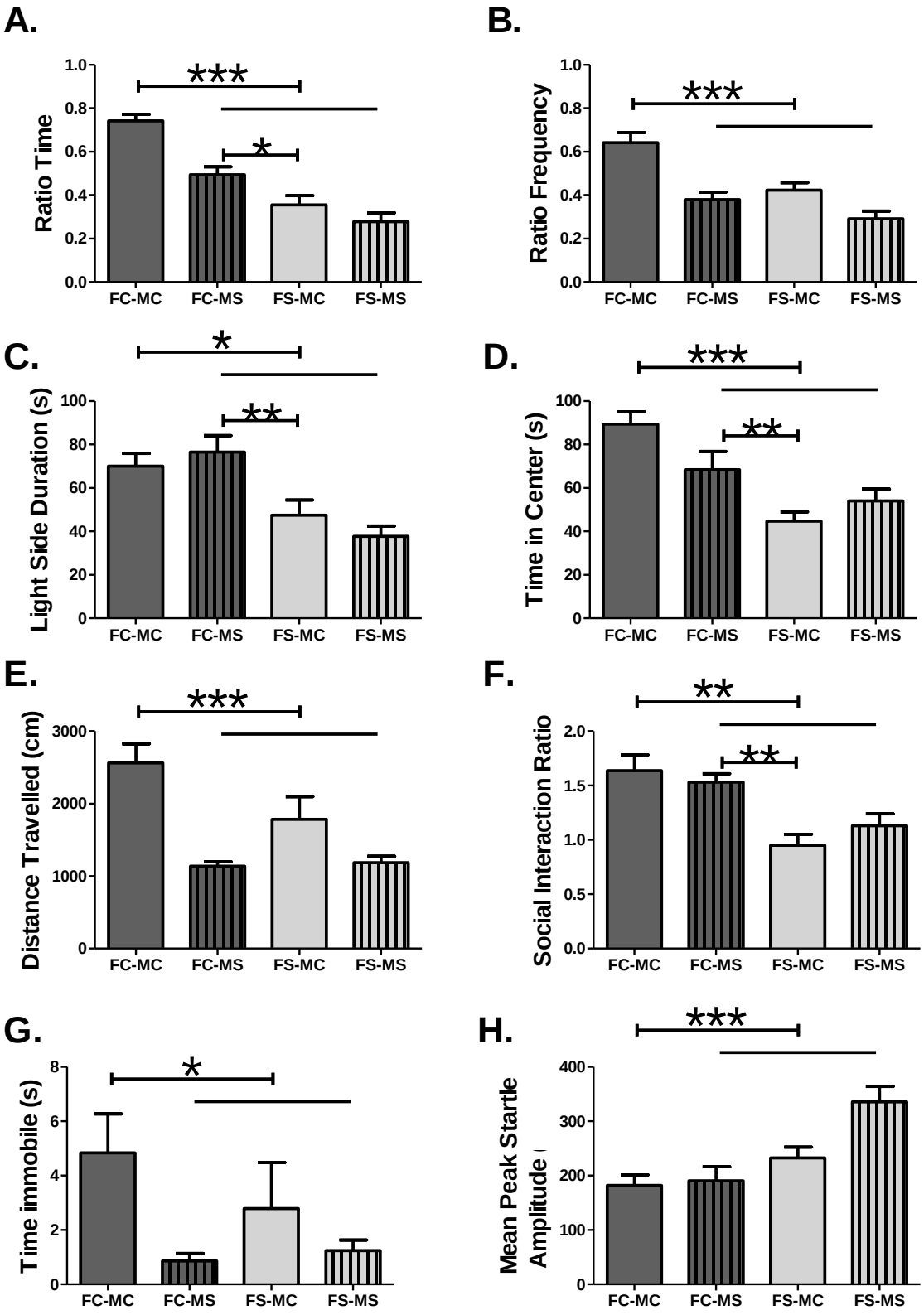


Figure 22. **Preconception predator stress increased anxiety-like behaviour in second filial (F2) adolescent mice.**

