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Resilience and Vulnerability in a Model of Early Life Stress

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Exposure to early life stress (ELS) is a major contributor to the development of psychiatric disorders, yet the neural pathways that translate these experiences into lasting vulnerability remain mostly unknown. In this thesis, the limited bedding and nesting (LBN) model in rodents was used to induce ELS by restricting the dams' access to nesting material during the early postnatal period. This manipulation is known to disrupt maternal care and expose pups to a stressful environment.

To assess the lasting impact of these early experiences, a behavioural battery was employed, which includes the Open Field Test (OFT), Elevated Plus Maze (EPM), Novel Object Recognition (NOR), a social interaction task (SI), and a cue-association task. Additionally, we assess the stress response induced by social interaction with an unfamiliar conspecific by measuring the levels of corticosterone.

Behavioural analysis in the OFT revealed that animals reared under LBN conditions spent significantly more time in the centre *($p < 0.05$)* compared to CTRLs. In contrast, no significant group differences emerged in the EPM. Interestingly, results in the two tasks, which commonly assess anxiety-like behaviour, were inversely correlated, underscoring the role of context-specific behavioural expression and highlighting interindividual variability. During SI, the LBN group exhibited an increased preference for unfamiliar conspecifics compared to the CTRL group (*$p < 0.05$).* Moreover, LBN males' corticosterone levels increased after the SI more strongly than seen in CTRL males (*$p<0.05$*).

These findings demonstrate that ELS can alter behaviour in a context-dependent manner and modulate stress reactivity in a sex-specific fashion. Upcoming experiments will further investigate these behavioural outcomes linked with neuronal recordings.

Key words: Psychiatric Disorders, Early Life Experiences, Behaviour, Stress Response

Table of contents

1 Introduction

1.1 Early-life Adverse Experiences as a Risk Factor for Mental Health Disorders

The environment plays a key role in shaping brain development by influencing gene expression and neural plasticity to allow for adaptive responses to changing conditions. Among environmental influences, early life experiences play a particularly crucial role in brain development. Positive experiences during this period have been shown to enhance resilience and emotional regulation later in life[1]. In contrast, negative early-life experiences, commonly referred to as early life stress (ELS), such as neglect, abuse, parental loss, or separation, can have profound and long-lasting effects on cognitive and emotional functioning and increase the risk for mental health disorders[2].

Mental health disorders are currently a huge global burden, affecting hundreds of millions of individuals and placing a strain on healthcare systems worldwide. According to the World Health Organisation report, in 2019, approximately 970 million people were living with a mental disorder[3]. In Europe alone, 3.1 million patients were discharged from hospitals with mental health diagnoses in 2021, and 3.6 % of all deaths in the European Union (EU) were attributed to mental disorders[4.]

Although effective treatments for mood disorders, including major depressive disorder, are available and benefit many patients, challenges remain. In about 30 % of cases, standard therapies fail to produce adequate improvement, a condition referred to as treatment-resistant depression (TRD)[5]. One key factor associated with TRD has been shown to be exposure to ELS [6], with epidemiological studies have strongly linked ELS and poor mental health outcomes. Approximately 50% of major depressive disorder patients report a history of early-life adversity [7], and those with such a history have a 1.5-2 times greater likelihood of developing TRD [8,9].

Unlike stress experienced in adulthood, which tends to have more transient effects and is often more responsive to treatment [10], ELS occurs during critical periods of brain maturation. This can lead to long-lasting neurobiological changes that increase vulnerability to psychiatric disorders and reduce the efficacy of conventional treatments later in life [11].

These findings underscore the need to understand how ELS contributes to the aetiology and treatment resistance of mental health disorders for developing more personalised and preventive

strategies. Early identification, as well as interventions that target neurodevelopmental trajectories, could help reduce the long-term burden of psychiatric illness and improve treatment outcomes.

1.2 Developmental Timing of Brain Areas and Sensitive Periods

The human brain continues to mature after birth, and prolonged activation of the stress-response system can affect areas crucial for cognitive function and emotional regulation. However, the effects and outcomes vary regarding developmental periods and environmental stressor adversity. Certain brain areas exhibit varying sensitivity to stress across different ages; for example, the hippocampus is particularly vulnerable to stress from birth until the age of two, while the frontal cortex has been shown to be more susceptible during adolescence[12].

Figure 1 Illustrates the developmental trajectories of key brain regions, highlighting their maturation timelines and periods of stress sensitivity[12]. These divergent trajectories may account for different impacts of ELS on long-term emotional and cognitive functions.

In the following sections, this thesis examines how ELS influences the maturation of the hypothalamic-pituitary-adrenal (HPA) axis, the amygdala, the frontal cortex, and the hippocampus.

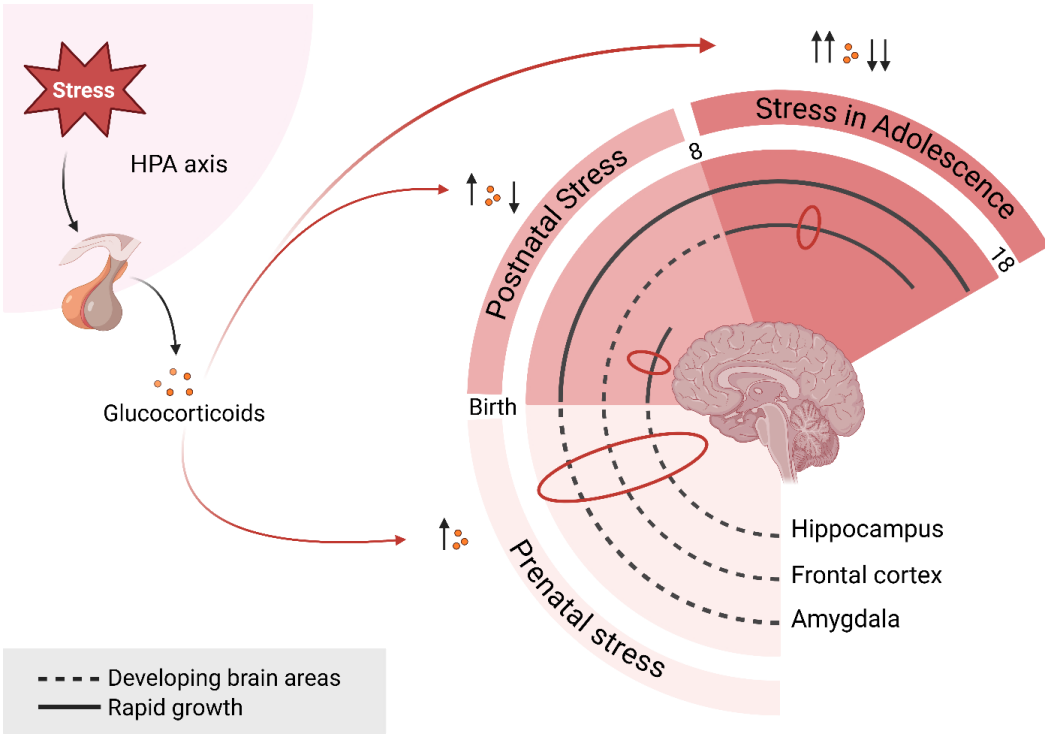

Figure 1. Developmental timing of brain region vulnerability to stress exposure. Illustrates the sensitivity of key brain regions and systems (amygdala, frontal cortex, hippocampus, HPA axis, and glucocorticoid regulation) to stress during developmental periods (prenatal, postnatal, and adolescence). During the prenatal stage, various brain areas are developing (indicated by dashed lines) and are particularly susceptible to stress. The amygdala undergoes the longest period of development, with particularly rapid growth during the postnatal and adolescent phases (solid lines). The frontal cortex matures during adolescence, making this period critical for its growth. The hippocampus experiences a phase of rapid development until about age 2, at which point it reaches full maturity. During the early postnatal phase, different environments can cause fluctuating levels of corticosteroids, resulting in diverse outcomes. Glucocorticoids have lasting effects throughout adolescence, and the frontal cortex remains highly sensitive to these alterations. *Adapted from Lupien, S. J., McEwen, B. S., Gunnar, M. R., & Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nature Reviews Neuroscience, 10(6), 434–445. https://doi.org/10.1038/nrn2639. Created with Biorender.com.*

1.2.1 HPA-axis: Regulating Stress and Brain Development

The HPA axis plays a central role in coordinating the body's response to stress, and its activity changes significantly throughout development (Figure 2). During development, other brain regions are particularly susceptible to HPA axis responses, such as glucocorticoid secretion[13]. Glucocorticoids are key regulators for brain development, as they initiate terminal maturation, structural remodelling of axons and dendrites, and cell viability, yet both suppressed and excessive levels of glucocorticoids can negatively impact the normal brain development and functioning (Figure 1) [14].

The HPA axis is particularly sensitive to environmental influences during the postnatal period, and disruptions in typical sensitive caregiving are linked to increases or prolonged activation of the HPA axis. These maternal changes often occur alongside maternal depression, and studies indicate that those offspring are at a higher risk of heightened HPA-axis activity and developing depression during adolescence[15–18].

In contrast, rodents experience a stress hyporesponsive period (SHRP) typically occurring in the first two postnatal weeks (P4-14). During this time, basal glucocorticoid levels remain low, and stress-induced activation of the HPA axis is reduced[19]. This phase is believed to safeguard the developing brain from excessive glucocorticoid exposure. However, when maternal care is disrupted, it can lead to an early end of the SHRP and cause long-term changes in stress responsiveness[20,21].

Following the SHRP, the adolescent period in rodents spans approximately P21 to P59, during which the HPA axis becomes fully functional; however, its response to stress remains developmentally distinct from that of adulthood. Adolescent rodents exhibit prolonged glucocorticoid responses to stressors, a slower return to baseline, and a lack of habituation to repeated stress exposures[22]. This phenomenon is thought to arise from immature neuroendocrine negative feedback mechanisms[23]. These changes indicate that adolescence constitutes an additional sensitive period during which the brain is particularly vulnerable to the effects of stress.

In humans, adolescence is similarly characterised by elevated basal and stress-induced HPA activity, likely influenced by increases in sex steroid levels during puberty[24]. However, translational comparisons must be made carefully, as developmental trajectories vary across species. For instance, while the rodent hippocampus continues to mature into adulthood, the human hippocampus is structurally mature by around two years of age (Figure 1). The frontal cortex, however, continues to develop in both species, with maturation especially prolonged in humans (Figure 1). Notably, glucocorticoid receptor (GR) expression in the PFC is high during adolescence, which may enhance sensitivity to glucocorticoid signalling. Adolescence is described as a critical window for the emergence of stress-related and other psychiatric disorders[25,26]. Furthermore, ELS can lead to persistent HPA dysregulation, with effects that often emerge or intensify during adolescence.

