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Cognitive and neural development in a rodent model of PTSD from maternal maltreatment

A thesis presented by

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ABSTRACT

Childhood neglect influences development and increases the risk for and severity of mental illness. Previous study has shown that early life stress (ELS) alters stress circuitry, elevates basal stress hormone, and impairs regulation of the HPA. In this study, we seek to understand the impacts of decreased quality of care and trauma on the cognitive and neural development of rats at adolescence. We used a 1-week limited bedding protocol to induce fragmented care in dams and a 10-minute exposure to fox odor to induce stress in offspring later. Memory function and patterns of brain activity following stressor exposure were assessed in order to characterize the impact of early life stress in male and female rats. The predator odor groups showed reduction in mobility and exploration time during and one week after exposure. The predator odor-exposed group without early life stress showed better memory performance. Female rats in the ELS groups are more susceptible to the fox odor and were less able to recognize novel objects, compared to the male counterparts. These findings also implied an interaction between ELS and predator odor, which requires further research to validate the impact ELS might have on later stressful events.

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1. INTRODUCTION

1.1 COVID-19 and Post-traumatic stress disorder (PTSD)

As the COVID-19 pandemic started to wind down, it left some indelible marks on the physical and mental health of anyone who went through it, especially young children.

COVID-19 is perceived as a disease affecting the elderly population, so the tolls on children are often overlooked. While for some, the impacts of COVID -19 may just be temporary, for many others, the pandemic has exposed them to a great source of stress, fear, and uncertainty. Over one million children who became orphans during the pandemic experienced tremendous adversity. The loss of a provider means a significant disruption of basic needs such as food, shelter, care, and love. For other children, parents losing their jobs during the economic downturn creates stressful situations at home and may lead to a decline in quality of care for the children. Whether children face the death of a loved one, or a lack of care which leads to fear and helplessness, such disruptions are also more likely to expose children to abuse, poverty, and mental health problems later in life (Bai et al., 2014; Bath et al, 2016; Chocyk et al., 2010; CDC, 2021).

Mental health conditions, such as depression, bipolar disorder, attention-deficit/hyperactivity disorder (ADHD), and post-traumatic stress disorder (PTSD), have a massive impact on economic and social stability and affect the quality of life of hundreds of millions of people (Alonso et al., 2004; Bastiaanssen et al., 2004). This study focuses on PTSD, a condition that falls under the spectrum of depression and anxiety-related disorders. People with this condition either experience or witness a traumatic event, which affects their thoughts, emotions, and behaviors, leaving them unable to function normally in daily life. Symptoms are categorized into four main clusters: intrusion, avoidance, negative alterations in cognition and mood, and alterations in arousal and reactivity. Intrusion included intrusive thoughts, frequent flashbacks to

negative events, and nightmares. Avoidance refers to the intentional avoidance of trauma-related events. Negative alterations in mood and cognition refer to social withdrawal, anhedonia, or negative self-perception. Alterations in arousal and reactivity include irritability, aggression, sleep disturbance and hypervigilance (Flandreau et al., 2018).

While much progress has been made in understanding how exposure to trauma initiates the development of PTSD, some researchers started to question if trauma is the main cause of the disorders. This is because only a subset of people who experience some type of trauma in their life eventually develop PTSD, while the rest will recover eventually (Richter-Levin, 2019). This suggests that there might be other factors - risk factors - that make some people more susceptible to develop PTSD and play a role in exacerbating the symptoms. The absence of these factors allow individuals to cope with stress effectively and return to a balanced mental state shortly after the traumatic event. Risk factors are organized into three main groups, including biological (sex or pre-existing mental health problems), genetic (hereditary or epigenetic), and environmental (childhood experience, social support, or additional life stress sources). In the context of COVID-19, the focus of this research is on environmental factors, which have been known to strongly modulate neuronal activity and can influence the development of neuropsychiatric disorders (DiGangi et al, 2013b; Meyer-Lindenberg et al., 2012; Robertson, 2004). For instance, a meta analysis done by Donohoe and Mothersill (2016) found that children and adults with early life adversity and social stress consistently show higher response in the amygdala, which is similar to brain activation reported in patients with psychiatric disorder (Mothersill, O., & Donohoe, G., 2016). Meanwhile, Heim's research group (2013) found that adults who experienced childhood sexual and emotional abuse show significant thinning in

specific areas of the cortex, suggesting early life stress has a long lasting effect on neural plasticity even to adulthood (Heim et al., 2013).

The aim of this study is to investigate the impact of environmental risk factors on the development of PTSD, especially on learning abilities and cognitive development in rodent models. To simulate parents' neglected or disruptive care during COVID-19, we will use the limited bedding protocol, which will be discussed in more detail in section 1.2. Section 1.3 will outline existing traumatic paradigms. Section 1.4 discusses how we evaluate the cognitive aspects of this model, including the behavioral and diagnostic test. Finally, in section 1.5, we explain the rationale, objectives of the current study and state our hypotheses.

1.2 Early life stress as an environmental risk factor

In animals ranging from rodents to non-human primates and humans, the perinatal period and childhood are developmental stages where the brain has significantly increased plasticity: massive creation of neurons comes to an end and brain areas start to differentiate. In primates, for instance, gene expression in progenitor cells and maturing neurons changes rapidly before birth. Meanwhile, layers and cortical areas acquire adult-like molecules in late postnatal development (Bakken, et al., 2016). In rodents, brains show a massive increase in axonal and dendritic density a week after birth, and peak in myelination rate and changes in neurotransmitter and receptors after 20 days, which corresponds to about 40 gestational weeks and 2.5 years in human time (Semple et al., 2013). Because of this malleability, abnormalities during development can lead to various neurological diseases. Early life stress (ELS) in the form of poor early life care has been shown to profoundly alter the development of stress-responsive circuitry (Francis et al., 2006; St. Cyr et al., 201; Zhang et al., 2013), leading to elevated basal stress

hormone levels (Rice et al., 2008; Rosenfield et al., 1991), and impaired regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Anisman et al., 1998). In sum, this stage is particularly vulnerable to environmental influences that act as stressors and can significantly influence the behavioral, physiological, and cognitive functions of individuals throughout their lives (Anderson, 2003; Chen & Baram, 2015; Roth & Sweatt, 2011).

Early life stress (ELS) can be studied through various methods, from prenatal stress by exposing mothers to stress or stress hormones to postnatal stress by manipulating the environment after birth. For prenatal stress, studies in both animals and humans have shown that exposure of pregnant females to stress or stress hormones leads to earlier activation of the fetus's HPA axis response, which puts stress on undeveloped systems, escalates the effect of risk factors, and increases their susceptibility to developing mental disorders later in life (Baydoun & Saftlas, 2008; Boersma & Tamashiro, 2015; Weinstock, 2008). In particular, Yeh and colleagues (2012) show that prenatal stress inhibits the regulation of brain derived neurotrophic factors (Yeh et al., 2012), which is required for proper development of neurotransmitters and learning and memory processes (Autry & Monteggia, 2012). Meanwhile, Chapillon et al. (2002) show that maternal stress leads to higher mortality of pups and reduced birth weight, a finding that Burgueño's group also reported (Burgueño et al., 2020). In humans, stress during pregnancy has been shown to have a long lasting effect on children, including sleep problems in infancy and toddlerhood (Connor et al., 2007), deficits in brain development, mood-related behaviors, and gut microbiota in offspring (Zhang et al., 2021).

To mimic the unstable, distracted parent-children relationship during the pandemic, this study focuses on the postnatal period, where the role of caregiver is crucial in forming healthy emotional and cognitive development. There are several studies that have investigated the effects

of postnatal stress in rodents, including changes in epigenetics, social and memory abilities, using different paradigms of early life stress. Amongst them, the maternal separation protocol has been widely used to establish many molecular and phenotypic characteristics in early-life stressed pups. This paradigm focuses on the quantity of care the pups receive by periodically isolating pups and their moms during critical periods. Like other mammals, during the first weeks after birth, rodent pups heavily depend on their mothers for food and heat. Therefore, an absence of the dams amounts to a number of adaptive behaviors, ranging from attempting to relocate the nest and vocalizing ultrasonic sound, to releasing of stress hormones such as corticosterone and inhibiting several anabolic pathways associated with development and growth (Kaffman & Meaney, 2007). These behaviors have short-term evolutionary advantages, such as ultrasound vocalization attracts mothers' attention and helps relocate separated pups. Or in the absence of food supply, corticosterone release helps promote gluconeogenesis and therefore preserve plasma glucose levels for other activities (Kuo et al., 2015). However, these behavioral changes can also induce long-lasting functional adaptations in adult rats. Recent studies have shown that early life stress in the form of maternal separation can significantly induce the functional adaptation of transcriptional factors and modify the expression of specific proteins, leading to enduring deficits in social and memory behaviors in rodents (Levine et al., 2012; Neigh et al., 2013; Wang et al., 2014).

While the maternal separation paradigm can provide valuable insights into the behavioral and genetic changes that occur in young individuals, they also have many limitations. For example, a prolonged absence of dams can lead to premature death in pups due to the lack of warmth. In some cases, pups have not developed olfactory bulbs to detect the absence of mothers (Walker et al., 2017). Over the past two decades, a new procedure - limited bedding paradigm -

has been developed to study early life stress in rodents and has started to gain support. The dams in this paradigm are not separated from their pups, but they do not receive enough bedding materials to build optimal nests and keep the pups warm and contained in the nest. This procedure produces a fragmented, unpredictable, and anxious caretaking style of mom, in an effort to mimic the quality of care that depressed human parents or the loss of parents can induce (Goodwill et al., 2019; Brunson et al., 2005; Gilles, Schultz, & Baram, 1996; Ivy et al., 2008; Walker 2017). Rodent pups experienced this paradigm show reduced corticosterone level (Avishai-Eliner et al., 2001; Brunson et al., 2005; Gilles et al., 1996; Moussaoui et al., 2016a), reduced CRF1 mRNA expression in CA1 and dentate gyrus (Avishai-Eliner et al., 2001), and reduced hippocampal expression of genes involved in mitochondrial biogenesis, energy metabolism, neurogenesis (Maniam et al., 2016). This protocol is chosen specifically for this research because of its translational validity and success of previous studies.