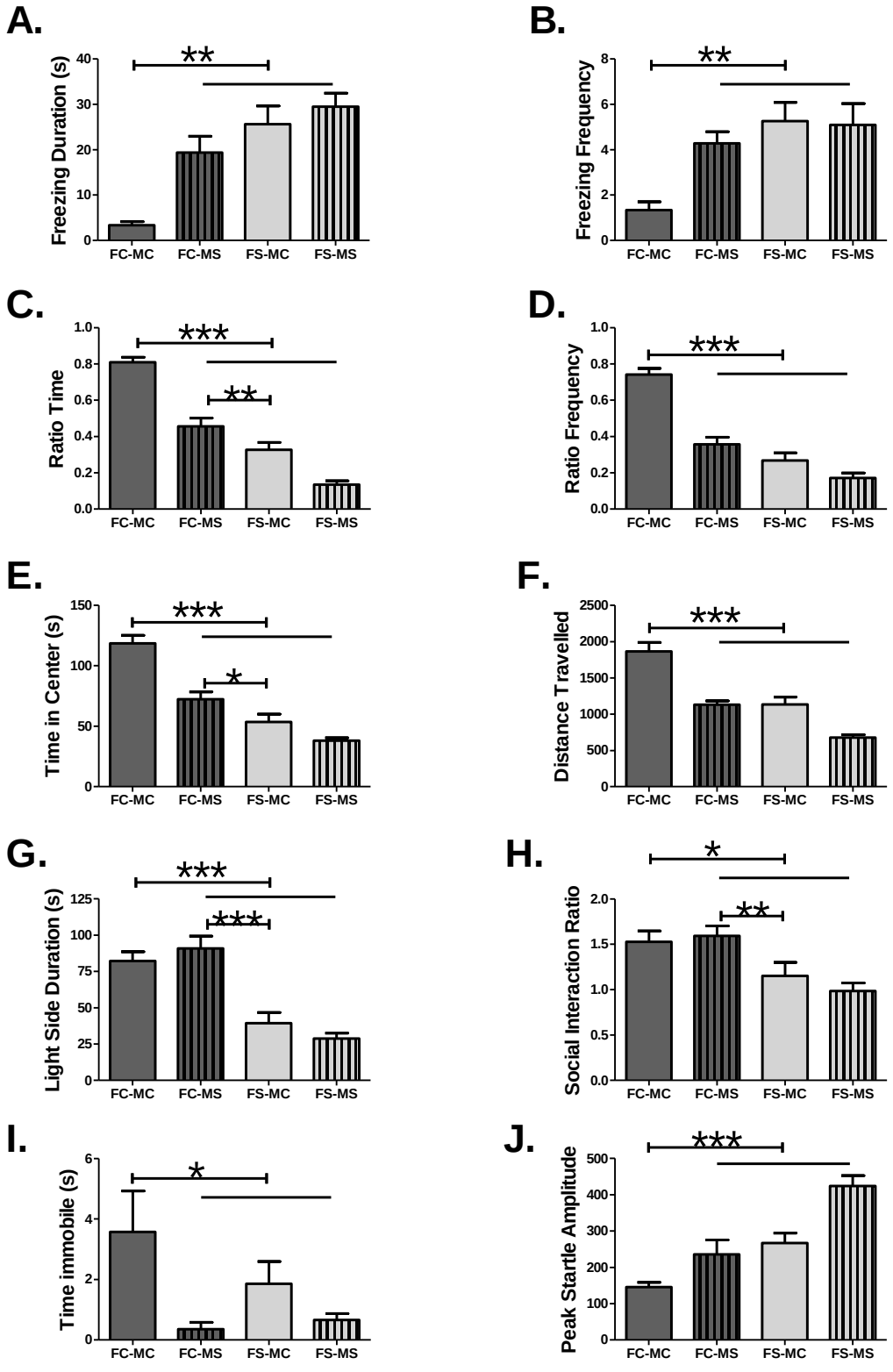


Figure 23. **Preconception predator stressed F2 mice show increased anxiety-like behaviour in the adulthood.**