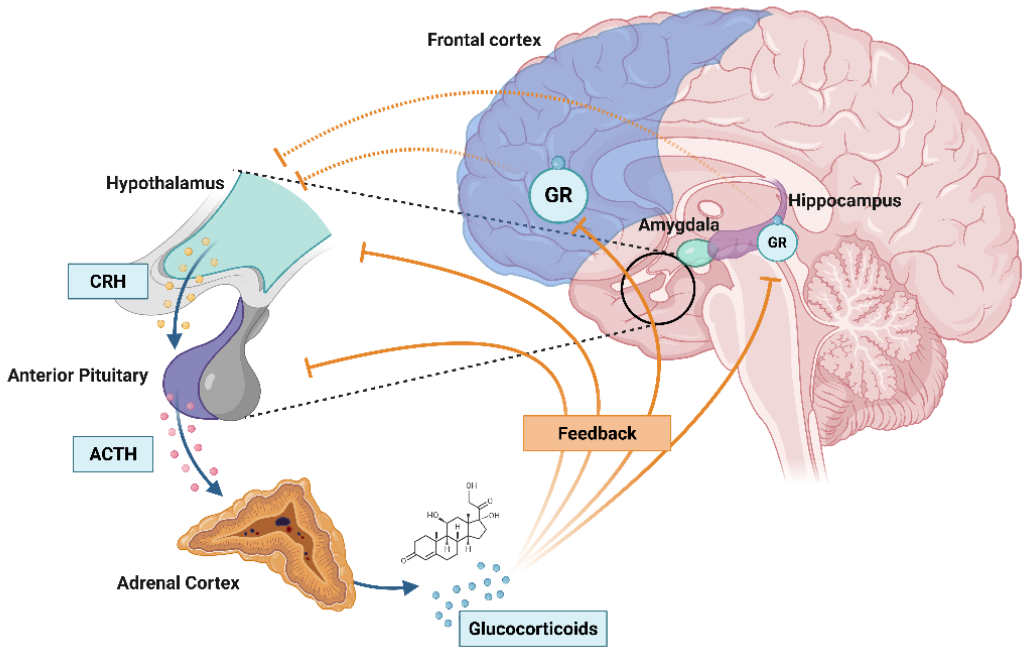

Figure 2. The human hypothalamus-pituitary-adrenal (HPA) axis in stress responses. The amygdala sends a stimulus to the hypothalamus to release corticotropin-releasing hormone (CRH), which triggers the pituitary gland to secrete adrenocorticotropic hormone (ACTH). This, in turn, leads to the production of glucocorticoids from the adrenal glands. Glucocorticoids can bind to glucocorticoid receptors (GR) and mineralocorticoid receptors (MR, not shown). Following receptor activation, a feedback regulation system (orange inhibitory lines) is generated that inhibits further glucocorticoid production. *Adapted from BioRender Template Library*[27]*. Created with BioRender.com.*

1.2.2 Amygdala: Effects of ELS on Fear and Stress Processing

The amygdala plays a crucial role in processing the emotional significance of stimuli, particularly in the context of fear learning and emotional memory. While it is most commonly associated with fear responses, it is also involved in other emotional states and in recognising emotional facial expressions. The amygdala consists of multiple interconnected nuclei, with the lateral and central nuclei being especially important for fear conditioning[28]. Fear conditioning is a form of associative learning in which a neutral stimulus becomes linked to an aversive event, such as a tone paired with a foot shock[29]. During this process, the amygdala acts as a central hub where sensory information about the conditioned and unconditioned stimuli converges, leading to learning-related synaptic changes in the lateral nucleus. This information is then transmitted through intra-amygdala pathways to the central nucleus, which coordinates the expression of fear responses (like freezing, autonomic changes, and stress hormone release) via its connections to the brainstem and hypothalamus[28].

The amygdala undergoes prolonged structural and functional maturation, as depicted in Figure 1. In humans, the amygdala continues to mature from infancy through late childhood. Structurally, its postnatal trajectory follows a pattern of rapid initial growth that gradually slows, reaching maximum volume around ages 9 to 11, after which synaptic pruning begins[30]. The highest sensitivity during amygdala development seems to occur around ages 10 to 11, aligning with significant transitions in functional connectivity patterns[30]. Research indicates that childhood adversity is associated with an increase in right amygdala volume, particularly when the stress is experienced during preadolescence[31,32]. Animal studies similarly indicate that ELS enhances dendritic arborisation in the amygdala, contributing to increased volume and potentially greater emotional reactivity [33,34].

1.2.3 Hippocampus: ELS-Induced Structural Alterations and Memory Impairments

The hippocampus plays a central role in declarative memory, spatial navigation, and the regulation of the stress response. It is especially vulnerable to the effects of ELS due to two main factors: its prolonged postnatal development and the high density of GRs within its structure (Figure 1). These receptors make the hippocampus highly sensitive to elevated stress hormones such as cortisol[35].

In humans, the maturation of the hippocampus extends significantly beyond birth, particularly during the critical first two years of life. This postnatal phase is characterised by rapid growth, synaptogenesis, and circuit refinement, rendering it a sensitive period during which the hippocampus is especially vulnerable to environmental influences[36,37]. In rats, hippocampal development persists into the early postnatal phase, where granule neurons in the dentate gyrus extend their axons, referred to as mossy fibres, to innervate hilar and CA3 pyramidal cells[38–40]. This critical period, particularly from postnatal days 1 to 21, coincides with the SHRP (see section 1.2.1). However, ELS can prematurely activate the HPA axis, thereby disrupting typical hippocampal development[41].

Studies on animals, using models such as MS or LBN, provide strong evidence of hippocampal vulnerability. For instance, maternal separation in neonatal rats leads to moderate learning impairments and a significant reduction in mossy fibre density in the adult hippocampus[41]. Moreover, a decrease in the number and density of granule cells in the dentate gyrus (DG),

alongside changes in dendritic structure, has been observed[42]. These structural disruptions are linked to cognitive deficits and emotional dysregulation later in life.

Findings in humans are in line with these findings: MRI-based assessments in adults with histories of childhood abuse and post-traumatic stress disorder consistently show reduced hippocampal volume, particularly in the left hemisphere[43]. For instance, women who experienced sexual abuse in childhood have been found to possess significantly smaller left hippocampal volume compared to controls[44]. Furthermore, a study by Humphreys et al. [45] underscores that the severity of ELS, rather than the quantity of adverse events or stress sustained through later childhood, is the strongest predictor of reduced hippocampal volume in adulthood.

In addition to causing significant structural alterations, ELS can impair neural plasticity in the hippocampus. When exposed to stress, the hypothalamus increases secretion of CRH, which acts on CRH receptors in various brain regions, including the hippocampus. Excessive CRH signalling in the hippocampus interferes with long-term potentiation (LTP), a crucial process for learning and memory. For instance, Ivy et al. [46] found that LBN rats exhibit heightened activation of CRH receptors in the CA1 and CA3 subfields, leading to deficits in spatial memory[46]. Administering a CRH antagonist effectively mitigated the memory loss, underscoring the role of excessive CRH signalling as a primary factor.

One critical target of ELS is adult hippocampal neurogenesis in the dentate gyrus, where new neurons are continuously generated throughout life and which has been described in both humans and animal models. During early development, the DG remains immature, and the infrapyramidal blade has yet to form. ELS during this sensitive period can significantly reduce neurogenesis and impair the integration of new neurons into hippocampal circuits. Additionally, ELS alters the expression and signalling of neurotrophic factors, particularly brain-derived neurotrophic factor, which is essential for synaptic plasticity and neuronal survival. Chronic glucocorticoid exposure, a common consequence of sustained HPA axis activation, disrupts BDNF-TrkB signalling and further impairs dendritic growth and complexity. [47]

1.2.4 Prefrontal Cortex: ELS Effects on Cognitive Function and Emotional Regulation

The prefrontal cortex is a vital brain region involved in a wide array of higher-order functions, including emotional regulation, social and motivational behaviours, perceptual processes, attention, working memory, and decision-making. It is central to executive function and cognitive control, providing top-down modulation of behaviour through mechanisms such as inhibitory control, goal-oriented planning, and behavioural flexibility. These functions rely on the intricate synaptic organisation and connectivity of PFC neurons with other cortical and subcortical regions.[48,49]

Although PFC neurons are generated prenatally, their functional maturation extends far beyond birth, particularly in humans. The differentiation of pyramidal and interneurons, the establishment of synaptic connections, and the development of cortical circuits continue into the third decade of life. In rodents, many of these processes take place during the first postnatal months (approximately P1 to P28), whereas in humans, analogous developmental milestones span from late gestation through early childhood[50]. For example, the mPFC undergoes rapid maturation during this period, with prenatal synaptogenesis peaking shortly after birth, followed by an extended phase of synaptic pruning and circuit remodelling. While the timing differs across species, these windows represent key phases of cortical development during which the brain is especially sensitive to environmental influences[50].

During early childhood, the human PFC experiences a two-to-threefold increase in dendritic spine density compared to adulthood, especially in the supragranular layers. This increase is attributed to a surge in axospine synapse formation, occurring from approximately two months before birth to two months after birth. A plateau phase follows, lasting until around three years of age, during which synaptic density remains relatively high before gradually declining. Notably, synaptic density in the human PFC peaks around 3.5 years of age, a relatively late timeline compared to other cortical areas. During this critical window, pyramidal neurons extend long-range projections to various cortical and subcortical targets, a process occurring primarily in the first postnatal year in humans (P0-P14 in rodents)[50].

Given its prolonged development and role in top-down regulation of emotional and cognitive processes, the mPFC is particularly susceptible to the effects of ELS. In rodents, ELS has been shown to interfere with synaptic organisation and plasticity in the mPFC, resulting in long-lasting structural and functional impairments [51] have demonstrated that ELS, such as MS, cause

significant atrophy of the basal dendritic tree and reduce spine density on both apical and basal dendrites of layer 2/3 pyramidal neurons. These structural alterations are accompanied by impairments in LTP, altered glutamatergic receptor expression, and increased anxiety-like behaviours, indicating a disrupted excitatory-inhibitory balance in mPFC circuits[52].