1.3 Predator odor as a traumatizing event

Different from depression and other neuropsychiatric disorders which often lack clear causes, PTSD is defined by an exposure to a traumatizing event that produces a whole range of dysfunctional responses. The goal of using animal models of PTSD is to mimic an originating, triggering event that has long-lasting impacts and is engraved into the memory of the survivor. Currently, there are several paradigms to model this type of event, even though the duration and the repeated exposure varies from test to test. Stressors can be either physical (such as forced swimming or electric foot shock), psychological (such as exposure to predator odor or social defeat), or a combination of both.

Different types of stressors provide different models of fear and anxiety. For instance, electric foot shock registers fear through repeated series of painful sensation over several days. This paradigm is used to model a pathway of unpleasant learned responses, but PTSD is not only caused by a learned pathway. It is possible, as Richter-Levin (2007) suggests, that a very traumatizing, disturbing event activates innate fearful responses and eventually leads to a pathological pathway. Real life examples include an experience of being sexually trafficked to a near death experience. In other words, one very impactful event is enough to break down a healthy person. If this is the case, then the electric foot shock paradigm and other stressors that assume learned responses are not suitable for studying PTSD.

Predator-scented stressors are often used, because they can bring out the fear responses that mimic near death experiences in a natural environment. Trimethylthiazoline (TMT), a synthetic compound isolated from fox feces, has been shown to elicit a number of fearful responses (Fendt, Endres, and Apfelbach, 2003; Hacquemand et al., 2013; Wallace et al., 2010) and elevate the corticosterone levels and dopamine metabolism in the medial prefrontal cortex and lateral/basolateral amygdala (Cohen et al., 2003; Morrow et al., 2000), in ways that are distinctive from conditioned fear. For this reason, in this research, TMT is employed to mimic a major stress event that happens at a later threshold of development. In using psychological stressor-like predator odor, researchers assume that PTSD is predisposed by a risk factor and is exacerbated by a severe trauma that leads to mental breakdowns and pathological responses.

When we use animal models to study human conditions, there are several challenges. Besides biological and physiological differences, animals cannot report symptoms, such as intrusive nightmares or disturbing thoughts that are one of the major symptoms in PTSD. The high degree of overlapping symptoms between PTSD and other anxiety-related disorders such as

depression or major anxiety disorders challenge whether we study PTSD and not other disorders. In addition, variability between scientists and different labs makes it difficult to establish a reliable protocol. Therefore, researchers incorporate different validity elements to make sure the model effectively captures the biological and behavioral symptoms observed in the disorders and can be replicated across labs. The common validities are Face (where similar animal behaviors are observed on some standard paradigms), Construct (where similar biological circuits are observed in human tissues and animals), and Predictive (similar expected outcome in clinical trials). At least one type of validity is established in a research to make sure the animal model has translational validity.

Besides general validities, each animal model for a specific disorder has to meet several requirements in order to be effective in translating human symptoms and become a reliable medium for drug testing and understanding pathological pathways. For the model of PTSD, the trauma must first be severe enough that a relative short amount of time can elicit stress responses from rodents. In this research, we assume near death experience in animals is severe enough that it elicits a wide range of stress responses. Second, the intensity of the trauma should predict the severity of the outcome. Third, there should be some considerable amount of variability between subjects to study risk or personal factors. Resilience or vulnerability factors expose people to different degrees of stress than in real life, so only a small percentage of people who experience trauma will eventually develop PTSD. Finally, the behavioral tasks should assess the incubation, extinction, or desensitization after a period of time. The DSM-V defines PTSD as long-lasting dysfunctional behaviors that last more than one month (American Psychiatric Associates, 2013). In real life, some symptoms of PTSD persist for several weeks and even increase with the time,

so longitudinal studies are necessary to ensure the subjects exhibit the symptoms of PTSD (Siegmund & Wotjak, 2006).

1.4 Assessment of PTSD: alterations in mood and cognition

As discussed, PTSD is highly comorbid with many psychiatric conditions (e.g mood disorder, substance abuse) or physical health problems (such as vascular disease), meaning that it is possible PTSD may directly or indirectly impact cognitive and physical performance (Quello et al., 2005; Zen et al., 2011). As mentioned in section 1.1, a hallmark symptom of PTSD is negative alterations in mood and cognition. This term refers not only to social withdrawal, anhedonia, or negative self-perception, but also indicates a change in cognitive processes, such as memory, attention, planning, or learning. Besides trauma-related memory, a meta-analysis done by Schuitevoerder and colleagues (2013) has shown evidence of lower scores on memory tests in the adult samples with PTSD than those without PTSD. People with high levels of PTSD symptoms show impaired attention and learning ability (Brandes et al., 2002; Burriss et al., 2007; Johnsen & Asbjørnsen, 2008; Lambert et al., 2019; Yehuda et al., 2004). Children who were exposed to traumatic events tend to have poorer academic performance (Saigh et al., 1997; Swab-Stone et al., 1995), lower verbal IQ (Saigh, et al., 2006; Carrion et al., 2010), and lower reading comprehension (Delaney-Back et al., 2002)

In healthy people, the hippocampus, part of the limbic system, is responsible for encoding and retrieving information (Fortin, Agster, & Eichenbaum, 2002; Tulving & Markowitsch, 1998). However, physiological arousal during and after the exposure to traumatic events might make memories challenging to regulate. Memories might be processed abnormally, leading to both overrepresentation of some memories, such as nightmares or fragmented and frequent

flashbacks, and suppression of some other types of memories, such as selective amnesia or poor learning abilities (Carrion & Wong, 2012).

Some researchers have hypothesized that the elevated level of corticosterone is the driving factor behind learning deficits in PTSD. It is known that stress enhances the activity of the hypothalamus-pituitary-adrenal (HPA) axis and results in increased secretion of corticosteroids from the adrenal cortex. Excessive release of corticosterone can lead to neurotoxicity in areas rich in glucocorticoid receptors such as prefrontal cortex and hippocampus, and thereby leads to learning and memory deficits in patients with PTSD. Long-term administration of corticosterone in rats has been shown to be correlated with learning deficits (Arbel et al., 1994). In humans, excess levels of corticosterone are associated with reduced hippocampal volume and memory dysfunction (Carrion et al., 2007; Starkman et al., 1992; Weems & Carrion, 2007). However, some studies have demonstrated that the role of cortisol might be beneficial for memory encoding. Sherman and colleagues have demonstrated that even though there was a dramatic increase in the levels of cortisol when the participants were stressed, this elevation enhances encoding of emotionally arousing memory (Sherman et al., 2023). This study agrees with other previous research with the hypothesis that cortisol shifts the encoding pathway and modifies interpretation of the events.

In this study, we will use the novel object recognition task to evaluate learning ability of control and experimental groups. We will also look at the brain tissues involved in learning and memory processes to understand the link between activation patterns and behavioral responses.

Behavioral assessment: Novel Object Recognition

While some features of PTSD are difficult to measure in rodents, the impact on memory can be assessed using a range of memory tasks. Many researchers used forced swim test, or Porsolt swim test, to assess depressive-like behavior or despair. Since the forced swim test has a stress component to it by increasing the corticosterone levels (Harrison et al., 2009), we do not want this added element to interfere with our assessment of early life stress and trauma later in life. The Barnes maze, a dry-land alternative to forced swim test, requires much longer training time for individual rats. Given our goal to assess memory within a short span of time during adolescence and adulthood, we therefore chose Novel Object Recognition, another memory-related task used frequently in behavioral testing (Ennaceur, A., & Delacour, J., 1988; Reger et al., 2009).

Humans often access their memories through spoken or written languages. In animals, assessment of cognitive abilities is done through experimental models of memory and learning. Amongst them, the Novel Object Recognition (NOR) task can be used to assess the learning and retention of animals, the preference for novelty, and test different effects of drugs. Developed in 1988 by Ennaceur and Delacour, the test is a simple, relatively short protocol that requires little training. The task procedure consists of three phases: habituation, familiarization, and test phase. In the habitual phase, the subjects freely explore an open-field arena with no object and then removed and placed in their cages. A purpose of this phase is to get the subjects used to the environment and not letting the environment be a confounding variable. During the familiarization phase, a single subject is placed in the arena with two identical objects, located in the opposite corners, and allowed to explore for a certain amount of time. After this phase and a period of retention, the animals are individually returned to the arena with two objects, one is

identical to the object in the familiarization phase and the other is novel (Antunes & Biala, 2012).

Rats are innately curious and exploratory; the NOR task relies on this propensity to measure the cognitive abilities to distinguish between new and familiar objects, in absence of rules or rewards. Rats often spend more time examining new objects but rats with cognitive problems cannot differentiate between the novel and familiar objects and therefore spend an equal amount of time examining both objects. Existing literature using the NOR task has shown that the predator stress paradigm has been shown to impair cognitive abilities (Diamond et al., 2006; Morrow et al., 2000). In another study, rats were exposed to fox odor during adolescence and were later evaluated on the NOR task. After one day, they found that TMT has shown to immediately disrupt rats' short-term memory, but have no effect after a month (Gao et al., 2021). To our knowledge, there is no current study that looks at the impact of predator odor/TMT on memory 7 days after the exposure, which is an equivalent amount of time for humans to be clinically diagnosed with PTSD.