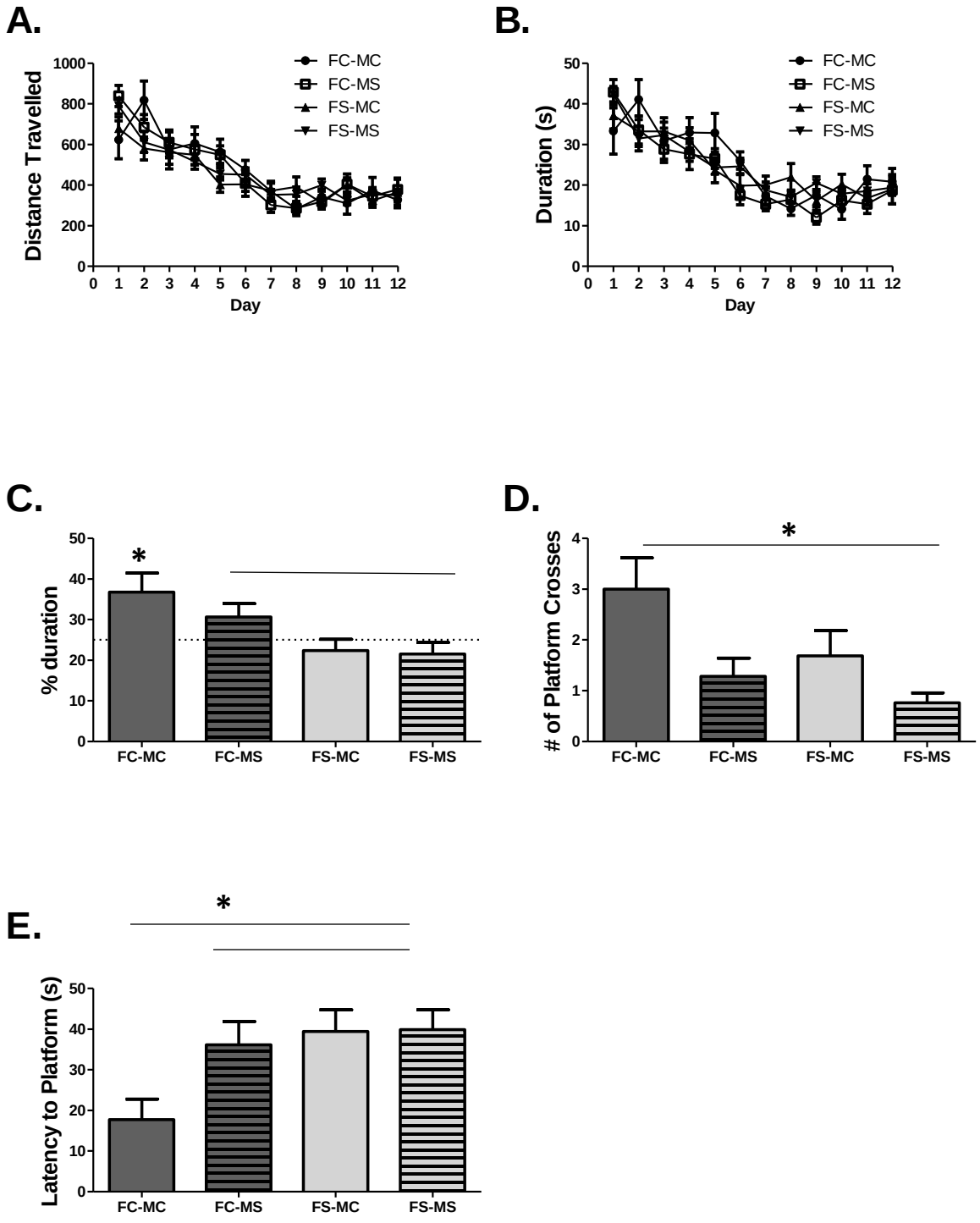


Figure 24. **Preconception predator stressed F2 mice show cognitive deficits in the adulthood.**

Tables caption

(In each table, differences between groups (control vs stressed) were considered significant when p values were < 0.05).

Table 1: Summary table for major experimental outcomes. Where,  = significantly increased in stress groups;  = significantly decreased in stress groups; NS= non-significant.

Table 2: Table represents mother-pup interaction, litter size, numbers of male and female pup and % of male pup's outcomes for normal and cross-fostering F1 generation experiments. In here, BMOM= biological mother, FMOM= foster mother, IT= interaction time, NS= non-significant.

Table 3: Descriptive statistical tab le for all behavioural outcomes including F0, F1 and F2 data. In here, RT= ratio time, RF= ratio frequency, FREEZDUR= freezing duration, FREEZFREQ=freezing frequency, OFTIM= open-field time in centre, OFDIS=open-field distance travelled, LDENT= LDB light side entries, LDTIM= LDB light side time, IMT= immobile time in force swim, SI= social interaction ratio time, PSA= peak startle amplitude.

Table 4: Table represents immunostaining outcomes for F0 and F1 mice (See abbreviations for this table in Page no. xviii).

Table 5A: Assessed the effect of stress in F2 generation (three stressed conditions vs control).

In here, **(1)** male F1 mice (from control F0 parents) were bred with female F1 mice (from control F0 parents) to generate F2 offspring labelled FC-MC (dad control-mom control); **(2)** male F1 mice (from predator stressed F0 parents) were bred with F1 females (from control F0 parents) to generate F2 offspring labelled FS-MC (dad stressed-mom control); **(3)** male F1 offspring (from control F0 parents) were bred with female F1 offspring (from predator stressed F0 parents) to produce F2 offspring labelled FC-MS (dad control-mom stressed); **(4)** male F1 mice (from predator stressed F0 parents) were bred with F1 females (from predator stressed F0 parents) to generated F2 offspring labelled FS-MS (dad stressed-mom stressed). To determine if there was a grandparental stress effect, planned comparisons were done comparing all three stressed groups (FC-MS, FS-MC and FS-MS) to the control group (FC-MC) across all behavioural measures (EPM,OF,LDB,AS,FS,SI) during adolescent(baseline behaviour, BEPMRT=baseline EPM ratio time, BEPMRF= baseline EPM ratio frequency, BLDENT= baseline LDB light side entries, BLDTIM= baseline LDB light side time, BOFTIM= baseline OF centre time, BOFDIS= baseline OF distance travelled, BOFVEL= baseline OF velocity , BSI=baseline social interaction ratio time, BFSW=baseline force swim immobile time, BPSAVG=baseline mean peak startle amplitude) and adulthood (after 2min RET, SEPMRT=after 2min RET EPM ratio time, SEPMRF= after 2min

RET EPM ratio frequency, SLDENT= after 2min RET LDB light side entries, SLDTIM= after 2min RET LDB light side time, SOFTIM= after 2min RET OF centre time, SOFDIS= after 2min RET OF distance travelled, SOFVEL= after 2min RET OF velocity , SSI= after 2min RET social interaction ratio time, SFSW= after 2min RET force swim immobile time, SPSAVG= after 2min RET mean peak startle amplitude).

Table 5B: FS-MC differs from FC-MS in F2 generation. To access whether there was a differential contribution from the grandmother and the grandfather to the grandchild's behavioural phenotype, planned comparisons were done comparing the FS-MC group to the FC-MS group across all behavioural measures (EPM,OF,LDB,AS,FS,SI) during adolescent(baseline behaviour, BEPMRT, BEPMRF, BLDENT, BLDTIM, BOFTIM, BOFDIS, BOFVEL, BSI, BFSW, BPSAVG) and adulthood (after 2min RET, SEPMRT, SEPMRF, SLDENT, SLDTIM, SOFTIM, SOFDIS, SOFVEL, SSI, SFSW, SPSAVG).

Table 6A: Assessed the effect of stress in F2 generation MWM probe (three stressed conditions vs control). To determine if there was a grandparental stress effect, planned comparisons were done comparing all three stressed groups (FC-MS, FS-MC and FS-MS) to the control group (FC-MC). Offspring who had at least one grandparent that was stressed (FC-MS, FS-MC, FS-MS) showed spatial learning deficits in the probe trial compared to offspring with both grandparents who were not stressed (FC-MC). During probe trial, offspring labelled FC-MC spent significantly more time in the target quadrant which had contained the hidden platform than three stressed conditions. Platform latency and number of platform location cross of FC-MC were significantly higher than stressed groups.