Further research reveals that ELS can lead to imbalanced inhibitory/excitatory activity within the mPFC, often coupled with altered functional interactions with other brain regions, such as the amygdala. This dysregulation is believed to contribute to both the heightened emotional reactivity and decreased cognitive flexibility observed in animals exposed to ELS. In particular, the impairment of cognitive flexibility has been associated with disrupted inhibitory signalling and aberrant mPFC-amygdala connectivity[53].

Human neuroimaging studies provide converging evidence for these findings. Structural MRI studies have reported that individuals with a history of early adversity exhibit reduced PFC volume, particularly in the medial and dorsolateral subregions[54,55]. Functional MRI studies show altered connectivity patterns, such as increased local connectivity in the left middle frontal gyrus and decreased functional coupling between the right dorsolateral PFC and regions like the left praecuneus and inferior parietal lobule[56]. These connectivity alterations are associated with cognitive impairments, including deficits in spatial working memory and emotional regulation. While such changes are not unique to ELS and can be observed in neurodegenerative diseases, they may still reflect characteristic neural adaptations following ELS exposure and contribute to long-term vulnerability.

1.2.5 Summary of Structural and Behavioural Consequences of ELS

Table 1. Summary of ELS effect on brain regions, function and behaviour

Brain Region	Primary Functions	Functional Consequences of ELS	Behavioural Outcomes
HPA-axis	Regulation of stress hormone secretion and the negative feedback loop	Dysregulated cortisol secretion (blunted or exaggerated), impaired feedback inhibition	Heightened stress sensitivity, emotional dysregulation
Amygdala	Processing of emotions, especially fear and threat detection and social memory	Hyperactivity and heightened responsivity to emotional stimuli	Elevated anxiety levels, fear generalisation, impaired social behaviour
Hippocampus	Memory formation, contextual processing, HPA axis feedback	Reduced volume, impaired neurogenesis, decreased glucocorticoid receptor expression/sensitivity	Memory deficits, dysregulated stress response
Medial Prefrontal Cortex	Top-down regulation of emotion, decision-making, and executive function	Impaired top-down regulation, impaired synaptic plasticity, altered glutamatergic receptor signalling, altered mPFC-amygdala connectivity	Increased anxiety, impaired stress regulation, and cognitive inflexibility
ELS: Early Life Stress; HPA-axis: Hypothalamic-Pituitary-Adrenal axis			

ELS exerts profound and enduring effects on both brain structure and behaviour. Despite differences in developmental timing and complexity between humans and rodents, a consistent picture emerges: ELS interferes with normal brain maturation, particularly in regions that are highly plastic and still developing. Table 1 summarises the findings across the HPA axis, amygdala, hippocampus, and prefrontal cortex.

In the following section, this thesis discusses rodent models of ELS, which provide insights into these processes and offer experimental tools to identify mechanisms underlying pathways of resilience and vulnerability.

1.3 Rodent Models of ELS

As discussed in the previous sections, human brain development continues long after birth. While the HPA axis is already highly responsive at birth, other brain regions, such as the hippocampus, amygdala, and frontal cortex, undergo substantial maturation during infancy, childhood, and adolescence. Similarly, rodents experience extended brain development. However, many neurodevelopmental processes that occur prenatally in humans take place during the early postnatal period in rodents, reflecting differences in the timing of maturation across species. [12]

Due to these species' differences, the timing of stress exposure produces different outcomes. For example, the first postnatal week in rodents is often considered roughly equivalent to the third trimester of human gestation in terms of certain neurodevelopmental processes, such as cortical and hippocampal maturation[12]. However, this equivalence does not apply to all physiological or behavioural systems. Processes such as independent breathing and early bonding begin after birth in both species, reflecting important developmental divergences.

Various rodent models have been developed to study the impact of ELS, including maternal separation, in which pups are removed from the dam for extended periods (e.g., 3 hours/day) during their early postnatal period. This model introduces intermittent, acute stress, often resulting in hyperactivation of the HPA axis[57–59].

This study employs the limited bedding and nesting paradigm, a model in which the dam remains present. Here, the environment is altered by reducing the availability of nesting and bedding materials during the critical postnatal window (typically P2-P9)[60,61]. This manipulation leads to fragmented and unpredictable maternal care, modelling chronic and subtle stress exposure without complete maternal deprivation[61–63]. The LBN is considered to more closely mimic the complex and often inconsistent caregiving environments experienced by human infants under stress. Additionally, LBN is more of a chronic stress than maternal separation is intermittent and acute stress [62–64] .

The translational value of the LBN model lies in its ability to simulate the subtle, chronic aspects of ELS in humans. Human infants often experience stress not through complete loss without any care, but through inconsistent, neglectful, or chaotic caregiving environments, which impair the development of secure attachment and affect the neuroendocrine function. Moreover, sensory cues from the dam, such as tactile stimulation and warmth, play a key role in regulating pup physiology and development. Disruptions in these cues during LBN mirror the sensory and emotional deprivation often experienced by children in neglectful environments. [63,65]

1.4 Current study

Building upon the translational relevance of the Research Domain Criteria (RDoC) framework[66] and the behavioural constructs accessible in rodent models, this study aims to investigate the long-term effects of ELS using a battery of behavioural tasks that probe anxiety-like behaviour, social interaction, reward learning, and working memory. The goal is to identify behavioural

phenotypes corresponding to stress-related domains, such as negative valence and cognitive systems, and explore behavioural alterations associated with ELS exposure.

A key objective is to determine whether ELS leads to consistent group-level differences compared to control animals across domains such as anxiety-like behaviour, learning, and social interaction. Additionally, we explore variability within the ELS group to identify potential markers of vulnerability or resilience, acknowledging that not all individuals exposed to early life stress show the same behavioural outcomes.

1.4.1 Link to Behavioural Domains and Introducing Transdiagnostic Framework

Studying mental health disorders is challenging due to the heterogeneity of symptoms and the comorbidity present among these disorders. Therefore, this study utilises the RDoC framework (see ***Figure 3***) to integrate various components and enhance the study's translational relevance[66]. The framework encompasses six key domains of human functioning, each containing subclasses known as constructs. These domains include emotion, cognition, motivation, and social behaviour, while the constructs facilitate our understanding of these domains through behavioural elements, processes, mechanisms, and responses. In our rodent model of ELS, the focus is on assessing selected constructs from these domains using established behavioural tasks. These constructs have been chosen because they reflect core symptoms observed in humans exposed to early-life adversity.

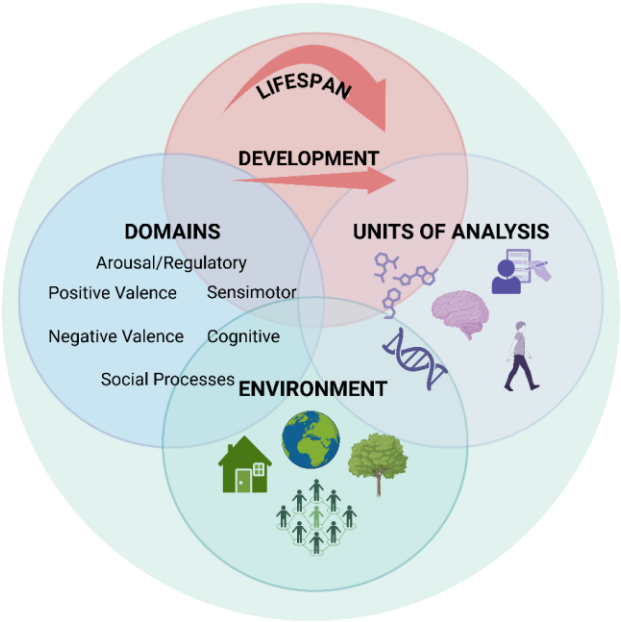

Figure 3. The Research Domain Criteria (RDoC) Framework. The RDoC framework, developed by the National Institute of Mental Health, provides a dimensional and integrative approach to understanding mental disorders. It organises research across multiple Domains of function (left circle), each of which can be studied at different units of analysis (right circle). These dimensions are influenced by the environment (lower circle) and are situated within a developmental trajectory across the lifespan (upper circle), emphasising the interaction between biology and context in shaping mental health and disease. Created with Biorender.com (reference: National Institute of Mental Health. Research Domain Criteria (RDoC). NIMH.) RDoC Matrix - National Institute of Mental Health (NIMH).

This study encompasses both negative and positive valences, social processes, and cognitive domains. Each will be prompted with the behavioural test battery: the Open Field test, elevated plus maze, novel object recognition, social interaction, and association cue task. Table 2 classifies and divides the domains of this study into constructs. It specifies whether a given construct can be studied using rodent models and how this study aims to assess it.

Table 2. Mapping of RDoC domains and constructs to behavioural tasks used in this study

RDoC Domain	Construct	Assessable	Task/Explanation
Negative Valence Systems	Acute Threat	Yes	**OFT, EPM**: avoidance of open arms/centre indicates innate fear
	Potential threat	Yes	**OFT, EPM**: centre/ open arm avoidance reflects anxiety-like behaviour
	Sustained threat	Yes	Chronic ELS induced changes in behaviour
Negative Valence Systems	Loss	No	Difficult to model directly in rodents
	Frustrative non-reward	No	Hard to isolate in current paradigms
	Reward learning (Reinforcement, habit)	Yes	**CAT**: assesses learning, habit formation after reversal

RDoC Domain	Construct	Assessable	Task/Explanation
Positive Valence Systems	Reward responsiveness (Anticipation, initial response)	Yes	**CAT:** Engagement during the tasks (how many trials per session)
	Reward valuation	No	Not available in our paradigms
Cognitive systems	Cognitive control	Yes	**CAT**: rule maintenance, reversal learning
	Working memory	Yes	**CAT and NOR:** Holding an auditory/ visual cue and using it to guide behaviour
	Perception (auditory, visual)	Yes	**CAT and NOR**: Discrimination of cues/objects
	Declarative memory (episodic memory)	Yes	**NOR**: assesses memory for previously encountered objects
	Attention	Partial	**CAT and NOR:** Not isolated, but required in both tasks for cue/object detection and response
	Language	No	Not possible in rodents
Social Processes	Affiliation and attachment	Yes	**SI:** preference for conspecifics
	Social communication	Yes	**SI**: Communication involves approaching and initiating interaction with each other (e.g., vocalisations, olfactory cues, body language)
	Perception and understanding of others	No	Limited in Rodents
	Perception and understanding of self	No	Not possible in rodents

ELS = Early Life Stress; EPM = Elevated Plus Maze; CAT = Cue Association Task; NOR = Novel Object Recognition; OFT = Open Field Test; SI = Social Interaction.