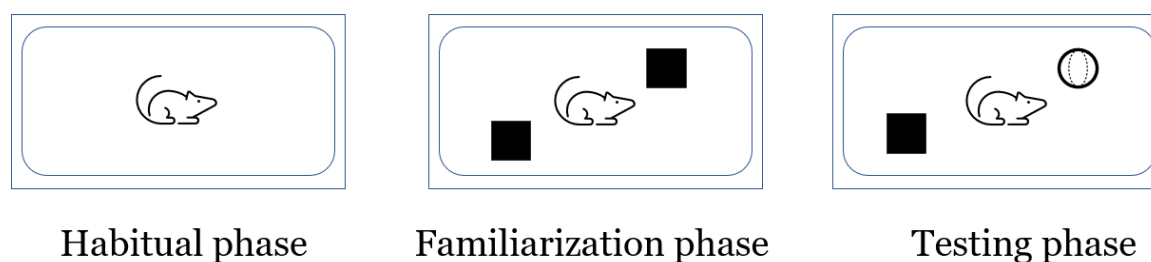


Fig. 1 Diagram of the Novel Object Recognition Task.

Neural assessment: Immunohistochemistry technique using c-Fos

C-fos is a proto-oncogene that is expressed within neurons following voltage-gated calcium entry into the cell. Once expressed, c-fos protein enters the cell nucleus and participates in protein complexes that interact with DNA. The immunohistochemical technique works by binding to these proteins, which then attracts labeled secondary antibodies and allows cells to be quantified. This way, the technique allows c-fos to be detected in neurons within 20-90 minutes after neuronal excitation (Bullit, 1990).

Immunohistochemistry technique allows researchers to detect the neural activity of specific brain areas in rodents' post-mortem tissues. As mentioned, hippocampus and prefrontal cortex are two main areas that are involved in the learning deficits in patients with PTSD. Prefrontal cortex (PFC) is located in the anterior frontal lobe, and has been known to play essential roles in thinking, planning, and attention mediation (Euston et al., 2012; Goto, Yang, & Otani, 2010). In healthy individuals, prefrontal cortex filters and suppresses information to direct attention to the relevant information and stimuli. In people with PTSD, heightened corticosterone level is significantly linked to the reduction of PFC gray matter (Carrion et al., 2010). On the other hand, even though the role of the hippocampus in learning ability is widely accepted, its specific role in object recognition is controversial. Some studies have shown that lesions in the hippocampus produce significantly impaired in memory retention (Clark, Zola, & Squire, 2000) but they are outnumbered by other studies that show lesions have no effect (Ainge et al., 2006; Forwood et al., 2004; Langston & Wood, 2009; Mumby, 2001). These findings suggest that even though the hippocampus is involved in memory processing, it might not directly affect the role of object recognition in our task. For this reason, we will investigate areas of medial prefrontal cortex and hippocampus.

While the hippocampus is involved with long-term recognition, the neighboring perirhinal cortex is responsible for object recognition in short term intervals. Specifically, experiments involving the targeted removal of perirhinal cortex have shown that it is essential in object recognition memory (Barker et al., 2004). Electrophysiological recordings have shown that the neurons in this area appear to code visual recognition memory in monkeys (Brown & Ziang, 1998; Brown et al., 2010) and rats (Zhu & Brown, 1995; Zhu et al., 1995). Aggleton and colleagues showed that rats with perirhinal cortex lesions perform much worse on the object recognition task (Aggleton et al., 2010). The results in these studies suggest the relevant role of perirhinal cortex in the NOR performance and its link with deficits in learning ability. Taken together with a broader work that focuses on neural circuits implicated in the novel object recognition task, we chose to investigate perirhinal cortex, medial prefrontal cortex, and hippocampus and their roles in recognition memory.

1.5 Rationale and Objectives of the Present Study

In this current study, the effect of early life stress, in the form of a short duration of decreased quality of maternal care, on cognitive and neural development of rats was studied. We hypothesized that animals in the experimental group will show a dysfunctional development and are more prone to develop atypical behavioral responses that can be compared to symptoms of human psychiatric disorders later in life. We used a 1-week limited bedding protocol to induce fragmented care in dams. This disruption in care is expected to impact the pups' stress responding from adolescence into adulthood. We will use a 10-minute exposure to fox odor to induce stress when rats reach adolescence and adulthood. A week following stressor exposure, memory function was measured through the novel object recognition task. The waiting period is

to ensure only animals manifesting behavioral and cognitive symptoms similar to those observed in PTSD patients are of interest. Patterns of brain activity through c-fos staining in regions of medial prefrontal cortex and perirhinal cortex will be assessed in order to characterize the impact of early life stress in male and female rats. To the researchers' knowledge, there are currently no studies that use early life stress in the form of limited bedding to study PTSD.

Based on previous studies, it has been hypothesized the fox-odor exposure group will show distressing behaviors in the predator odor chamber and impaired memory on the NOR task, evidenced by an equal amount of time spent on exploring both new and old objects. It was also hypothesized that a similar effect would be observed in the early life stressed groups, but early life stressed and predator odor group would perform significantly worse than the predator odor group alone because of the impact of early stresses on the undeveloped system. These results will support the hypothesis that early life stress leads to a pathological activation of the stress system and predisposes individuals to develop behaviors that mimic neuropsychiatric disorders later in life.

Data from c-fos immunohistochemistry will provide further evidence for changes in activation in the area of medial prefrontal cortex, and perirhinal cortex, driven by the exposure to perinatal stress and predator odor. As a result of the pathological activation system, the researchers expect to see decreased activation throughout the medial prefrontal cortex and perirhinal cortex. In addition, the researchers propose that the individual performance in the exposure chamber and the NOR will correlate with the brain activation patterns observed in the interested brain areas. The result from immunohistochemistry data will support the roles of these areas in the memory component of this task.

We aim to understand the relationship between behavior and brain function throughout the developmental periods and how the decreased quality of care during neonatal period modulates this development. This research hopefully can provide meaningful interpretations from the animal studies to inform future effective drug therapy and other interventions.

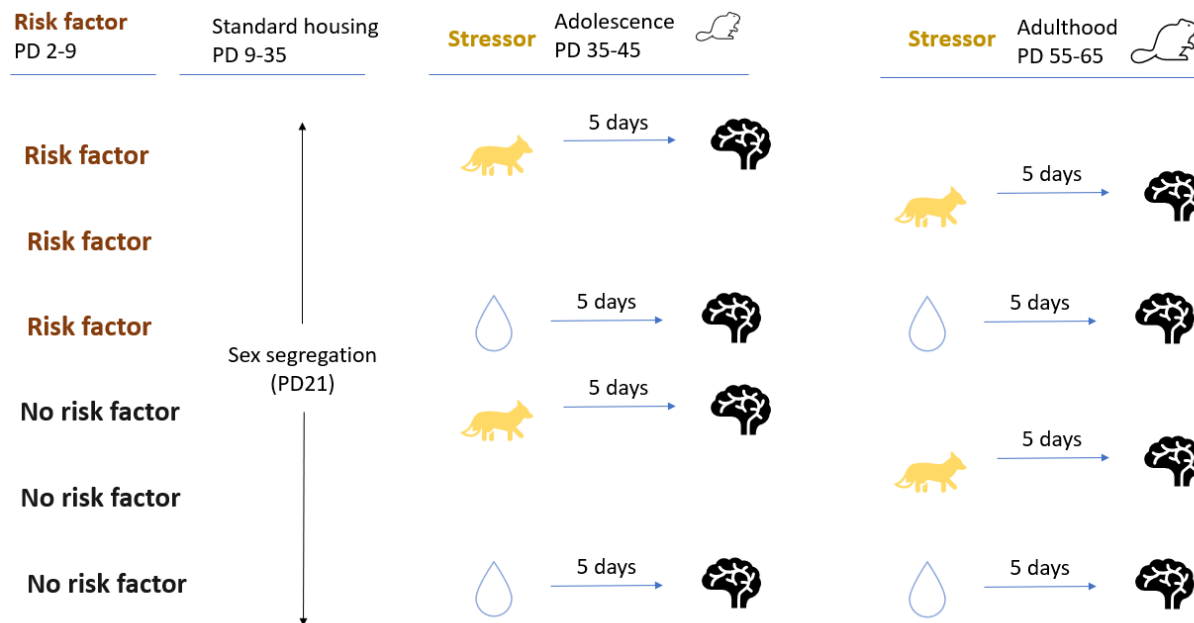


Fig. 2 Experiment design of control and experimental groups.

2. METHOD

2.1 Research Design

This research is an experimental, between-subjects design, with two main groups Early life stress vs. Control groups (limited bedding v. normal bedding) with an second independent variable, namely two acute stress conditions (fox odor v. no odor). Sex is used as a covariate, so each condition will have similar numbers of male and female rats, but we are not planning to use sex as a grouping variable. All experiments were approved by the Connecticut College Institutional Animal Care and Use Committee. To minimize the stress of animal handling, all of the following were conducted by two researchers: animal colony maintenance, breeding, prenatal stress, and behavioral tests.

Experimental Condition	Stress Condition
Acute stressor at adolescence	
Limited Bedding (n ~ 12)	Fox odor (n ~ 7)
	No odor (n ~ 5)
Normal Bedding (n ~ 15)	Fox odor (n ~ 9)
	No odor (n ~ 6)
Total number of rats	27

Table 1: Experimental and Control groups

2.2 Materials

Nesting material Abundant dye-free cotton fibers were given to the dams two days prior to birth.

Novel Object Recognition (NOR) Rats are placed in an observation chamber to acclimate to the environment for 10 minutes on 3 consecutive days. On the fourth day, the rats are placed in the observation chamber and allowed to explore for 5 minutes. This is the familiarization phase. One hour after the familiarization phase, there is a 5-minute test in which one object is novel and one is familiar. Exploring is defined as time spent actively engaged with the object, sniffing it, looking at it, grabbing it. Running around the object, sitting or climbing on it was not recorded as exploration.