Table 6B: FS-MC differs from FC-MS in F2 generation MWM probe. In here, FS-MC = male F1 mice (from predator stressed F0 parents) were bred with F1 females (from control F0 parents) to generate F2 offspring labelled FS-MC (dad stressed-mom control); FC-MS= male F1 offspring (from control F0 parents) were bred with female F1 offspring (from predator stressed F0 parents) to produce F2 offspring labelled FC-MS (dad control-mom stressed).

To access whether there was a differential contribution from the grandmother and the grandfather to the grandchild's behavioural phenotype, planned comparisons were done comparing the FS-MC group to the FC-MS group. There were no significant effects found in this comparison.

Table 7: Selective human studies supporting the role of DNA methylation in PTSD. **Abbreviations:** adenylate cyclase-activating polypeptide Type I receptor gene (*Adcyap1r1*),

amplification of inter-methylated sites (*Aims*), BARX homeobox 1 (*Barx1*), brain-derived neurotrophic factor (*Bdnf*), catechol-O-methyltransferase gene (*Comt*), community and domestic violence (CDV), corin, serine peptidase genes (Corin), cystic fibrosis transmembrane conductance regulator (*Cftr*), FK506 binding protein 5 (*Fkbp*), nuclear receptor subfamily 3 group C member 1 gene (*Nr3c1,* glucocorticoid receptor gene), insulin like growth factor 2 (*Igf2*), interleukin like chemokines (*Il8, Il16, Il18*), nuclear receptor subfamily 3 group C member 2 gene (*Nr3c2,* mineralcorticoid receptor gene), SET and MYND domain containing 3 (*Smyd3*), solute carrier family 6 member 4 (*Slc6a4*).

Table 8: Selective rodent studies supporting the role of DNA methylation in predator stress model. **Abbreviations:** amplification of inter-methylated sites (*Aims*), arginine vasopressin (*Avp*), brain-derived neurotrophic factor (*Bdnf*), disks large-associated protein 2 (*Dlgap2*), FK506 binding protein 5 (*Fkbp5*), nuclear receptor subfamily 3 group C member 1 gene (*Nr3c1*, glucocorticoid receptor gene).

Table 9: Total number of mice used in this thesis.

Table 1: Summary table for major experimental outcomes.

F0 mice	**Anxiety-like behaviour after 5 min RET: (Control vs. Stress)** FREEZDUR: ↑ FREEZFREQ: ↑ EPM: RT in open arm: ↓ RF in open arm: ↓
F1 mice	**Anxiety-like behaviour after 2 min RET during adulthood: (Control vs. Stress)** FREEZDUR: ↑ FREEZFREQ: ↑ EPM: RT in open arm: ↓ RF in open arm: ↓ OF: time in center: ↓ distance travelled: ↓ LDB: light side entries: ↓ light side duration: ↓ AS: ↑ FS: **NS** SI: **NS** MWM: ⊗ **Cross-fostering experiments: Anxiety-like behaviour during adulthood after 2min RET:** **(Biological stressed offspring fostered either with stressed or control mother Vs. control offspring)** FREEZDUR: ↑ FREEZFREQ: ↑ EPM: RT in open arm: ↓ RF in open arm: ↓ OF: time in center: ↓ distance travelled: **NS** LDB: light side entries: ↓ light side duration: ↓ AS: ↑ FS: **NS** SI: ↓ MWM: ⊗

F2 mice	**Anxiety-like behaviour after 2 min RET during adulthood offspring who had at least one grandparent that was stressed vs. control)** FREEZDUR:  FREEZFREQ:  EPM: RT in open arm:  RF in open arm:  OF: time in center:  distance travelled: **NS** LDB: light side entries: **NS** light side duration:  AS:  FS: **NS** SI:  MWM: 
F0 mice	**Molecular measures: (Control vs. Stress)** Plasma corticosterone:  **Immunostaining:** c-FOS:  in DENTRH; DENTLH; VDLH; CA1RH; VCA1RH; VCA1LH. GR:  in DENTRH; DENTLH; CA1RH; CA1LH and Hypotha.  in CA2RH; CA2LH; CA3RH; CA3LH; Thla and BLA. FKBP:  in DENTRH; CA2RH; CA3LH; and BLA.

F1 mice	**Molecular measures: (Control vs. Stress)** Plasma corticosterone:  **Immunostaining:** c-FOS:  in DENTRH; DENTLH; VDRH; VDLH; CA1RH; CA1LH; VCA1RH; VCA1LH. GR:  in DENTRH; DENTLH; CA1RH; CA1LH; CA3LH; Thla and Hypotha. FKBP:  in DENTRH; DENTLH; CA1RH; CA1LH; Thala and BLA.  in CA2RH; CA2LH; CA3RH; and CA3LH.

Table 2: Table represents mother-pup interaction, litter size, numbers of male and female pup and % of male pup's outcomes for normal and cross-fostering F1 generation experiments.