1.4.2 Fear and Anxiety: The Impact of Early Life Stress on Negative Valence

Negative Valence Systems are responses that emerge after aversive situations, such as fear and anxiety. In the human RDoC framework, this domain includes constructs such as acute threat (Fear), Potential Threat (Anxiety), sustained threat, loss and frustrative non-reward (i.e., negative responses when an expected reward is withheld after repeated effort). Individuals with a history of ELS exhibit increased anxiety, altered threat sensitivity, and blunted physiological stress responses, consistent with impairments in the negative valence domain [67–69].

While not all constructs can be fully modelled in rodents, several core components are accessible through behavioural assays. Rodent models are particularly well-suited to investigate acute threat, typically manifested as innate fear responses; potential threat, often reflected in anxiety-like behaviours such as increased risk assessment or avoidance; and sustained threat,

which refers to a prolonged aversive emotional state resulting from persistent internal or external stressors[66]. While rodents do not experience emotion in the same way humans do, sustained behavioural and physiological alterations, in this case ELS, after chronic stress exposure, are often interpreted as animal correlates of this domain.[70] ELS can be reflected in long-term increases in anxiety-like behaviour, freezing or reduced exploratory behaviour across multiple behaviour tasks, such as the EPM and the OFT [71,72].

1.4.3 Reward Processing: The Impact of Early Life Stress on Positive Valence

Positive Valence systems refer to the processes involved in responses to positive motivational situations, including reward-seeking, anticipation, and reward learning[66]. Exposure to ELS in humans has been shown to reduce reward anticipation, impair reward learning and alter valuation [73,74], which may contribute to the anhedonia or motivational deficits commonly observed in stress-related disorders [75,76].

Several constructs from this domain can be experimentally assessed in rodent models, including reward responsiveness, reward learning, and reward valuation. The cue-association task (see Methods section) used in this study primarily targets reward learning, including reinforcement learning and habit formation, as animals learn to associate specific cues with the availability and location of rewards. Over time, their performance reflects their ability to learn, adapt, and consolidate these associations, especially when the reward contingencies change.

Reward responsiveness can be further divided into reward anticipation and the initial response to reward. In the context of the cue-association task, reward anticipation is reflected in the animal's motivation to initiate trials or approach the correct location following a cue. The initial response to reward could be observed through behaviours immediately following successful trials, such as sighs of heightened engagement (e.g. number of trials).

Reward learning encompasses probabilistic and reinforcement learning subconstructs, along with habit, which we can model in our rodents. Through reinforcement learning, rodents can learn to associate cues with rewards. We aim to test whether ELS affects the motivation or overall performance of the learning process. Habit refers to behaviour that becomes automated and less sensitive to changes in outcomes. In the cue-association task, if animals continue to respond based on previously learned rules despite a change in reward contingencies (e.g., during rule reversal), this may indicate habitual behaviour. Emerging evidence suggests that ELS may

promote this type of rigid, habitual responding [77,78], making the individuals less able to adapt when reward rules change.

1.4.4 Memory: The Impact of Early Life Stress on Cognitive Systems

Although certain aspects of human cognition, such as language, abstract reasoning, or complex social cognition, cannot be directly modelled in rodents, many fundamental cognitive processes are accessible through well-established behavioural tasks. In this study, we focus on cue-association learning and NOR, which assess core subconstructs such as associative learning, memory and cognitive flexibility. Moreover, rodents are capable of demonstrating forms of abstraction, such as generalisation, making them suitable for probing key components of cognitive systems despite species-specific limitations.

Cognitive systems encompass a range of brain functions involved in processing information, including perception, attention, working memory, learning and cognitive control. In the RDoC framework, this domain includes constructs such as attention, declarative memory and working memory (Table 1). In humans, ELS has been linked with deficits in cognitive domains, with studies reporting poorer executive functioning, difficulties in attentional control, and memory impairments[79–82].

While cognitive systems are more challenging to model in rodents due to species-specific differences in higher-order functions, such as language, abstract reasoning, and complex social cognition, several core subconstructs are still accessible through behavioural tasks (cue-association and novel object recognition).

The cue association task is designed to assess key cognitive functions, such as goal selection, rule maintenance, and behavioural flexibility in response to changing reward contingencies. This task requires animals to discriminate between auditory cues, engage working memory, and adapt to changing reward contingencies.

The NOR task primarily probes recognition memory but also engages a range of cognitive subdomains. The animal's ability to distinguish novel from familiar objects reflects relational memory processes, which are considered rodent analogues of human episodic memory. Visual discrimination of objects is essential, as animals typically rely on visual cues to detect novelty, although other sensory modalities may contribute. When delays are introduced between object

exposures and testing phases, the animal must temporarily retain object-related information to guide exploration behaviour. [83]

While neither task is designed to isolate attention, the NOR and association tasks require animals to detect and process specific stimuli (novel objects or auditory cues), making attentional engagement necessary for successful performance.

1.4.5 Social Behaviour: The Impact of Early Life Stress on Social Processes

The systems for the social processes domain include constructs such as social communication, perception of others, and affiliation and attachment[66]. In humans, ELS increases the risk of developing psychiatric disorders, and social dysfunctions are often comorbid with these disorders[84].

While some components of social behaviour, such as complex communication or higher-order perspective-taking, are uniquely human, rodent models allow us to assess core elements of social processing, particularly those related to affiliation and attachment. The SI task targets these constructs by measuring a rodent's motivation to engage with a familiar or novel conspecific. Rodents naturally seek social contact, making this task a robust tool for detecting changes in affiliative behaviour following ELS[85].

2 Results

2.1 Anxiety-Like Behaviour

2.1.1 Open Field Test

The OFT is a widely used paradigm to assess anxiety-like behaviour and exploratory tendencies in rodents. The key parameters include avoidance of the centre zone (used as a proxy for anxiety) and locomotion activity (stillness, walking pace, and high-speed behaviour). Here, the effects on behaviour were investigated, with particular attention given to sex differences.

To probe the anxiety-like and avoidance behaviour, the avoidance of the centre zone and the number of entries into the centre zone were assessed. Additionally, sitting/stillness, walking pace, and running speed time were examined based on movement velocity thresholds: Stillness/sitting time was calculated if the rats moved under 2 cm/s. Speeds greater than 20 cm/s are interpreted as running and escape-like behaviour or high arousal behaviour. Normal

movement ranges from 2 to 20 cm/s [86]. The total distance travelled was analysed to assess general activity levels.

Data were assessed for normality using the Shapiro–Wilk test. When data were normally distributed, independen*t*-sample *t*-tests were used to compare groups. For non-normally distributed data, non-parametric Mann–Whitney U tests were applied.

Table 3. Group means, standard deviations, and statistical comparisons for movement and anxiety-like parameters in the OFT and EPM

Parameter	CTRL Mean (SD)	LBN Mean (SD)	Group Comparison	Sex Effects
OFT (Open Field Test)				
Total Distance (cm)	63,014.93 (22,093.24)	65,986.98 (18,528.75)	*p=0.68* (*t*-test)	No effects (One-way ANOVA *p= 0.2*)
Average Velocity (cm/s)	151.82 (70.24)	136.66 (55.92)	*p= 0.505* (*t*-test)	**LBN ♂ > LBN ♀ (*p= 0.0017*)**
Centre Entries	30.13 (19.63)	36.25 (17.50)	*p =0.34* (*Mann-Whitney U*)	No effects (One-way ANOVA *p= 0.43*)
Time in Centre (s)	36.10 (26.88)	63.24 (39.80)	***p =0.0438*** (*Mann-Whitney U*)	No effects (One-way ANOVA *p= 0.27*)
Latency to Centre (s)	15.73 (44.63)	57.53 (120.68)	*p = 0.061*	No effects (One-way ANOVA *p= 0.12*)
Stillness Time (s)	215.84 (190.72)	205.02 (142.50)	*p = 0.72*	**LBN ♂ > LBN ♀ *(p < 0.0001*)**
High Speed Time	318.14 (86.16)	267.66 (77.25)	***p= 0.02*** (*Mann-Whitney U*)	-
Normal Time (s)	31.92 (9.16)	52.22 (26.70)	***p=0.04*** (*Mann-Whitney U*)	-
EPM (Elevated Plus Maze)				
Total Distance (cm)	20,555.47 (5,208.96)	16,966.24 (3,214.26)	***p = 0.027 (Mann-Whitney U)***	CTRL ♀ > LBN ♀ (p = 0.02)
Average Velocity (cm/s)	68.48 (56.52)	17.37 (10.69)	***p = 0.027 (Mann-Whitney)***	CTRL ♀ > LBN ♀ (p = 0.02)
Stillness Time (s)	149.62 (22.99)	166.96 (23.58)	**p = 0.04 (*Mann-Whitney U*)**	LBN ♀ > CTRL ♀ (p = 0.04)

Parameter	CTRL Mean (SD)	LBN Mean (SD)	Group Comparison	Sex Effects
OFT (Open Field Test)				
High-Speed Movement Time (s)	120.00 (17.14)	106.72 (19.58)	p = 0.05 (Mann-Whitney)	CTRL ♀ > LBN ♀ (p = 0.03)
Arm Entries (total)	43.00 (22.00)	44.00 (20.00)	P= 0.90 (*t*-test)	–
Latency to First Open Arm Entry (s)	8.29 (11.11)	10.33 (10.14)	P= 0.59 (*Mann-Whitney U*)	–
Time in Open Arms (s)	7.28 (9.17)	5.28 (4.76)	P= 0.69 (Mann-Whitney)	–
Normal-Speed Movement Time (s)	30.55 (9.14)	26.48 (5.51)	P=0.14 (*t*-test)	-

CTRL = Control group; LBN = Limited Bedding and Nesting Group; SD = Standard Deviation.
– indicates that there is no statistical difference between groups.

2.1.1.1 Centre Zone Avoidance Reflects Reduced Anxiety-like Behaviour in LBN Group

The LBN group spent significantly more time in the centre compared to the controls ($p < 0.05$) (Figure 4A), suggesting reduced anxiety-like behaviour in animals exposed to ELS. In contrast, there were no significant group differences in the number of centre entries (Figure 4B). However, LBN females exhibited the highest mean entries, followed by LBN males. CTRL animals, on the other hand, showed fewer entries with less variability, whereas LBN males displayed a wider spread, possibly indicating individual differences in stress resilience.