2.3 Subjects

Timed-pregnant Sprague-Dawley rats and their offsprings served as subjects in this experiment. They were housed individually with free access to standard lab chow and water, and kept in a 12-12 hour light/dark cycle (lights on at 700). 12 offspring (6 males, 6 females) from one dam served in the early life stress group, and 14 offspring (4 males, 10 females) from another dam served as a control group. After weaning, all rats were separated based on sex and housed in groups of 4. All procedures were approved by the Connecticut College Institutional Animal Care and Use Committee.

2.4 Experimental Procedure.

Maternal maltreatment (limited bedding) Two timed-pregnant Sprague-Dawley dams are obtained from Charles River Laboratories (Wilmington, MA) a week prior to parturition. The dams had ad libitum access to food and water and were kept in a 12-12 hour light/dark cycle

(lights on at 700). In addition, they were given abundant bedding materials. They were monitored twice daily for the birth of pups.

The day of birth is considered postnatal day 0 (PD0). One dam delivered 12 pups, and the other delivered 15 pups, which amounted to the total of 27 rats used in the study. From PD2 to P9, the dam and pups in experimental groups were taken half of the bedding materials. Control groups are housed in cages with abundant bedding. During this time, the cages were changed once because a water bottle leaked onto the bedding materials. Both groups resumed normal bedding environments, starting from PD9. They remained in the cages with the dam through PD20. During this period, the pups were checked daily and given new cages every third day. After the early life stress period, on PD21, rats were weaned and divided into groups of four same-sex weanlings. Every three days, they were handled and changed cages. Reference (Figure 1) for the time of behavioral procedure for this experiment. The procedure was adopted by ... and modified to fit the goal of the study and the equipment of the facility.

Stress Exposure On PD30, rats reached adolescence period. They were exposed to a single 10-minute session of either water or 5 μ L of 2,5-dihydro-2,4,5-trimethylthiazoline (TMT, fox feces component) in a chamber measuring 19 in x 9 in x 8 in. After the exposure, they were returned to the housing and remained in their cages for 5 days. Stimulus contact and direction changes were measured based on video footage.

Cognitive testing On PD37, Novel Object Recognition (NOR) task was used to assess the cognitive abilities of the rats. The NOR arena is a small, open, red plexiglass chamber (12 $\frac{1}{4}$ in x 22 in) with a detachable floor. The task procedure consists of three phases: habituation, familiarization, and test phase. In the habituation phase, each rat was allowed to freely explore the open area in the absence of any objects for 10 minutes on 3 consecutive days. During the

familiarization phase on the fourth day, a single rat was placed in the arena containing two identical sample objects for 5 minutes. After a retention interval of 5 hours, during the test phase, each animal was returned to the arena and allowed to explore two objects, one is identical to the sample and the other one is novel. In both familiarization and test phases, the animals were placed in the same experimental context, and the objects were located in opposite and symmetrical corners of the arena. After each session of NOR, the arena and objects were thoroughly cleaned with ethanol. Exploration in this task is defined as direct orientation of the animal's snout toward the objects or direct interaction (sniffing/grabbing). Running around or climbing on the objects are not considered as exploration activities.

Tissue Preparation Rats were deeply euthanized with carbon dioxide approximately 1.5h after performance on NOR task. Rats were perfused transcardially with physiological saline, followed by 4% paraformaldehyde in 0.1 M PBS. Brains were extracted and placed in the same 4% paraformaldehyde overnight and then transferred to a 30% sucrose solution a day later. They were stored at 4°C in cryoprotectants.

Cryosectioning Sections measuring 40 μ of the hippocampus and prefrontal cortex were obtained corresponding to AP 2.28 (rostral), 3.24 (mid) and 4.44 (caudal) hippocampus (Paxinos & Watson, 2009) in a -20°C cryostat and were stored at 4°C in cryoprotectant until immunolabeling.

Immunohistochemistry Immunohistochemical staining for c-Fos were conducted in three brain areas, namely medial prefrontal cortex, hippocampus, and perirhinal cortex. Brain slices were washed three times in phosphate buffered saline (PBS) for 10 minutes each prior to incubation with a 1:50 dilution of Fos primary antibody (Santa Cruz #LotD0822) in a blocking solution containing 1% bovine serum albumin, 0.30% Triton-X, and normal goat serum. After

two days, a series of PBS washes were performed on the sections, followed by a 2-hour incubation in biotinylated goat anti-rabbit secondary antibody (Jackson Laboratories) diluted 1:200 in blocking solution. The slices were then treated with avidin-biotin-horseradish peroxidase complexes (Vectastain Elite ABC) kit in PBS for 1 hour after several PBS washes. Following the treatment, the slices were given several washes with phosphate buffer (PB) for 10 minutes each, then soaked in a solution containing cobalt chloride, nickel ammonium sulfate, glucose oxidase, ammonium chloride, diaminobenzidine (DAB), and glucose solution in phosphate buffer for 15 minutes. The experiment ended with several washes of PBS and let dry overnight.

After the staining, brain sections were dehydrated in a series of alcohols and defatted with HistoClear. Slides were coverslipped with Permount, and analyzed under microscope 20x magnification. Each of the three sections for each rat were assessed for the number of Fos-stained nuclei (dark brown/black circles).

2.5 Data analysis

The dependent variables were the recognition index for the NOR task and the number of Fos-stained nuclei for the immunohistochemistry technique. Shapiro-Wilk and Bartlett tests were used to determine if data were normally distributed. If all data within one experiment passed both normality tests, then the statistical test that assumes a Gaussian distribution was used. Otherwise, the statistical test that assumes a non-Gaussian distribution was used. All statistical tests were two-tailed with an alpha of 0.05. Gender effect was inspected by two-way or three-way ANOVA. Hat values and Cook's distance test were used to evaluate evaluate high leverage and influential points in the response values. Points detected by these tests were excluded from regression analysis, and the results from models with and without these points are reported in the result

sections. The details of all statistical tests, numbers of replicates, and P values were reported in the Supplementary Section. Statistics were performed using RStudio version 4.0.

3. RESULT

3.1 Behavioral Data

Predator odor is associated with reduced locomotion during acute stress exposure and one week after exposure

To test if ELS contributes to the development of PTSD-like behaviors, we subjected the Sprague-Dawley newborn rats to 7 days of maternal stress from PD2 to PD7. When subjects reached adolescence, they were exposed to acute stress and were administered the novel object recognition (NOR) test. Locomotions (changes in direction and stimulus contact) during stress exposure and time spent examining objects on NOR task were scored by a single researcher to minimize variability.

During the stress exposure, fox odor groups were less likely to change directions or come in contact with the stressor, compared to the water group (Fig. 3A&B). Specifically, the fox odor groups showed more direction changing ($t(18) = -4.0228$, $p < 0.01$), but did not show significant differences when they contacted the stimulus ($W(18) = 47.5$, $p = 0.1$). No effect of ELS and sex was found during the stressor exposure.

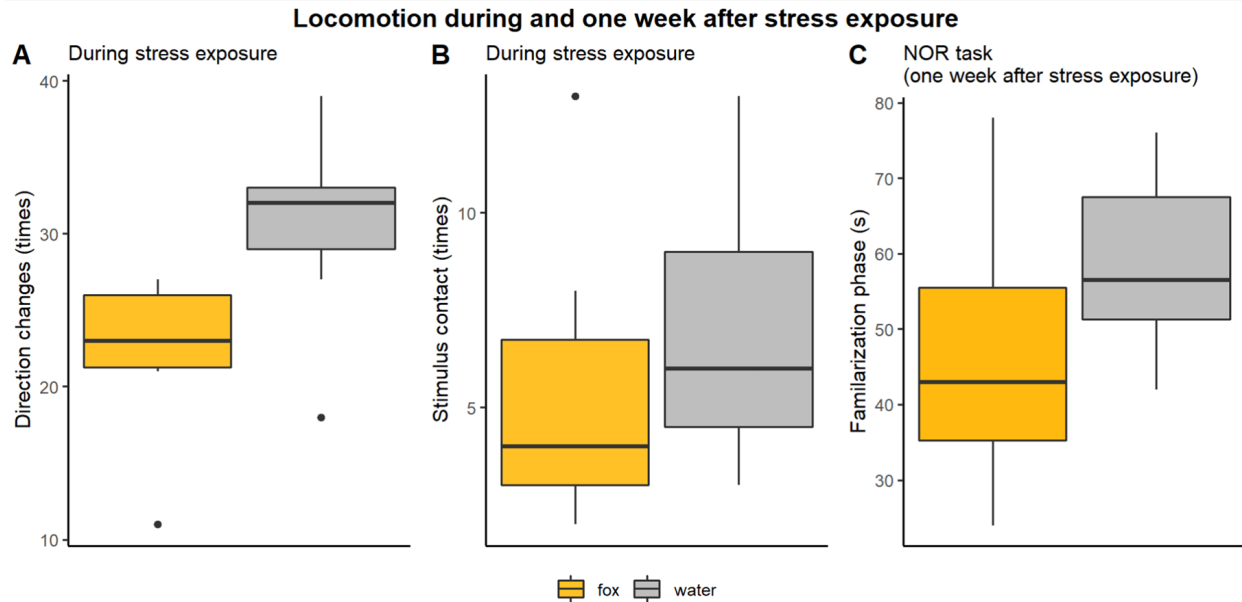


Fig. 3 Locomotion in terms of direction changes, stimulus contact, and exploration time of fox odor and water groups during stressor or water exposure. ELS and control groups were divided into subgroups, with one group exposed to predator odor and one group exposed to water. **A & B** Fox odor groups were less likely to change directions or touch the stimulus, compared to the water group. **C** Time spent during the familiarization phase in the novel object recognition (NOR) task.

Reduced exploratory time in predator odor-groups

In the NOR test, there are three phases: habituation, familiarization, and testing. The rats were tested on this behavioral paradigm 7 days after the stress exposure. The fox odor exposed groups spent less time exploring overall and have more variability. Initial statistical analysis showed no difference between groups. Hat value measures indicated no leverage points, but Cook's distance tests returned three values that are highly influential in the analysis. The removal of these points revealed a remarkable difference in exploratory behavior ($t(17) = -2.27, p = 0.043$) between fox odor and water exposure groups (Fig. 3C). No effect of ELS, sex, or the interaction of fox and ELS was observed.