		Mom-pup interaction time (IT)					Number of live pups born		
Comparison		**PND5**	**PND7**	**PND9**	**PND12**	**LITTER SIZE**	**Male pups**	**Female pups**	**% of male pups**
A) Normal condition									
Control vs Stress	BMOM-pup interaction time (IT)								
	(within 30 min):								
	i) NS main effect stress for IT	F(1,17)=2.694, p=0.119				t(17)=0.015,p=0.988	t(17)=-0.68,p=0.947	t(17)=0.082,p=0.935	t(17)=-0.294,p=0.773
	ii) Linear trend analysis revealed	F(1,17)=0.082, p=0.777							
	NS age*stress effect								
B) CF condition									
Control vs Stress	FMOM-pup interaction time (IT)								
	(within1hr):								
	i) NS main effect stress for IT	F(1,12)=2.416, p=0.146				t(12)=-816,p=0.430	t(12)=-1.044,p=0.317	t(12)=0.212,p=0.836	t(12)=-0.774,p=0.454
	ii) Linear trend analysis revealed	F(1,12)=0.385, p=0.547							
	NS age*stress effect								

Table 3: Descriptive statistical table for all behavioural outcomes including F0, F1 and F2 data (See supplementary excel Table link below, to open this Google.doc excel link- right click on this link and open hyperlink**).**

https://docs.google.com/spreadsheets/d/1332ZLWIQtTJFdD-OK0b6LgywS-O7-ICH/edit#gid=361262621

Table 4: Table represents immunostaining outcomes for F0 and F1 mice.

Area	Generation	p-value	t-value	Adjust p-value
c-FOSDENRH	F0	0.0000009	20.425	0.0000081
c-FOSVDENLH	F0	0.00004753	10.352	0.00021389
c-FOSDENLH	F0	0.0000535	10.14	0.0001605
c-FOSVCA1RH	F0	0.00015516	8.399	0.00034911
c-FOSCA1RH	F0	0.000159	8.362	0.0002862
c-FOSVCA1LH	F0	0.01171341	3.575	0.01757012
c-FOSVDENRH	F0	0.05380574	2.393	0.06917881
c-FOSBLA	F0	0.7037065	0.399	0.79166981
c-CA1LH	F0	0.7757	0.298	0.7757
c-FOSCeA	F0	0.7626	0.32	0.763
c-FOSCA1RH	F1	0.00000005	32.846	0.00000045
c-FOSDENLH	F1	0.00000047	20.253	0.00000212
c-FOSCA1LH	F1	0.00000299	16.655	0.00000897
c-FOSVCA1RH	F1	0.00000349	16.222	0.00000785
c-FOSVDENRH	F1	0.000005	15.258	0.000009
c-FOSVCA1LH	F1	0.00004102	10.622	0.00006153
c-FOSVDENLH	F1	0.00004506	10.449	0.00005793
c-FOSDENRH	F1	0.0017193	8.246	0.00193421
c-FOSBLA	F1	0.20597479	1.418	0.20597479
c-FOSCeA	F1	0.9752	0.03	0.9753
GRCA2LH	F0	0.00000001	34.302	0.0000001
GRCA2RH	F0	0.0000026	15.287	0.000013
GRCA1RH	F0	0.00000225	11.924	0.0000075
GRCA3RH	F0	0.0000024	11.802	0.000006
GRCA3LH	F0	0.00000253	11.749	0.0000051
GRHYPOTH	F0	0.00000342	11.283	0.0000057
GRDENTLH	F0	0.00000492	10.755	0.000007
GRBLA	F0	0.000006	10.382	0.0000075
GRCA1LH	F0	0.0001	7.11	0.0001111
GRDENTRH	F0	0.000209	6.44	0.000209
GRTHAL	F0	0.00523	3.8	0.0047545
GRMPFC	F0	0.8563	0.187	0.7135833
GRDENTRH	F1	0.00000022	16.108	0.0000026
GRHYPOTH	F1	0.00000024	15.928	0.0000014
GRTHAL	F1	0.0000003	15.483	0.0000012
GRCA1RH	F1	0.00000063	14.076	0.0000019
GRCA1LH	F1	0.00000626	10.416	0.000015
GRDENTLH	F1	0.00000907	9.512	0.0000181
GRCA3LH	F1	0.00815	3.494	0.0139714
GRCA3RH	F1	0.03547	2.526	0.053205
GRMPFC	F1	0.0954	1.897	0.1272
GRCA2RH	F1	0.1065	1.818	0.1278
GRCA2LH	F1	0.2483	1.261	0.2708727
GRBLA	F1	0.985	0.019	0.985
FKBPBLA	F0	0.00000039	14.972	0.0000047
FKBPCA2RH	F0	0.0000445	7.978	0.000267
FKBPDENRH	F0	0.0012384	4.871	0.0049536
FKBPCA3LH	F0	0.0041	3.973	0.0123
FKBPTHAL	F0	0.0266	2.71	0.06384

FKBPCA2LH	F0	0.042	2.417	0.084
FKBPCA3RH	F0	0.0435	2.395	0.0745714
FKBPCA1RH	F0	0.0652	2.135	0.0978
FKBPCA1LH	F0	0.0784	2.017	0.1045333
FKBPHYPOT	F0	0.083	1.98	0.0996
FKBPMPFC	F0	0.499	0.708	0.5443636
FKBPDENLH	F0	0.615	0.522	0.615
FKBPDENLH	F1	0.000001	39.441	0.000012
FKBPCA2LH	F1	0.00000055	14.327	0.0000033
FKBPCA1LH	F1	0.00000326	11.354	0.000013
FKBPCA3RH	F1	0.00002303	8.737	0.0000691
FKBPBLA	F1	0.00002691	8.553	0.0000646
FKBPTHAL	F1	0.00003373	8.291	0.0000675
FKBPCA3LH	F1	0.00022685	6.324	0.0003889
FKBPDENRH	F1	0.000416	5.776	0.000624
FKBPCA1RH	F1	0.001058	4.996	0.0014107
FKBPCA2RH	F1	0.019056	2.928	0.0228672
FKBPHYPOT	F1	0.45	0.794	0.4909091
FKBPMPFC	F1	0.8	0.259	0.8