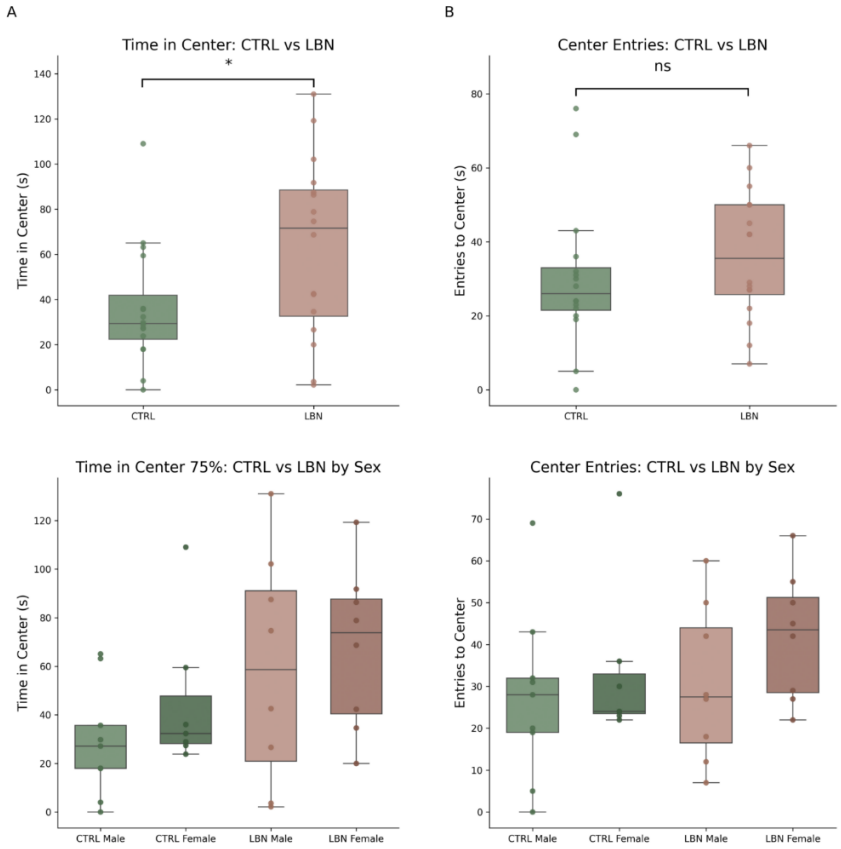

Figure 4. Time spent and entries to the centre. A) Column shows time spent in the centre by boxplots. Upper boxplots are divided by group, and lower boxplots are divided by group and sex. Statistical T-tests were calculated between groups, and ANOVA was used to compare sex differences. * = p <0.05. B) Same as A but shows entries to the centre. N=32.

Overall, the CTRL group was more homogeneous, while the LBN subgroup had a wider spread, suggesting different coping mechanisms. The possibility that increased centre time was driven by hyperactivity rather than reduced anxiety-like behaviour prompted a more detailed investigation of general locomotion and velocity patterns (see next section).

2.1.1.2 Locomotor Activity Patterns Show Sex-Specific Differences

Total distance travelled and average velocity did not significantly differ between the LBN and CTRL groups (Figure 5A-B). However, sex differences were apparent, with LBN females showing significantly higher velocity than LBN males (**$p < 0.01$, Figure 5B).

To further characterise these differences, movement was divided into walking pace (2-20cm/s), high-speed movement (>20 cm/s), and stillness (<2 cm/s for > 1 s). As shown in Figure 5C,

LBN females moved more at normal speed and high speed on average than other sex groups. Specifically, normal walking movement time was significantly higher in LBN females compared to CTRL females ($p<0.05$), while LBN males exhibited significantly more stillness than LBN females (***$p<0.001$).

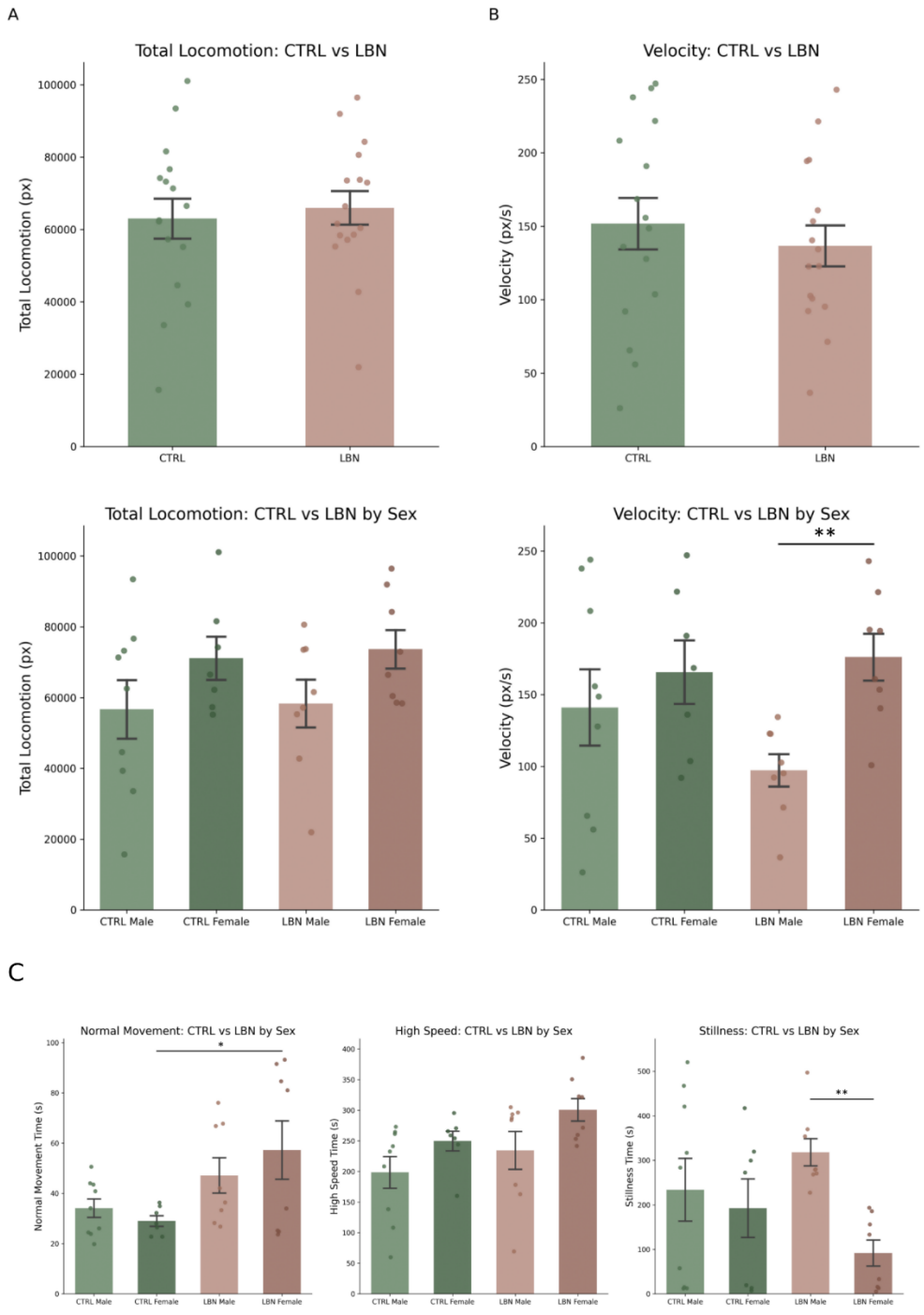

Figure 5. Total Locomotion and Velocity during the OFT. A) Total distance travelled by group (top) and by group and sex (bottom). B) Average velocity by group (top) and group and sex (bottom). C) Time spent in different movement types: normal movement (2-20 cm/s), high-speed movement (>20

cm/s), and stillness (<2 cm/s). Bars represent mean ± standard deviation (SD). Significance was calculated using ANOVA with post*t*-hoc comparisons. *p<0.05, **p < 0.01, ***p < 0.001

These findings prompted further exploration of how different movement parameters relate to one another and to centre zone avoidance (next section).

2.1.1.3 Correlations Between Movement and Anxiety-like Behaviour

Pearson correlations were calculated to see if there is a correlation between the movement behaviour and the anxiety-like behaviour. The analysis revealed a strong negative correlation between time spent in the centre and stillness (r = –0.61, p = 0.000; Figure 6A), showing that animals exhibiting less anxiety-like behaviour tend to move more. Moreover, time spent in the centre positively correlated with high-speed movement (r = 0.67, p = 0.000; Figure 6B), possibly indicating that exploratory behaviour is associated with increased arousal or escape-like activity.

A moderate negative correlation was observed between total locomotion and stillness time (r = –0.41, p = 0.019; Figure 6C), further supporting the notion that greater activity is linked to reduced anxiety-like behaviour. Complementing this, the time spent in the centre positively correlated with total locomotion, number of centre entries and average speed, confirming that animals that explored the arena more extensively also entered the centre more often and moved at higher speeds (Figure 6D-E).

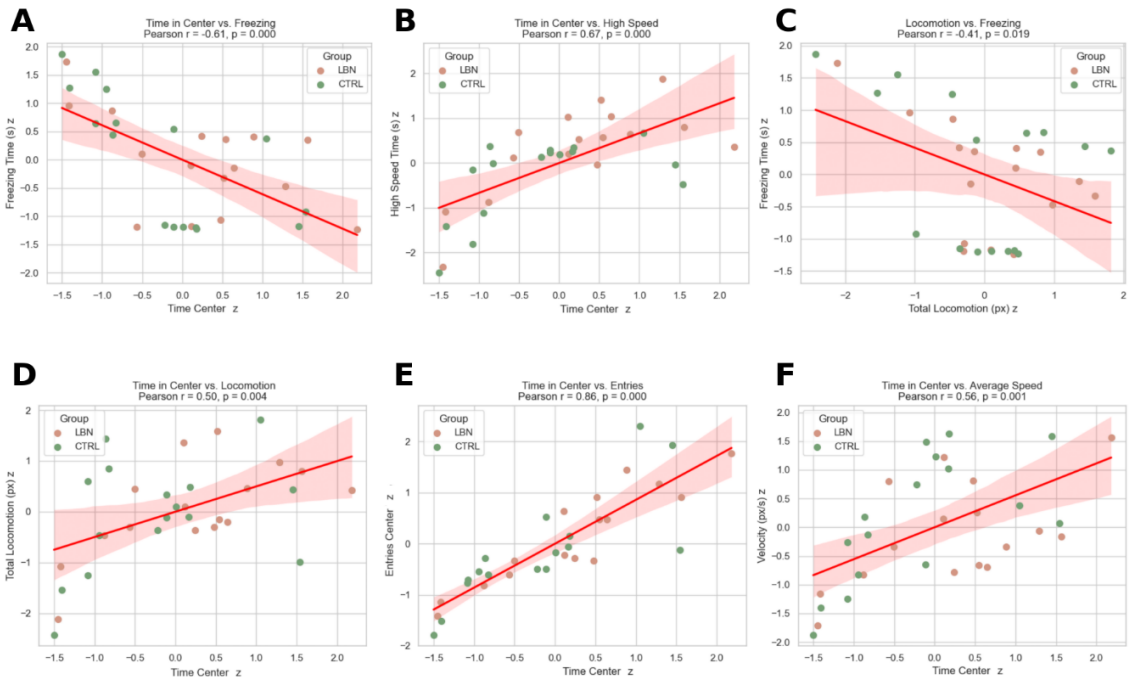

Figure 6. Correlations between centre explorations and movements. Scatter plots display Pearson's correlations between Z-scored behaviour metrics across all animals (CTRL: green; LBN: orange; n=32). Each point represents one subject. A) Increased time spent in the centre vs. stillness time. B) Time in centre vs. time spent at high speed. C) Total locomotion vs. Stillness time. D) Time in centre vs. Total locomotion. E) Time in centre vs. number of centre zone entries. F) Time in centre vs. Average speed. Linear regression lines with 95% confidence intervals (shaded areas) are shown for each correlation. Pearson's *r* and *p*-values are reported above each panel.