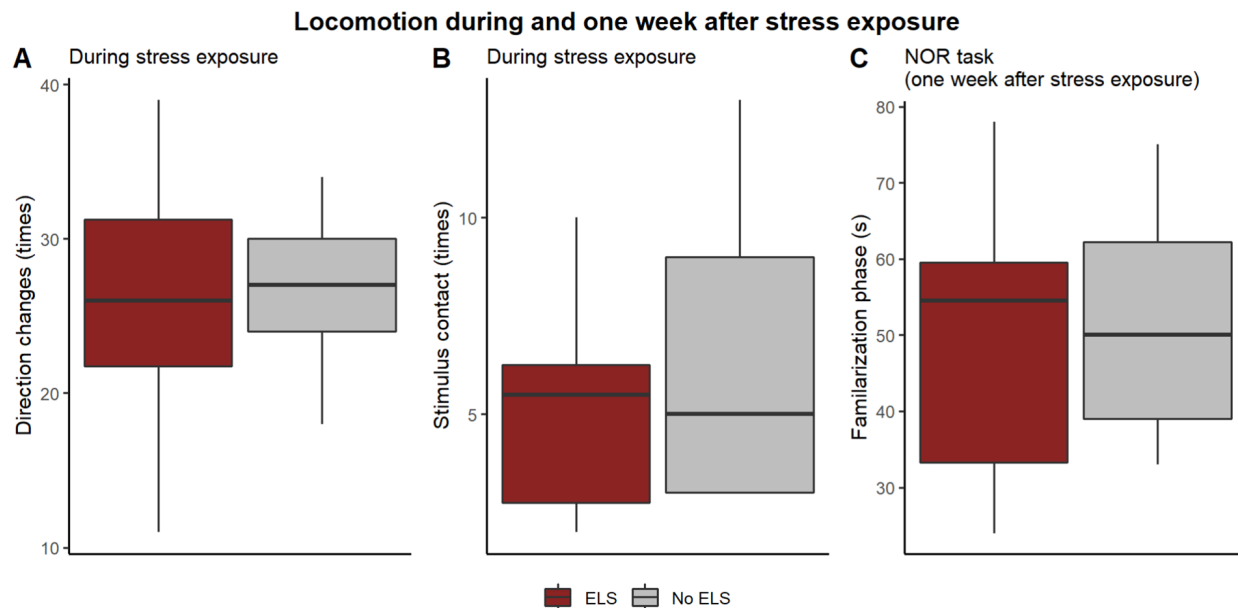


Fig. 4 Locomotion, measured by changes in directions, times contacting the stimulus, and time exploring objects in ELS and no ELS groups **A** During stress exposure, ELS stress group tended to move less often, compared to the no ELS group **B** During stress exposure, ELS stress contacted the stimulus more often than the ELS groups **C** One week after exposure, ELS group showed more activities on NOR than than ELS stress.

Better memory performance was observed in predator odor with no early stress group

In the NOR test, the recognition index was calculated as the time exploring new objects divided by the total time exploring both new and old objects (Antunes & Biala, 2012). A recognition index of 1 indicates a clear distinction between the time spent on old objects and new objects, while a recognition index of 0.5 shows rats spend equal amounts of time on both new and old objects and thus showed no recognition. A week after the stress exposure, increased recall ability was observed in fox odor exposed rats compared with water exposed rats ($t(24)=2.1652, p=0.04$) (Fig. 5B). Further analysis shows that better memory performance was observed in fox odor with no early stress groups (Fig.6). Fox odor-exposed rats with early life stress perform similar to the water exposed rats.

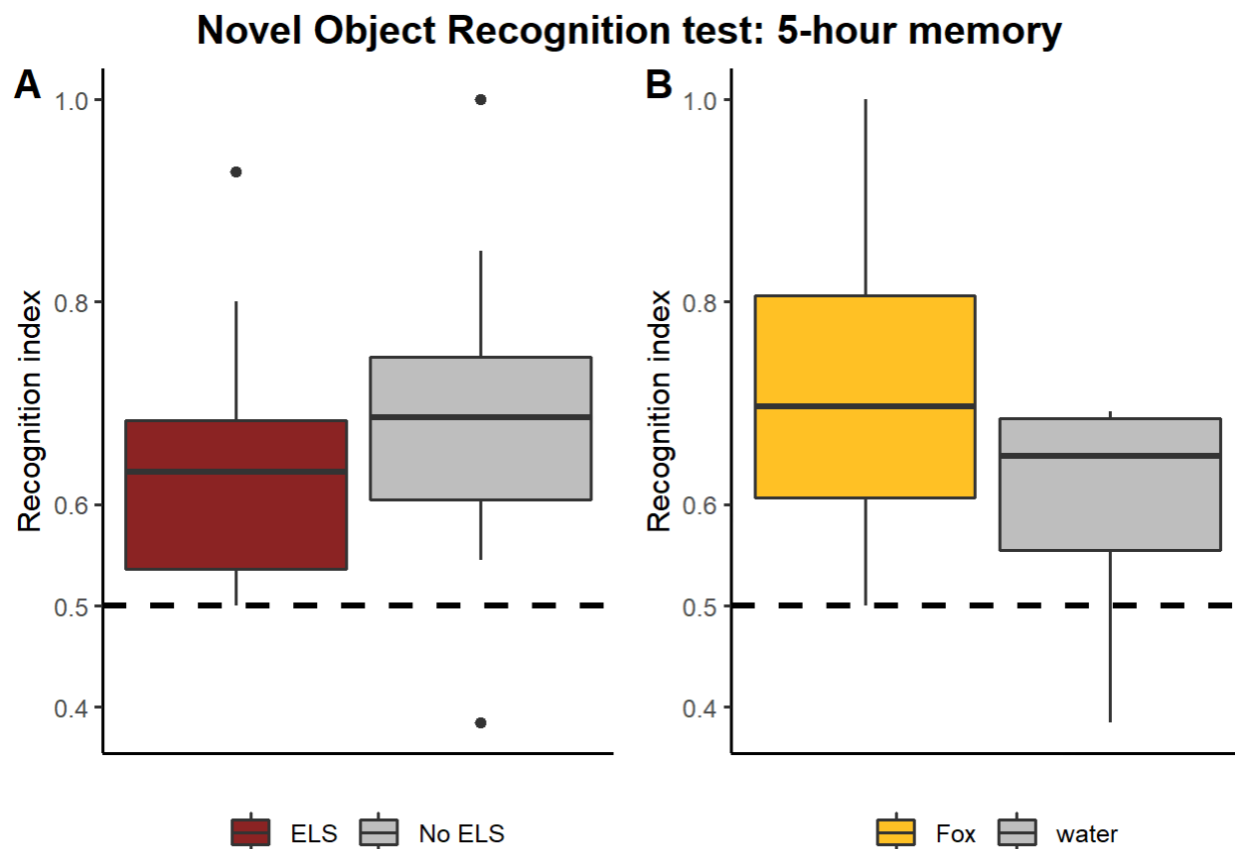


Fig. 5 Comparisons of the recognition index using different variables. Index was calculated as the time spent on new object divided by total time spent on new and old objects. Index of 1 indicates a clear discrimination between old and new objects, whereas index of 0.5 indicates no recall ability. **A** ELS groups on average showed less recognition. **B** Predator odor groups showed better memory performance. Significance is denoted as followed: * $p < 0.05$, ** $p < 0.01$.

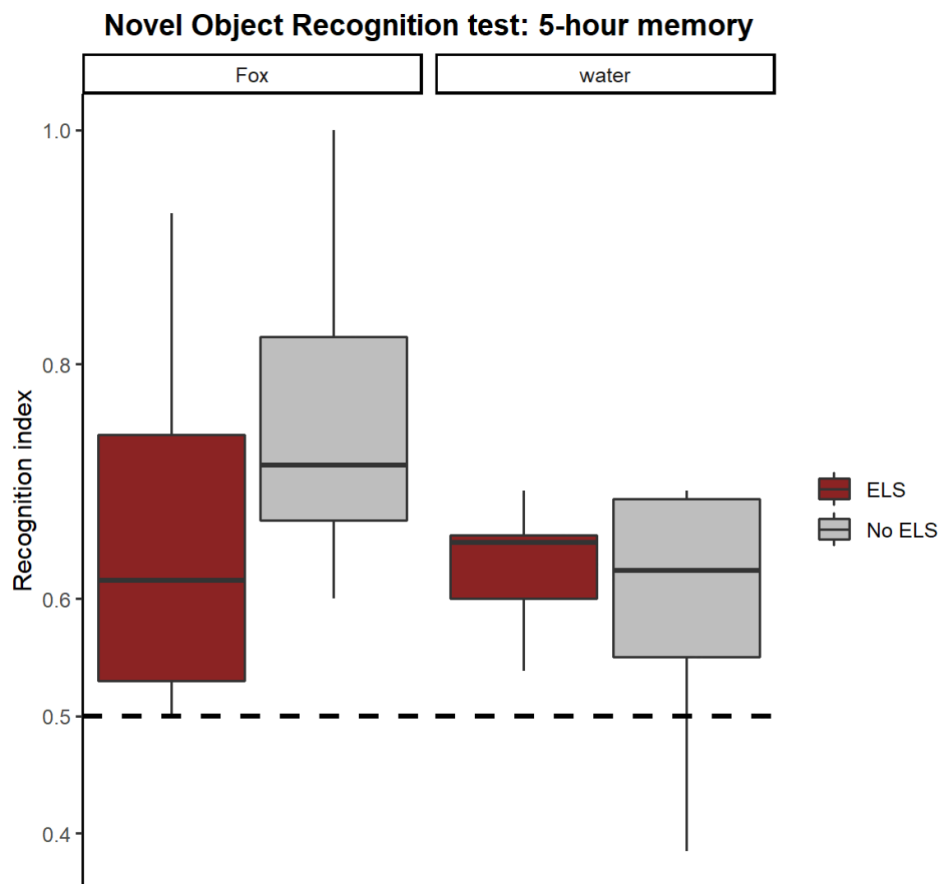


Fig. 6 Recognition index, broken down by the type of early life stress. Water-exposed groups did not show differences between the type of early life stress, whereas there was a clear difference in the predator odor groups. The Fox odor-exposed group showed better memory performance when they did not experience early life stress.