Table 5A: Assessed the effect of stress in F2 generation (three stressed conditions vs control)

Measures	F -value	df	DS-MS MEAN	DC-MS MEAN	DS-MC MEAN	DC-MC MEAN	p-VALUE
BEPMRT	68.54	(1,71)	.278 **(N=21)**	.493 **(N=14)**	.356 **(N=20)**	.742 **(N=24)**	0.0001
BEPMRF	34.39	(1,71)	.291 **(N=21)**	.379 **(N=14)**	.422 **(N=20)**	.641 **(N=24)**	0.0001
BLDENT	3.2674	(1,70)	4.1904 **(N=21)**	7.7142 **(N=14)**	6 **(N=19)**	7.2083 **(N=24)**	0.0750
BLDTIM	5.593	(1,70)	37.71 **(N=21)**	76.57 **(N=14)**	47.42 **(N=19)**	70 **(N=24)**	0.0208
BOFTIM	27.35	(1,71)	54.027 **(N=21)**	68.361 **(N=14)**	44.703 **(N=20)**	89.415 **(N=24)**	0.0001
BOFDIS	33.904	(1,71)	1185.648 **(N=21)**	1135.463 **(N=14)**	1782.968 **(N=20)**	3215.847 **(N=24)**	0.0001
BOFVEL	34.395	(1,71)	4.289 **(N=21)**	4.118 **(N=14)**	6.046 **(N=20)**	10.879 **(N=24)**	0.0001
BSI	9.271	(1,70)	1.130 **(N=21)**	1.530 **(N=14)**	.9504 **(N=19)**	1.636 **(N=24)**	0.0033
BFSW	5.234	(1,70)	1.2453 **(N=21)**	.8659 **(N=14)**	2.7902 **(N=19)**	4.838 **(N=24)**	0.0252
BPSAVG	7.1626	(1,71)	336.143**(N=21)**	190.468**(N=14)**	232.922 **(N=20)**	182.144 **(N=24)**	0.0092
AFTER 2MIN RET							
SEPMRT	218.117	(1,70)	.1344 **(N=21)**	.4551 **(N=14)**	.3269 **(N=19)**	.80921 **(N=24)**	0.0001
SEPMRF	1806.409	(1,70)	.1723 **(N=21)**	.3574 **(N=14)**	.2687 **(N=19)**	.7420 **(N=24)**	0.0001
SLDENT	0.4326	(1,70)	4.333 **(N=21)**	9.214 **(N=14)**	7.526 **(N=19)**	7.708 **(N=24)**	0.5129
SLDTIM	18.011	(1,70)	28.761 **(N=21)**	90.928 **(N=14)**	39.368 **(N=19)**	82.291 **(N=24)**	0.0001
SOFTIM	100.844	(1,70)	38.081 **(N=21)**	72.274 **(N=14)**	53.467 **(N=19)**	118.508 **(N=24)**	0.0001
SOFDIS	61.90	(1,70)	675.659 **(N=21)**	1129.538 **(N=14)**	1134.88 **(N=19)**	1863.470 **(N=24)**	0.0001
SOFVEL	787.814	(1,70)	2.252 **(N=21)**	4.190 **(N=14)**	3.891 **(N=19)**	6.337 **(N=24)**	0.0001

SSI	5.166	(1,70)	.9845 **(N=21)**	1.5933 **(N=14)**	1.1509 **(N=19)**	1.5274 **(N=24)**	0.0261
SFSW	6.0939	(1,70)	.6613 **(N=21)**	.3585 **(N=14)**	1.861 **(N=19)**	3.570 **(N=24)**	0.0160
SPSAVG	33.349	(1,71)	423.818 **(N=21)**	236.103 **(N=14)**	266.831 **(N=20)**	146.232 **(N=24)**	0.0001
FREEZDUR	47.341	(1,70)	29.476 **(N=21)**	19.357 **(N=14)**	25.631 **(N=19)**	3.375 **(N=24)**	0.001
FREEZFREQ	197.97	(1,70)	5.095 **(N=21)**	4.2857 **(N=14)**	5.2631 **(N=19)**	1.3333 **(N=24)**	0.0001

Table 5B: DS-MC differs from DC-MS **in F2 generation**

Measures	**F -value**	**df**	**DC-MS MEAN**	**DS-MC MEAN**	**p-VALUE**
BEPMRT	5.501	(1,71)	.493 **(N=14)**	.356 **(N=20)**	0.0218
BEPMRF	0.4636	(1,71)	.379 **(N=14)**	.422 **(N=20)**	0.4982
BLDENT	3.522	(1,70)	7.7142 **(N=14)**	6 **(N=19)**	0.0647
BLDTIM	10.3428	(1,70)	76.57 **(N=14)**	47.42 **(N=19)**	0.0020
BOFTIM	7.726	(1,71)	68.361 **(N=14)**	44.703 **(N=20)**	0.007
BOFDIS	2.389	(1,71)	1135.463 **(N=14)**	1782.968 **(N=20)**	0.1266
BOFVEL	1.995	(1,71)	4.118 **(N=14)**	6.046 **(N=20)**	0.1622
BSI	9.393	(1,70)	1.530 **(N=14)**	.9504 **(N=19)**	0.0031
BFSW	1.065	(1,70)	.8659 **(N=14)**	2.7902 **(N=19)**	0.3056
BPSAVG	1.468	(1,71)	190.468**(N=14)**	232.922 **(N=20)**	0.2297