2.1.2 Elevated Plus Maze

2.1.2.1 Anxiety-like parameters

Parameters such as time spent in open arms, latency to first open arm entry, and overall number of arm entries were analysed to assess anxiety-like behaviour in the EPM. No significant group differences emerged for these classic anxiety-like measures (Figure 7A-C), and no sex differences (Table 3).

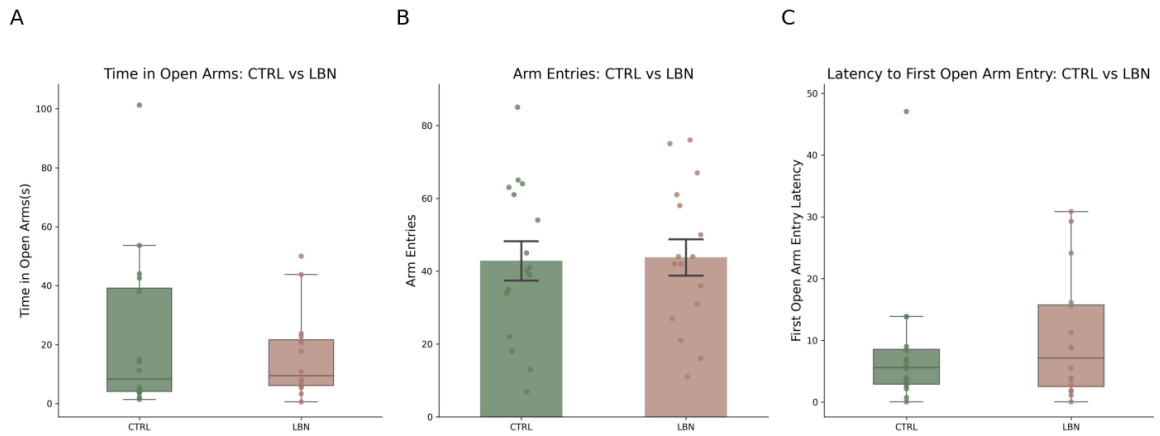

Figure 7. Group differences in anxiety-like behaviour during the EPM task. Group comparisons between the CTRL and LBN groups for three anxiety-like behaviour parameters: time spent in open arms, latency to the first open arm entry, and overall arm entries. The plots compare group differences in open-arm activity, with median, interquartile ranges, and outliers displayed. (A) The boxplot illustrates the effect of group on time spent in the open arm. The plot shows data separated by group (CTRL, LBN). The data represent the median, interquartile range, and outliers. (B) Barplot shows group differences in arm entries. (C) Boxplot of latency to the first arm entry by group. Data distribution was assessed using the Shapiro-Wilk test. For normally distributed data, an independent *t*-test was used, while for non-normally distributed data, the non-parametric *Mann-Whitney U* test was applied. N=32.

Despite no strong effects in the classic anxiety-like parameters, a more detailed analysis of locomotor patterns revealed some significant findings. CTRL animals, on average, moved more in the maze compared to LBN animals (p=0.03, Figure 8A, Table 3). CTRL females had the highest total distance travelled. Notably, LBN females showed increased stillness behaviour (p=0.04), while CTRL females spent more time in high-speed movement (p=0.03). CTRL males showed the highest proportion of normal-speed exploratory behaviour. These findings could demonstrate that group or sex differences in anxiety-like exploration might be masked by underlying differences in locomotor activity.

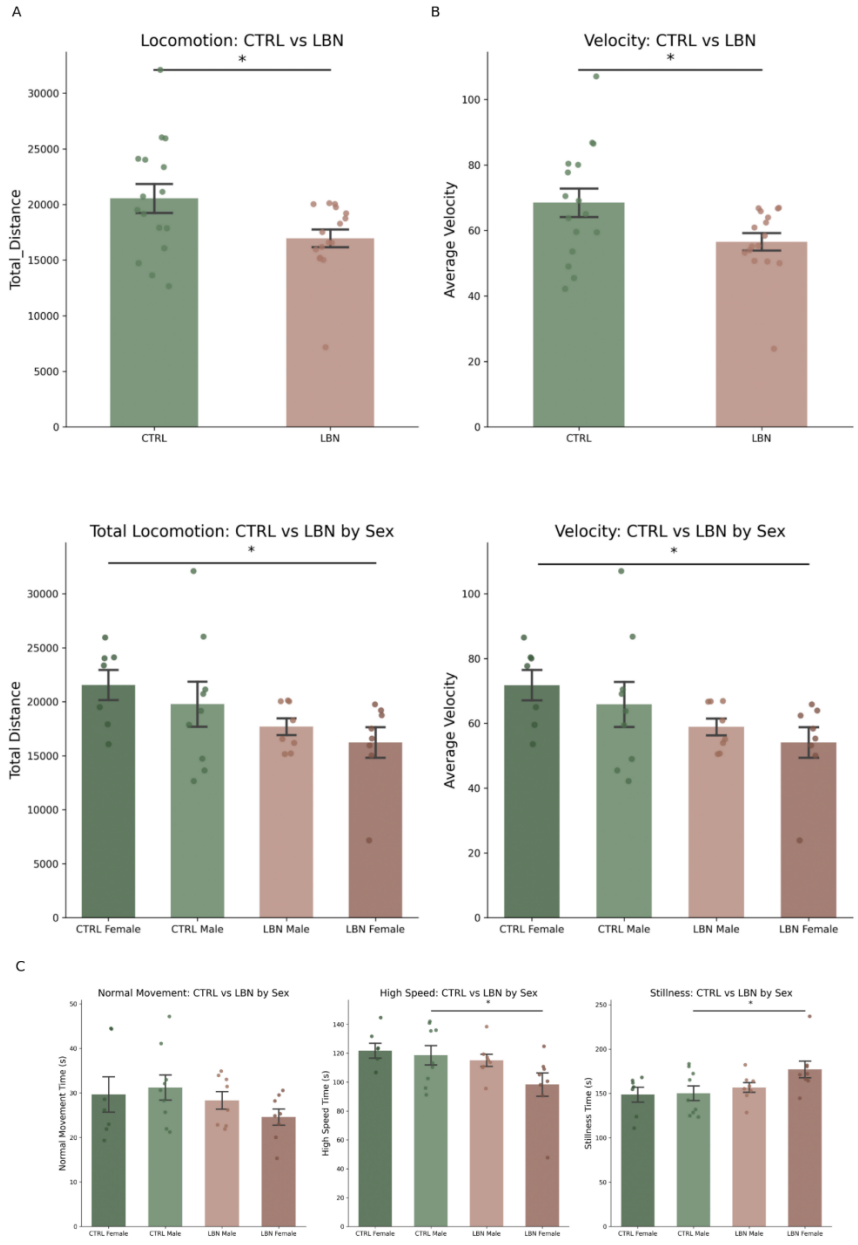

Figure 8. Total Locomotion and Velocity during the EPM. A) Total distance travelled by group (top) and by group and sex (bottom). B) Average velocity by group (top) and group and sex (bottom). C) Time spent in different movement types: normal movement (2-20 cm/s), high-speed movement (>20 cm/s), and stillness (<2 cm/s). Bars represent mean ± standard deviation (SD). Significance was calculated using ANOVA with pos*t*-hoc comparisons. *p<0.05, **p < 0.01, ***p < 0.001

2.1.3 Cross-Test Summary of Anxiety-Like Behaviour

To compare anxiety-like behaviours across tasks, a composite *Anxiety Index* was calculated using Z-scores derived from key parameters: time in open arms, stillness time, and open arm entries in the EPM; and time in centre, centre entries, and stillness time in the OFT. Z-scores were reversed when necessary, so higher values always reflected increased anxiety.