Learning-deficit phenotype was sex specific, with females are most sensitive to the effect of acute stressor and early life stress

Sex differences and ELS effects were analyzed, but no difference was observed. After the removal of two influential points from Cook's distance test, a three-way ANOVA revealed a main effect of sex, with females showing a greater degree of learning deficit, but only in the acute stressor group ($F(1,21) = 4.977, p=0.036$) (Fig. 7). Further analysis of interactions between three variables showed significant interaction between stressor and sex ($F(1,17) = 5.079, p=0.037$), while the effect of sex difference became more robust, accounting for changes in the type of stressor and early life stress ($F(1,17) = 3.310, p=0.022$). There was a trend between sex and ELS ($F(1,17) = 4.144, p=0.057$), but no significant interaction was observed between ELS and sex ($F(1,17)=0.057, p=0.8137$).

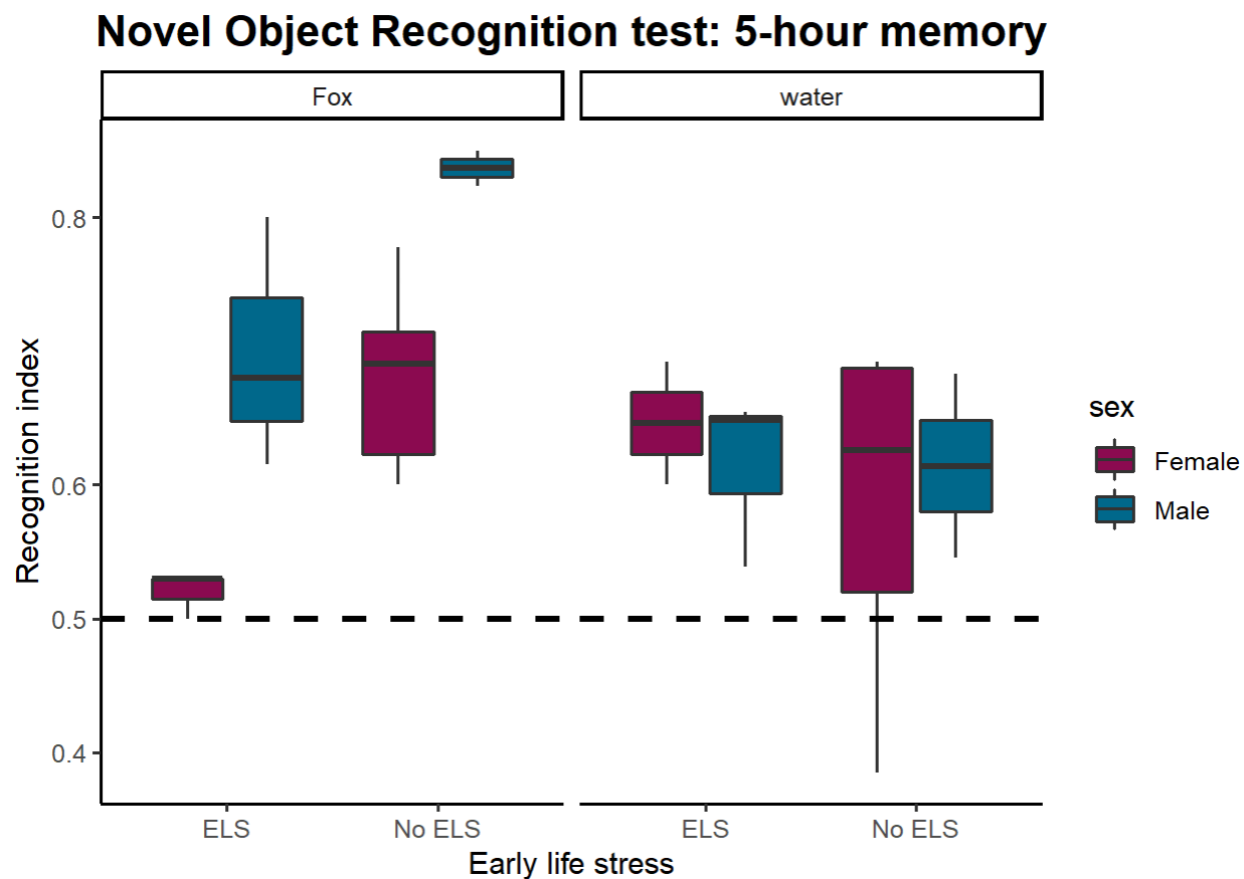


Fig. 7 Comparisons of the recognition index were made by each ELS group, stressor group, and sex. Index was calculated as the time spent on new object divided by total time spent on new and old objects. Index of 1 indicates a clear discrimination between old and new objects, whereas index of 0.5 indicates no recall ability. Sex differences in the acute stressor group were observed.

Male population's recognition index is sensitive to fox odor, but no differences were observed for the females

Separate data analysis for males and females were done on recognition index to analyze the effect of sex on stress odor and ELS (Fig. 8). Average values of recognition index were analyzed for each sex. Female population showed no discrimination in memory recall, whether they are in the acute stressor or water group ($t(1,13) = 13.339$, $p = 0.24$) or in the ELS/no ELS groups ($t(1,13) = 9.7431$, $p = 0.59$). In contrast, fox-odor exposed male subjects show clear differences in recognition index, compared to the water group ($t(1, 6.9478) = 2.5792$, $p = 0.036$). The effect becomes more robust when controlled for the ELS variable ($F(1,6) = 8.182$, $p = 0.028$). No effect of ELS was observed in the male groups. Hat value measure and Cook's tests show there are no leverage or highly influential points that might skew the analysis.

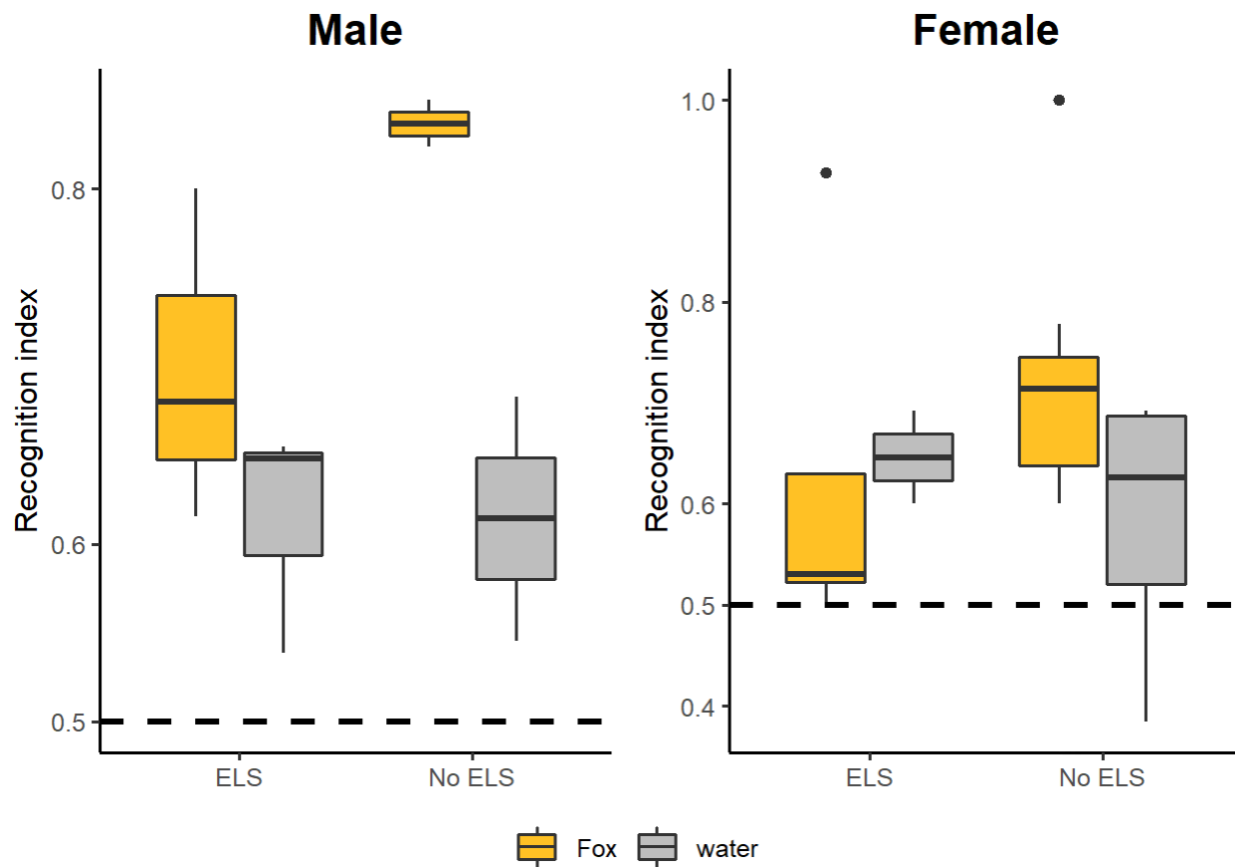


Fig. 8 Recognition index analyzed separately for males and females. For males, predator odor groups tended to perform significantly better compared to the control on the NOR task. No effect of predator odor or ELS were observed for females.