AFTER 2MIN RET					
SEPMRT	7.9705	(1,70)	.4551 **(N=14)**	.3269 **(N=19)**	0.0062
SEPMRF	2.945	(1,70)	.3574 **(N=14)**	.2687 **(N=19)**	0.0906
SLDENT	1.4877	(1,70)	9.214 **(N=14)**	7.526 **(N=19)**	0.2267
SLDTIM	31.522	(1,70)	90.928 **(N=14)**	39.368 **(N=19)**	0.0001
SOFTIM	4.927	(1,70)	72.274 **(N=14)**	53.467 **(N=19)**	0.0297
SOFDIS	0.00127	(1,70)	1129.538 **(N=14)**	1134.88 **(N=19)**	0.9717
SOFVEL	.7913	(1,70)	4.190 **(N=14)**	3.891 **(N=19)**	0.3763
SSI	7.050	(1,70)	1.5933 **(N=14)**	1.1509 **(N=19)**	0.0098
SFSW	1.1394	(1,70)	.3585 **(N=14)**	1.861 **(N=19)**	0.2894
SPSAVG	.6827	(1,71)	236.103 **(N=14)**	266.831 **(N=20)**	0.4115
FREEZDUR	2.325	(1,70)	19.357 **(N=14)**	25.631 **(N=19)**	0.1318
FREEZFREQ	.7952	(1,70)	4.2857 **(N=14)**	5.2631 **(N=19)**	0.3756

Table 6A: Assessed the effect of stress in F2 generation MWM_ probe (three stressed conditions vs control)

	F -value	df	DS-MS MEAN	DC-MS MEAN	DS-MC MEAN	DC-MC MEAN	p-VALUE
PRBDIS	9.862	(1,55)	745.102 **(N=23)**	769.164 **(N=14)**	884.897 **(N=16)**	1092.83 **(N=10)**	0.0027
PRBVEL	.1937	(1,55)	14.371 **(N=23)**	14.53 **(N=14)**	16.569 **(N=16)**	19.721 **(N=10)**	0.6616
PRBFREQ	12.318	(1,55)	0.6956 **(N=23)**	1.285 **(N=14)**	2.333 **(N=16)**	3 **(N=10)**	0.0009
PRBLAT	28.230	(1,55)	41.634 **(N=23)**	36.138 **(N=14)**	39.423 **(N=16)**	17.755 **(N=10)**	0.0001

Table 6B: DS-MC differs from DC-MS **in F2 generation _MWM_ probe trial**

Measures	F -value	df	DC-MS MEAN	DS-MC MEAN	p-VALUE
PRBDIS	0.9762	(1,55)	769.164 **(N=14)**	884.897 **(N=16)**	0.3275
PRBVEL	1.452	(1,55)	14.53 **(N=14)**	16.569 **(N=16)**	0.2334
PRBFREQ	3.519	(1,55)	1.285 **(N=14)**	2.333 **(N=16)**	0.0660
PRBLAT	0.1760	(1,55)	36.138 **(N=14)**	39.423 **(N=16)**	0.6765

Table 7: Selective human studies supporting the role of DNA methylation in PTSD

Reference	Candidate gene	Epigenetic changes	Sample size	Generation study carried out through
Koenen et al., 2011	*Slc6a4*	Lower *Slc6a4* methylation levels in blood tissue and higher number of traumatic events increased risk for PTSD	100	F0
Ressler et al., 2011	*Adcyap1r1*	*Adcyap1r1* methylation levels were observed in the peripheral blood of PTSD subjects compared with control	94	F0
Rusiecki et al., 2013	*Il8, Il16, Il18*	Increased serum *Il18* methylation in combat veterans who developed PTSD, but decreased *Il18* methylation levels in veterans without PTSD	150	F0
Norrholm et al., 2013	*Comt*	Higher blood level methylation of *Comt* are associated with impaired fear inhibition	270	F0
Yehuda et al., 2013	*Nr3c1, Fkfbp5*	Pre psychotherapy *Nr3c1* 1F promoter methylation in blood tissue positively correlated with improvements in symptoms, while decreased *Fkbp5* methylation occurred concomitantly with recovery from PTSD	16	F0
Labonté et al., 2014	*Nr3c1*	Increased *Nr3c1*mRNA expression and decreased overall *Nr3c1* 1B and 1C promoter methylation levels in individuals blood with lifetime PTSD	46	F0
Vukojevic et al., 2014	*Nr3c1*	In the peripheral blood, methylation of *Nr3c1* 1F promoter is linked to traumatic memories and PTSD risk in male survivors of the Rwandan genocide	152	F0