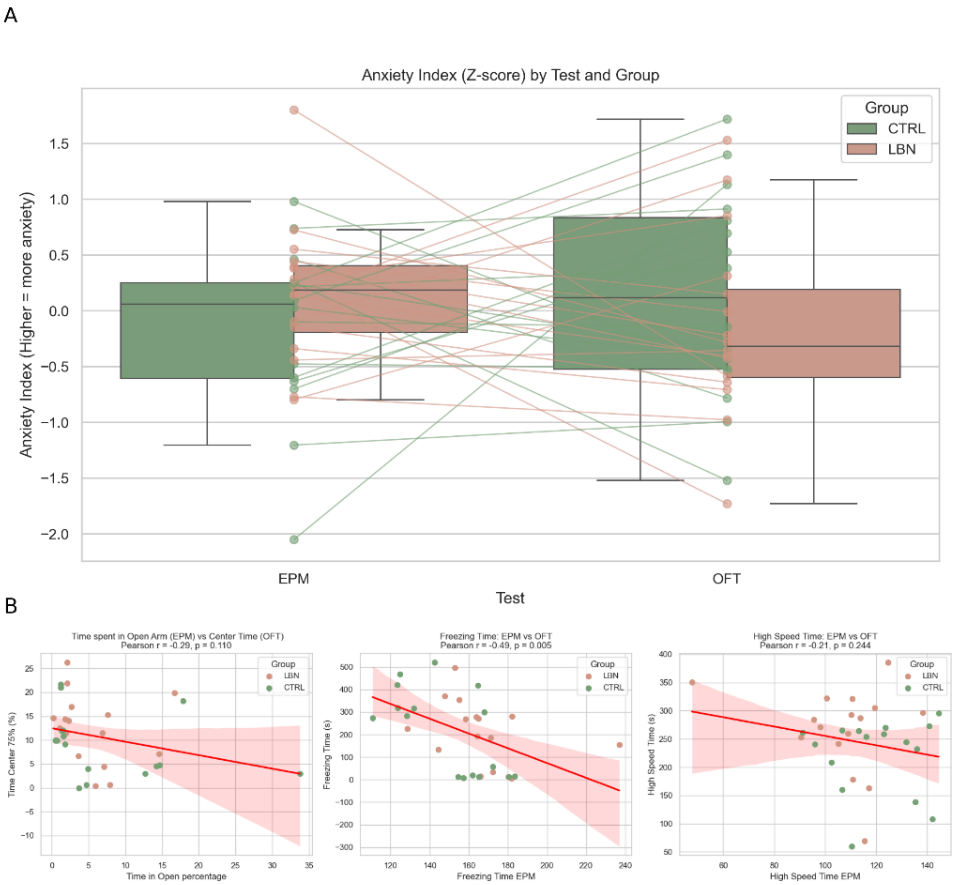

Figure 9. Anxiety-like behaviour across two tests and correlation of key parameters. A) Anxiety Index Z-scores from the EPM and OFT, separated by group (CTRL vs. LBN). Each line represents one animal across both tests. Higher values indicate greater anxiety-like behaviour. B) Pearson's correlations between complementary behavioural parameters from the EPM and OFT: Left shows time spent in the open arms of the EPM vs. centre time in the OFT. The middle shows Stillness time in EPM vs. OFT. On the right is shown high-speed movement time in the EPM vs. OFT. Linear regression lines with 95% confidence intervals (shaded areas) are shown for each correlation. Pearson's *r* and *p*-values are reported above each panel. (CTRL: green; LBN: orange; n=32).

Interestingly, individual anxiety profiles often diverged across tests. Some animals with higher anxiety indices in the EPM showed lower indices in the OFT and vice versa. Group-wise, the LBN group displayed a slightly elevated anxiety index in the EPM but a lower index in the OFT – implying that context and task structure may differentially engage anxiety-like responses (Figure 9A).

Cross-test parameter correlations (see Figure 9B) revealed a negative correlation between time spent in the open arms (EPM) and time in the centre (OFT), indicating that animals that explored

the centre more were less likely to explore the open arms. Stillness time also showed a negative cross-task correlation, further supporting the idea of tes*t*-specific coping styles or anxiety manifestations.

2.2 Recognition Memory in the Novel Object Recognition Task

2.2.1 Discrimination Performance

To assess recognition memory, the discrimination index (DI) was calculated (see Methods) during the second NOR session (NOR2), where one object was replaced with a novel one. While no significant group or sex differences were found (Figure 10A), individual variation was notable, and several animals showed poor performance with DI values near or below zero, indicating a lack of novelty preference.

Exploration time (Figure 10B) did not differ significantly between groups either, revealing that total time spent with the objects was not group dependent. However, total exploration time was relatively low overall, approximately 25 to 50 seconds, despite the task duration being 10 minutes. Nonetheless, individual variability was again present, which may relate to underlying behavioural differences.

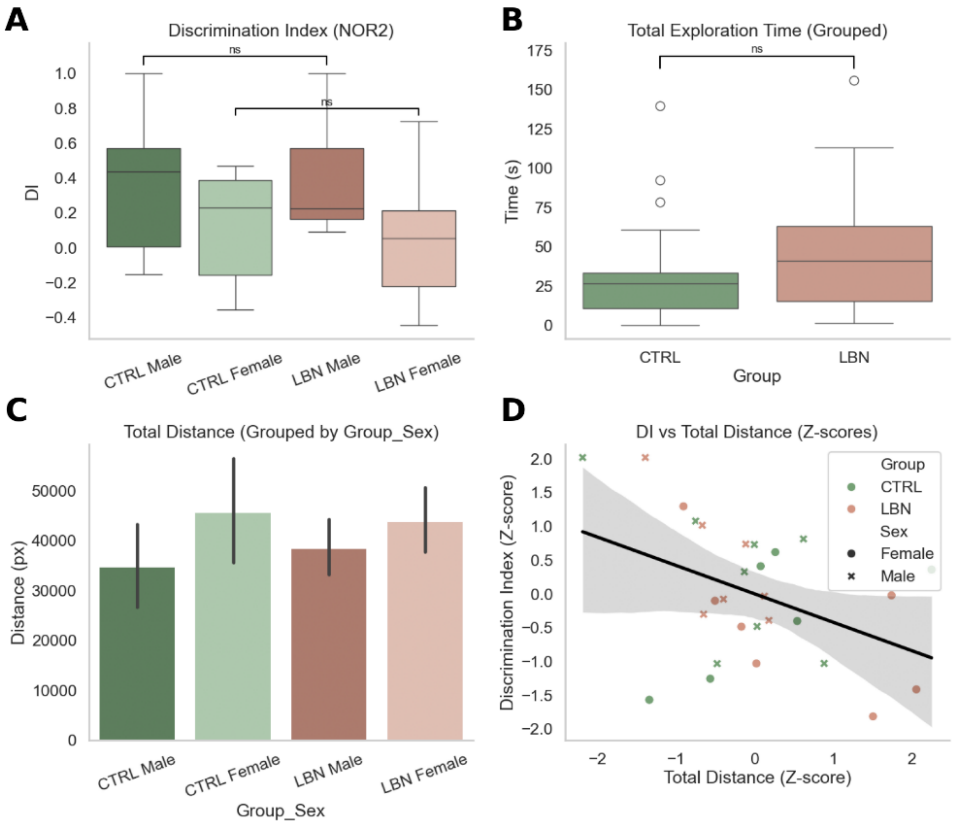

Figure 10. Performance in the Novel Object Recognition Task. A) Discrimination Index by group and sex. B) Total exploration time for novel and familiar objects by group. C) Bar plot of total distance moved during the task by group and sex. D) Correlation between total distance (Z-score) and DI. Ns=non significance

2.2.2 Movement and stillness patterns

A closer look at movement behaviour during both NOR1 and NOR2 sessions revealed high levels of sitting/stillness across animals, especially in NOR1 (Figure 11A). This was particularly pronounced in the LBN group (Figure 11B) and could have influenced object exploration and recognition performance.

Sitting time decreased from NOR1 to NOR2 but remained high overall, especially in LBN animals. High-speed movement was generally low but increased slightly in NOR2 in CTRL animals. Walking pace movement remained consistent across sessions and groups.

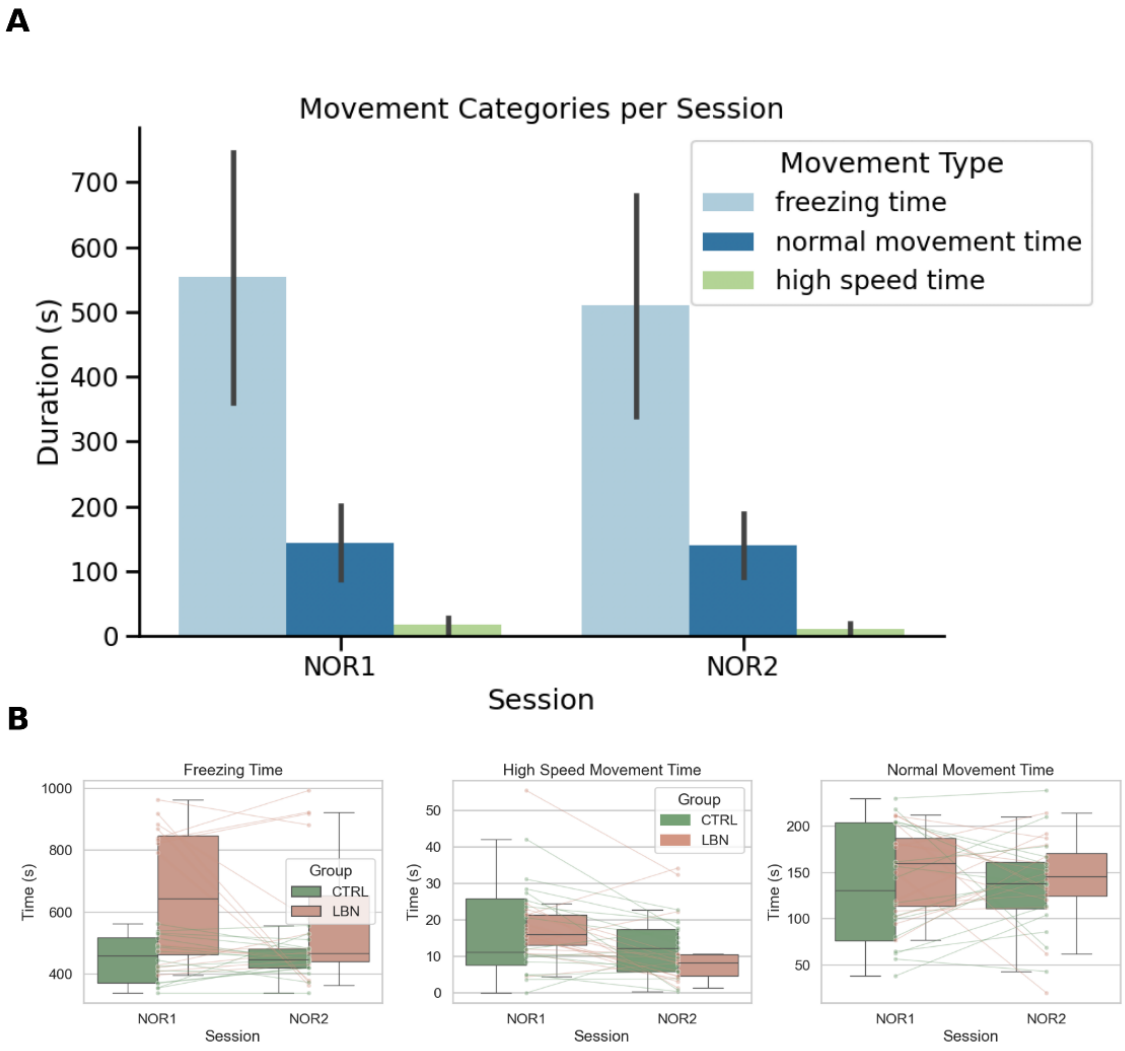

Figure 11. Movement behaviour across NOR sessions. A) Duration of stillness, normal, high-speed movement during NOR1 and NOR2. B) Boxplot shows group comparisons for each movement category across sessions, with individual lines.