Control groups are susceptible to predator odor, but ELS show no difference

Another separate analysis was done on recognition index for early life stress (ELS) and control groups. For ELS groups, no effect of predator or sex was found on the recognition index ($t(1,8) = 0.42, p = 0.68$; $t(1,11) = -0.34, p = 0.73$). For the control group, the main effect of fox odor was revealed, with the fox odor exposed groups showing better performance in the ability to learn new objects ($t(1,11) = 2.4199, p = 0.03$). No interactions between sex and predator odor were found for either group nor any high leverage and influential points (Fig. 9).

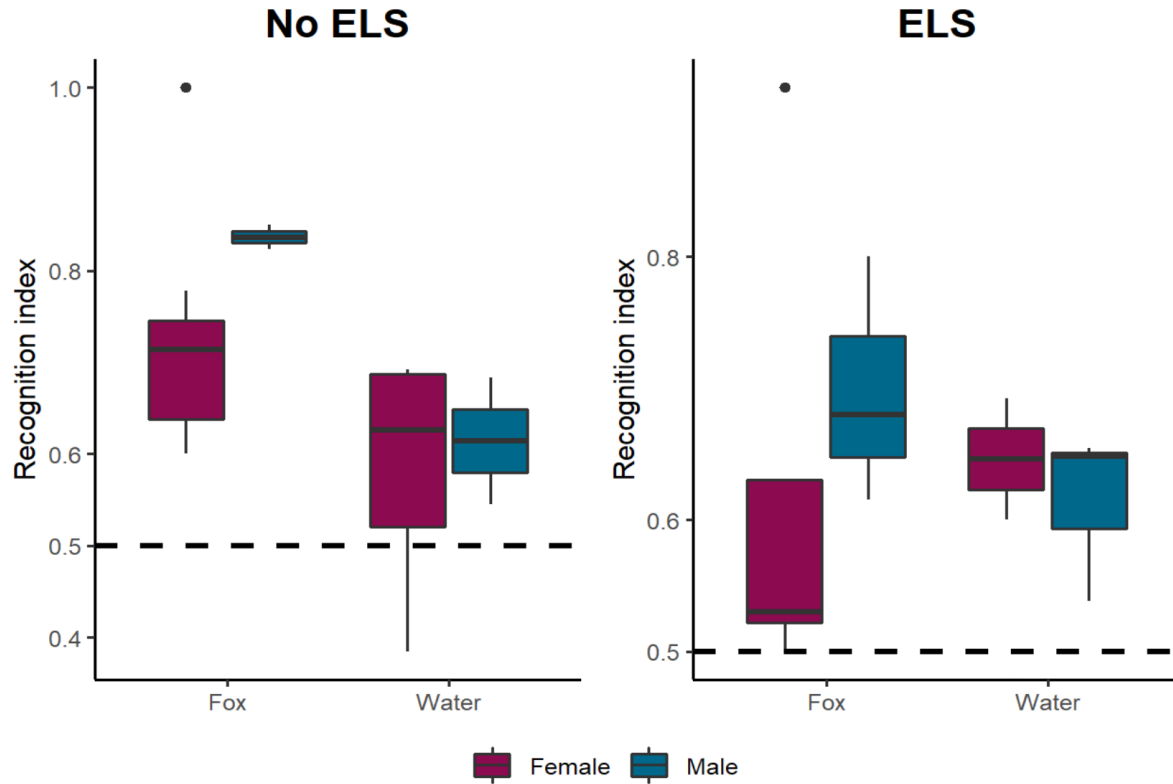


Fig. 9 Recognition index analyzed separately for early life stress and control groups. For the control group, the main effect of predator odor was found, with stressed groups memorizing objects better than the non-stressed groups. No effect of predator odor or sex were observed for the ELS group.

3. 2 Immunohistochemistry Data

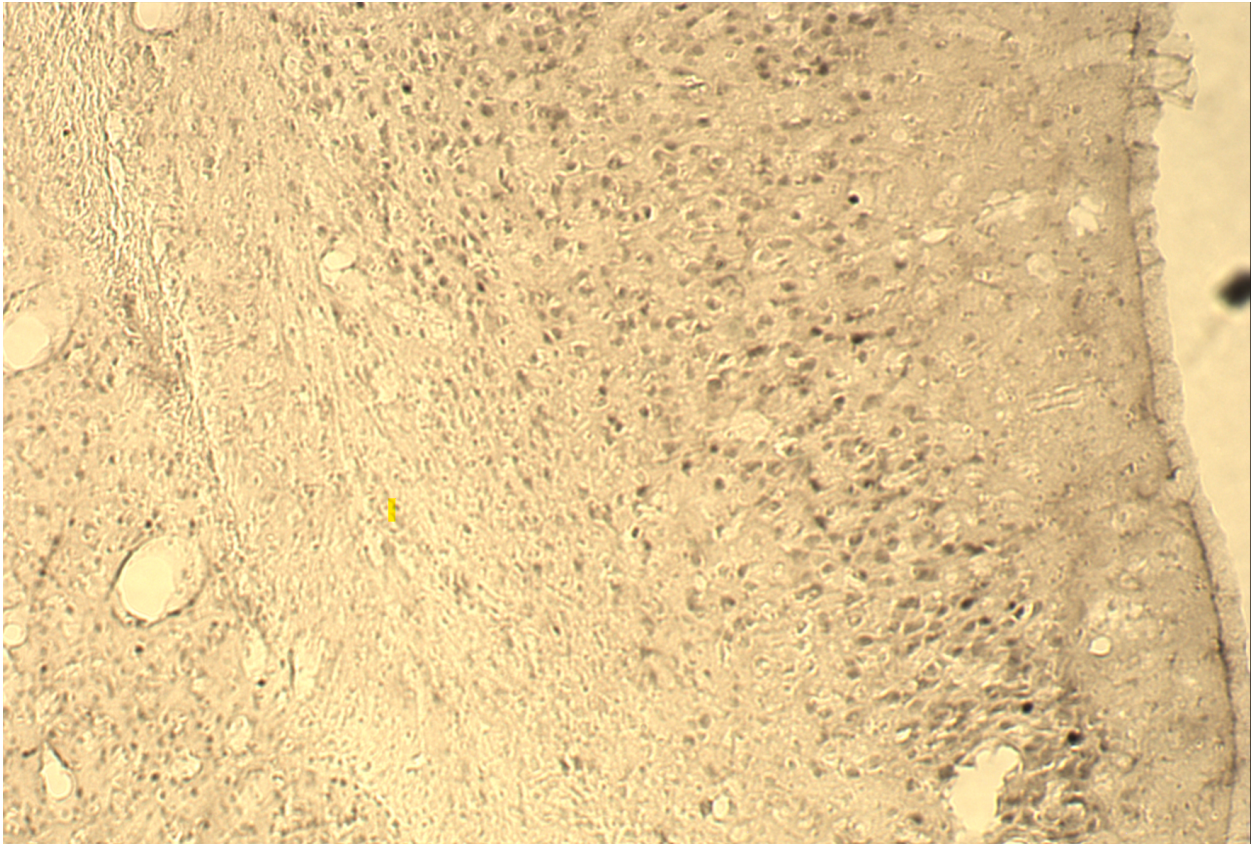


Fig. 10 Images of c-fos expression in tissue in perirhinal cortex (dark brown/black oval objects).

Due to technical problems, the prepared tissues did not show clear Fos-stained nuclei. Future research may involve the preparation and examination of samples.

A portion of this data provides evidence that ELS in the form of fragmented maternal care can lead to PTSD-like symptoms in adolescence periods. We showed that predator odor groups displayed remarkably reduced locomotion during the stressor exposure, and this effect still remained 7 days after. Surprisingly, the predator odor exposed groups on average showed a higher recognition index. Breaking this pattern down by the type of early life stress, we see fox

odor groups show better memory only when they did not experience early life stress. Further analysis revealed the main effect of sex, with male subjects tend to perform better on the novel object recognition task than females. However, there are no sex differences in the control groups. Within-group analysis showed the effect of predator odor disappeared in the female population, and the effect of sex was no longer apparent in the early life stress or control groups.

4. DISCUSSION

In this study, we sought to answer three main questions:

- 1) Does early life stress alone induce fearful behaviors and learning-deficit phenotypes?
- 2) Does the fox odor alone induce fearful behaviors and learning-deficit phenotypes?
- 3) Does the combination of early life stress and fox odor make subjects more vulnerable to develop PTSD-like phenotypes?

We will address each question in the following sections.

Does early life stress alone induce fearful behaviors and learning-deficit phenotypes?

We observed no significant behavioral differences between early life stress and control groups. ELS groups did not show reduced locomotion nor significant impairment in the ability to distinguish between new and old objects. This is contrary to previous research, which has shown that early life stress (ELS) can lead to the development of stress-associated disorders such as major depressive disorder (MDD), anxiety, and posttraumatic stress disorder (Molet et al., 2014; Yan et al., 2017; Orso et al., 2020) and worse performance on cognitive tasks (Chen & Baram, 2015; Cui et al., 2016; Opala, Liu, Long, & Walker, 2016). There are many possible explanations for this pattern, including sample size and the experimental procedure. Compared to other research, the experimental group in our study is limited in size (n=13). The small sample size is related to higher variability, which makes it challenging for diagnostic tests to detect outliers. We did not observe the main effect of ELS in our analysis, but we found a trend toward interaction between predator odor and early life, which we will discuss in later sections.