Yehuda et al., 2014	*Nr3c1*	*Nr3c1* 1F promoter methylation inversely correlated with symptoms severity in the blood tissue of combat veterans with PTSD	122	F0
Yehuda R, Daskalakis NP, Lehrner A, 2014	*Nr3c1*	Offspring with paternal PTSD showed higher *Nr3c1* 1F promoter methylation in the blood tissue if maternal PTSD was not present. Offspring with maternal and paternal PTSD showed lower methylation	95	F0, F1(Intergeneration)
Perroud et al., 2014	*Nr3c1, Nr3c2*	Higher plasma levels methylation at promoter 1F for *Nr3c1* in female survivors of genocide and their offspring compared to controls, but no differences observed in *Nr3c2* methylation	25	F0, F1(Intergeneration)
Yehuda et al., 2016	*Fkbp5*	Holocaust survivors showed increased methylation of the promotor region for *Fkbp5*, while their offspring showed the opposite in their blood tissue	71	F0, F1(Intergeneration)
Kertes et al., 2017	*Bdnf*	Maternal experiences of war trauma were associated with higher *Bdnf* methylation in umbilical cord blood, placental tissue, and lower methylation in maternal venous blood.	24	F0, F1(Intergeneration)
Serpeloni et al., 2017	*Barx1, Cftr, Corin, Smyd3*	Grandmaternal exposure to CDV during pregnancy was significantly associated with decreased methylation in *Corin*, *Smyd3*, and *Barx1*, and increased methylation of *Cftr* in grandchildren's saliva samples	121	F0, F1, F2,F3 (Intergeneration)
Kim et al., 2017	*Bdnf*	Subjects with PTSD showed a higher methylation *Bdnf* promoter I region in their blood tissue compared with those without PTSD	248	F0
Voisey et al., 2019	*Bdnf*	Decreased methylation at three *Bdnf* sites were observed in combat exposed PTSD veterans blood compared with control.	96	F0

Table 8: Selective rodent studies supporting the role of DNA methylation in predator stress model

Reference	Candidate gene	Epigenetic changes	Sample size	Generation study carried out through
Chertkow-Deutsher et al., 2010	*Dlgap2*	Higher *Dlgap2* methylation and reduced mRNA expression in predator odor exposed rat's hippocampus.	Not mentioned	F0
Bowen et al., 2014	*Avp*	Predatory stress was associated with decreased *Avp* promoter methylation in the medial amygdala	48	F0
St-Cyr and McGowan, 2015	*Bdnf*	Female offspring of mice exposed to predator odor during pregnancy had decreased *Bdnf* transcript abundance positively correlated with a concomitant decrease in methylation of *Bdnf* exon IV in the hippocampus	42	F0 , F1(Intergeneration)
St-Cyr et al., 2017	*Nr3c1, Fkfbp5*	Female offspring from prenatal predator odor-exposed dams showed increased transcript abundance of both the glucocorticoid receptor gene (*Nr3c1*; on the day of birth) and *Fkfbp5* (in adulthood) in the amygdala	24	F0, F1(Intergeneration)

Table 9: Total number of mice used in this thesis

Control female (n=30), control male (n=30), stressed female (n=33) and stressed male (n=33) were used to generate total number of F1 mice (control mice n= 240 and stressed mice n= 264).

a) In this thesis F1 generation mice were used: Total number of F1 mice (control n= 157 and stressed n= 171 were used for behaviour, corticosterone assay, immunostaining (GR and FKBP5) and breeding to generate F2 mice.

Control F1 mice:

Experiments	**Mice number**
F1 Behavioral experiments:	117
Molecular experiments (Cort assay, immunostaining: c-Fos, GR and FKBP5):	29
F1 mice were used to generate F2 mice:	11
Total:	157

Stressed F1 mice:

Experiments	**Mice number**
F1 Behavioral experiments:	133
Molecular experiments (Cort assay, immunostaining: c-Fos, GR and FKBP5):	29
F1 mice were used to generate F2 mice:	9
Total:	171

b) Cross foster experiment: Control Mother (F0): n=7, Stressed mother(F0): n=7, Control Father(F0): n=7, Stressed mother(F0): n=7. Total (n=110) F1 babies used in cross fostering experiment.

Control Mom's babies crossfosterd to control mom **(Number of F1 babies= 34)**	Control Mom's babies crossfosterd to PS mom **(Number of F1 babies= 23)**
PS Mom's babies crossfosterd to control mom **(Number of F1 babies= 23)**	PS Mom's babies crossfosterd to PS mom **(Number of F1 babies= 30)**

c) F2 generation experiment: Control F1 mice [n=11, male F1 mice (n=5), female F1 mice (n= 6)] , Stressed F1 mice [n=9, male F1 mice (n=5), female F1 mice (n= 4)] were used to generate total (n=79) F2 mice.

Control M X Control F offspring from F1 generation (Number of F2 babies = 24)	**Control F X Stressed M offspring from F1 generation (Number of F2 babies= 20)**
Control M X Stressed F **offspring from F1 generation (Number of F2 babies= 14)**	Stressed M X Stressed F **offspring from F1 generation (Number of F2 babies= 21)**

Appendix (For Immunostaining)

1. 0.2 M Phosphate Buffer (2X)

4 litres

Sodium phosphate dibasic	92.00 g
Sodium phosphate monobasic	20.96 g

Dissolve in 3800 ml dH_2O. pH to 7.4 using 10N NaOH (to increase pH) or conc HCl (to decrease pH). Top up with dH_2O to final volume (4000ml)

2 litres

dH_2O	1900 ml
Sodium phosphate dibasic	46.0 g
Sodium phosphate monobasic	10.48 g

2. PBS

1 liter

0.2 M phosphate buffer	500 ml
dH_2O	500 ml
sodium chloride	9 g

3. Saline

100 ml

Sodium chloride	0.9 g
dH_2O	100 ml

4. 20% Sucrose

For brain storage and cryoprotection:

100 ml

1. Add 20 g sucrose to a graduated cylinder.
2. Fill to 50 ml line with dH_2O
3. Add 50 ml 0.2 M phosphate buffer
4. Stir (small stir-bar to fit grad cylinder) until dissolved and store @ 4°C

500 ml

1. 100 g Sucrose
2. 180 ml dH_2O
3. 250 ml phosphate buffer

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