Another possible explanation for this lack of effect is the development stages at which we exposed the subjects to stress. Previous research has already started to establish the role of risk factors during early developmental stages. Disruption during this sensitive period might alter developmental trajectories and lead to pathological outcomes later in life. Much research has focused on the molecular pathways and symptoms manifesting during adulthood. For instance, many groups have found early life stress can lead to impairment in learning and related cognitive tasks in adult animals (Naninck et al., 2014), accelerated maturation of hippocampus cells (Bath et al., 2016), and increased pain sensitivity and more sensitive nociceptors in adult rats (Green et al., 2011; Prusator & Meerveld, 2014). On the other hand, very few studies have followed the growth of subjects during their course of development, from adolescence to young adulthood. Studying subjects at different life stages gives insight into the development mechanism of certain disorders. One study done by Molet and colleagues (2016) shows that after exposure to limited bedding, even though adolescent rats showed less socialization and sucrose preference, they do not show fear-like or other distressing behaviors until they reach adulthood. In another study, symptoms of depression manifest little during adolescence, but become prominent during young adulthood (Goodwill et al., 2019). In our study, the original plan included the exposure of ELS and control subjects to an acute stressor at adolescence and young adulthood. Due to a limited research window, the experiment ended when the subjects reached adolescence. It is possible that subjects might not have shown any distressing symptoms until they reached adulthood. Thus, the null results here do not mean the paradigm is invalid. Moreover, the small number of variables we used might not be sufficient to capture multi-faceted symptoms of PTSD, including anhedonia, losing appetite, and reduced preference for social time. It could be the case that the

subjects show some of these behaviors and this study failed to capture it. Future research can replicate this protocol with different times of maturation and include more variables.

Does the fox odor alone induce fearful behaviors and learning-deficit phenotypes?

Here, we show that exposure to predator odor is linked to a noticeable decrease in the subjects' mobility during the exposure period and continues to have an effect 7 days after exposure. Animal models of traumatic stress and PTSD that have been developed using exposure to predator scent as a stressor have shared similar results. Study by Fendt's group (2003) and several other reports showing that rats (Wallace & Rosen, 2000) and mice (Hebb et al., 2004; Hacquemand et al., 2013) display reduced mobility and freezing in the presence of TMT. Furthermore, a study has shown that TMT produces early and long lasting gene expression of FKBP5, GRM5 and CNR1, which have all been implicated in PTSD (Tyler et al., 2020). It is possible that TMT indirectly reduces locomotion in rats by inducing lasting behavioral consequences relevant to traumatic disorders.

Notably, predator odor-exposed groups experienced better recall ability, which is not consistent with our hypothesis that predator odor-exposed groups experience impaired memory on the NOR task. This is surprising, since a comparable study has found the contrary. For instance, Gao and colleagues show fox odor-exposed groups performed significantly worse compared to the control. In both of our experiments, the rats were exposed to fox odor during adolescence and were later evaluated on the NOR task. After one day, Gao's group found that fox odor-exposed groups performed significantly worse compared to water-exposed groups. However, one month later, the effects of TMT exposure on the novel object recognition memory disappeared (Gao et al., 2021). The results of our study have demonstrated that predator odor

exposure has positive effects on cognitive ability after a week. Although we do not have direct comparison with Gao's results at one-day and one-month time points, there are many explanations for the differences in our results. It is possible that one day after the exposure, the rats are still under immediate impact of predator stress and perform worse on cognitive tasks, while given time to rejuvenate, they become hypervigilant of the surrounding environment and learn better. Furthermore, in our analysis, when we break this pattern down by the type of early life stress the pups experience, we observed a big difference in performance between early life stress and no early life stress groups. Specifically, the fox odor-exposed group shows higher recognition index only when they do not experience stress during the postnatal period. Meanwhile, the group that experience stress both early and later in their life perform about the same as the water-exposed groups. This might suggest one-time stress might be beneficial for learning, but the combination of early life stress and stress later in life might take a greater toll on the adaptive stress responses and learning ability.

Current research offers mixed opinions on the pathway behind the learning deficits in PTSD because the effects of stress on learning and memory in rats are complex and depend on various factors. Kitraki et al. (2004) found that 21 days of restraint stress affected hippocampal plasticity, neurogenesis, and spatial memory in rats. Reduced glucocorticoid receptor (GR) immunostaining was detected in the hippocampi of rats after 21 days of restraint stress, which suggests that stress-induced changes in GR status may affect learning and memory processes. Some researchers posit that the elevated level of corticosterone released during stress exposure may be the driving factor. Excessive release of corticosterone has been shown to lead to neurotoxicity in areas rich in glucocorticoid receptors such as prefrontal cortex and hippocampus, and thereby may lead to reduced glucocorticoid receptors and impair learning and

memory ability. On the other hand, some studies suggest that cortisol may have a beneficial role in memory encoding. For instance, Buchanan and Lovallo (2001) proposed that elevated cortisol levels during memory encoding enhances long-term recall performance because emotionally arousing events, as a result of heightened cortisol levels, are better encoded than neutral information. Their theory is consistent with animal research, showing that cortisol plays an integral role in the neurobiology process for learning (McGaugh, 2000).

The two views are not entirely independent however. Lynch (2004) found that high concentrations of circulating corticosterone, consistent with marked stress, inhibited long-term potentiation (LTP) in the hippocampus, while low concentrations of corticosterone enhanced LTP. The studies imply the level of cortisol might impact the effects of stress on learning and memory in rats. It is possible that the types of stressor used in different studies elicit distinctive fearful responses and corticosterone release levels, thereby giving us different interpretations of the role of stress on learning. The timing of the stress event is also important, as the time of exposure may improve or impair learning and memory processes (Aguayo et al., 2018).

Taken together with the broader work trying to understand PTSD, this report suggests that predator odor exposure produces behavioral changes indicative of a stress response, but shows a remarkable memory performance. Future research can replicate this protocol to study the impact of stress and the level of cortisol before, during, and after the stress events to elucidate the role of this hormone on memory and learning.

Does the combination of early life stress and fox odor make subjects more vulnerable to develop PTSD-like phenotypes?

The data shown here demonstrated that ELS in the form of limited bedding led to some sex-specific learning deficits in the groups that were exposed to trauma. Interestingly, sex differences are only observed in predator odor-exposed groups, with adolescent female rats tending to recall less than their male counterparts. We also found a trend towards interaction between predator odor and ELS. Our study suggests that sex differences in the recognition ability become apparent when we take account of predator odor and stress. There might be a link between ELS stress and predator odor, but it requires further research to reach a conclusive answer.

With women being nearly two times more likely than men to be affected by mood and anxiety disorders (But & Stein, 2002; Garter et al., 1998; Weisman et al., 2001), the role of sex differences becomes increasingly important in understanding the vulnerability of each sex. At molecular levels, according to McCarthy and Arnold (2011), sex chromosomes cause sex difference via genetic differentiation in gonads, which produces different types and levels of gonadal hormones. The fluctuating levels of hormones directly impact cell proliferation, cell specific responses, and cell to cell communications in developing brains. It is logical that these different molecular formations will lead to different behavioral responses. However, what makes it challenging to understand the differential vulnerability of each population is that on the same prototypes, each sex does not exhibit consistent responses. For example, many researchers have reported that females tend to be protected from the impact of ELS, while the males' phenotypes change significantly. Using limited bedding protocol, Naninck and colleagues found lasting changes in adult neurogenesis that impairs learning and memory were observed only in males

(Naninck et al., 2014). In contrast, the results from this study add to a growing body of literature on the sex differences in models of early life stress, with females being more vulnerable to stress and early life experiences. Gao and colleagues also noted a clear difference in female and male offspring' cognitive ability, with impairment only observed in female mice (Gao et al., 2021). Agawar et al. found a similar pattern using maternal separation and chronic stress: females tend to exhibit worse memory performance and show more behavioral vulnerability (Agarwal et al., 2020).

The bases for differences amongst studies are not clear. It is possible that these varying results are due to the type of tests used to evaluate symptoms. For instance, female animals have been shown to have higher levels of recognition for novel objects (Bettis & Jacobs, 2012; Sutcliffe, Marshall, & Neill, 2007), so the reported results can be attributed to the task itself and not the sex difference. In addition, variability in protocols between labs and lab members can also contribute to varying results. To reach a common understanding requires researchers finding an optimal task for both sexes, and achieve consistent results among some standardized protocols.

This study is not without limitations. First, the immunohistochemistry portion of this research was not finished, thus we cannot understand the link between the brain and actions. Future research can include this part to understand the link between brain activation patterns and behavioral characteristics. Second, with only about 8 animals per subgroup, the sample size in this study is small; the analysis and interpretation therefore are limited. We propose the use of larger sample sizes at both adolescence and adulthood to understand the impact of early life stress over the course of development. Third, the unfamiliarity with this early life stress protocol also posed a challenge for the researchers. During the experiment, the bottles in one cage leaked

and dampened the bedding; we had to remove the bedding and change both cages. It is possible that due to this interruption, the results might be modified in ways that we did not expect.

Another direction this research can take is to explore the relationship between emotions and cognitive learning in the context of PTSD. An assumption behind the limited bedding protocol is that parents and children's relationships are pivotal for children's healthy emotional and cognitive development. Therefore, negative emotions can have a detrimental impact on cognitive function, including memory, attention, planning, and problem-solving. With the dams experiencing stress, a fragmented, anxious caretaking style of the pups was expected to develop, leading us to expect that the pups did not develop normal cognitive and emotional management. While this study focuses on the cognitive part of PTSD, the direct relationship between cognition and emotions is left unexplored. It would be beneficial to understand how the affective impacts relate to learning and cognitive functions. With better understanding and more experience with the protocol, for future studies, we can measure a wider set of variables, including more biological markers (corticosterone, for instance) and behavioral responses (anhedonia/sucrose preference/social preference) on different paradigms, to capture the multi-faceted symptoms of PTSD and broaden the scope of research.

In conclusion, the results of the present study have provided evidence that exposure to predator odor has a lasting impact on stress responses, evidenced by reduced mobility and exploration time during and one week after exposure. The predator odor group, however, shows better memory performance but only when they do not experience early life stress. These findings also implied an interaction between ELS and predator odor, which requires further research to validate the impact ELS might have on later stressful events. When accounting for

ELS and predator odor, we found clear sex differences, with the females being most sensitive to the impact of predator odor.

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