These results show that reduced mobility, particularly in the form of sitting, may have impacted the expression of novelty recognition.

2.2.3 Correlation Between Movement and Recognition

To better understand this relationship, a correlation analysis was conducted between total distance moved and DI. A negative correlation was found (Figure 10D), indicating that animals with lower exploration distances also tended to perform worse in the NOR task. This supports the notion that sitting or reduced mobility levels might be linked to impaired recognition memory or reduced engagement with the task.

Table 4. Group Means and p-values for Novel Object Recognition Task Parameters

Parameter	CTRL Mean (SD)	LBN Mean (SD)	Statistical Comparison	Sex Effects
Total Exploration Time	29.81 (29.51)	45.56 (36.83)	p = 0.0709 (*Mann-Whitney U*)	No significant sex differences
Stillness Time	449.46 (65.54)	613.83 (221.65)	**p = 0.0101** (*Mann-Whitney U*)	CTRL_M < LBN_M (p = 0.0238)
Total Distance (px)	39615.78 (19854.73)	41523.82 (16692.62)	p = 0.6788 (*t*-test)	-
Average Speed (px/s)	66.00 (33.10)	57.34 (26.08)	p = 0.2496 (*t*-test)	-
High-Speed Time	14.00 (10.01)	14.77 (11.02)	p = 0.8826 (*Mann-Whitney U*)	-
Normal Movement Time	136.78 (56.84)	147.44 (46.88)	p = 0.4165 (*t*-test)	-
Novel Time	22.93 (21.80)	26.25 (24.36)	p = 0.6644 (*Mann-Whitney U*)	
Familiar Time	15.61 (19.61)	20.09 (18.40)	p = 0.3956 (*Mann-Whitney U*)	
DI	0.25 (0.39)	0.14 (0.49)	0.49	-

2.3 Social Interaction and Corticosteroid Levels

2.3.1 Social Novelty Preference Index

To evaluate social behaviour and recognition of social novelty, the Social Novelty Preference Index (SNPI) was calculated for each animal as the proportion of time spent interacting with the stranger over total interaction time (Stranger/ [Stranger + Cage mate]).

Across groups, a significant increase in SNPI was observed in LBN animals compared to CTRL animals (Figure 12A). The increase was particularly strong in LBN females compared to CTRL females ($p<0.01$, Figure 12B). When SNPI scores were analysed by group and sex, LBN females showed the highest score, followed by LBN males, and CTRL females had the lowest score. A significant sex comparison was found when comparing LBN females to CTRL females.

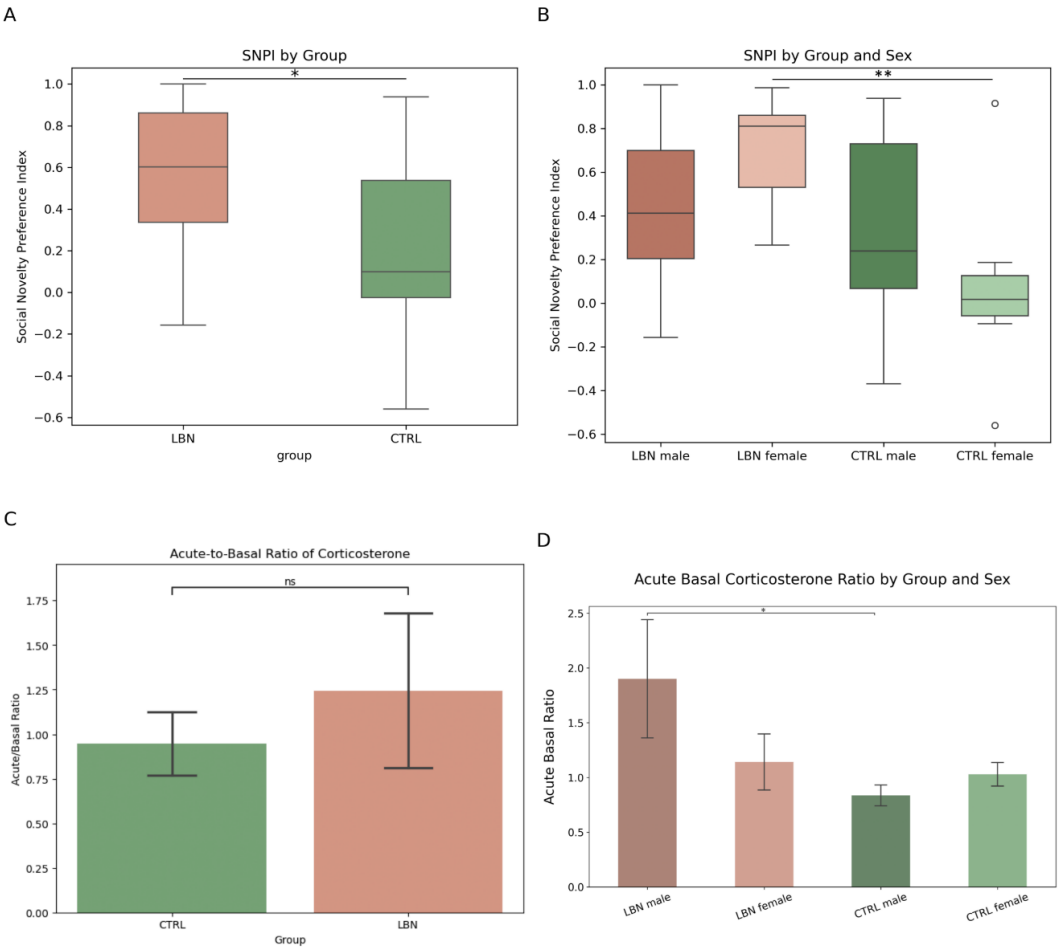

Figure 12. Social Novelty Preference Index and corticosteroid levels during SI. A) SNPI scores comparing two groups: LBN vs. CTRL ($p<0.05$). Significance was calculated using a t-test. B) SNPI scores by group and sex. Different groups of the same sex were tested against each other, with LBN females having a significant ($p<0.01$) result from CTRL females. Data is presented using boxplots, with the median, interquartile range, and whiskers indicating the data range. C) Basal-to-Acute Ratio group comparisons. D) Basal-to-Acute Ratio group and sex comparisons.

These results point toward an increased social memory or curiosity toward novel interaction. To assess the stress response following the novel interaction, corticosterone levels were analysed in relation to recent social experiences.

2.3.2 Corticosterone Response Patterns

To assess the stress reactivity, plasma corticosterone levels were measured under basal conditions and following the social interaction with a novel conspecific (acute condition). A basal-to-acute ratio was calculated to show how the corticosteroid changed from its basal level. Group comparisons revealed that LBN animals had a higher ratio, yet it didn't reach

significance (Figure 12C, Table 5). When sex differences are compared between groups, LBN males show a significantly higher ratio compared to CTRL males (Figure 12D, Table 5).

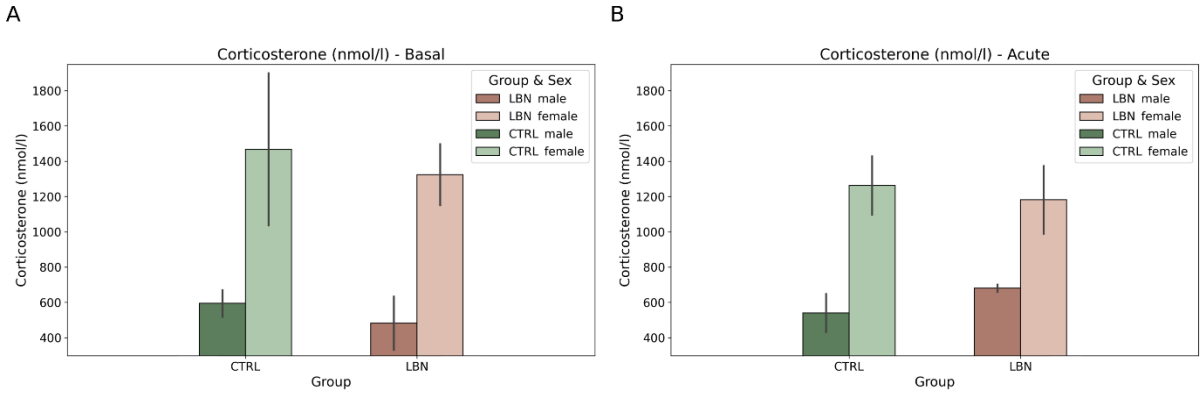

Figure 13. Basal and Acute Corticosteroid Levels. A) Basal corticosterone divided by group and sex. B) Acute corticosterone levels divided by group and sex.

Basal and acute corticosteroid levels were significantly higher in both CTRL and LBN females, with concentrations ranging from approximately 1200 to 1400 nmol/l, whereas males in both groups exhibited lower levels, ranging from approximately 400 to 600 nmol/l (Figure 13A-B)

Table 5. Group Means and p-values for Social Interaction Task Parameters

Parameter	CTRL Mean (SD)	LBN Mean (SD)	Group	Sex
SNPI	0.21 (0.45)	0.56 (0.36)	***p=0.026***	LBN ♀ > CTRL ♀ (***p < 0.01***)
Basal corticosteroid	782.51 (350.31)	841.25 (475.89)	-	LBN ♀ > LBN ♂ (***p<0.05***) CTRL ♀ > CTRL ♂ (***p<0.05***)
Acute corticosteroid	746.74(369.25)	895.33(289.98)	-	LBN ♀ > LBN ♂ (***p<0.05***) CTRL ♀ > CTRL ♂ (***p<0.05***)
Acute to basal ratio	0.948(0.176)	1.245(0.435)	-	LBN ♂ > CTRL ♂ (***p<0.05***)
Abbreviations: CTRL = Control group; LBN = Limited Bedding and Nesting Group; SD = Standard Deviation; SNPI = Social Novelty Preference Index. – indicates that there is no statistical difference between groups.				

2.4 Learning and Cognitive Flexibility in the Association Task

2.4.1 Task Overview and Cohort Structure

The association task was designed to evaluate learning and cognitive flexibility using a two-sound cue-based reward paradigm. The tasks involved learning to associate specific auditory cues with distinct reward locations. They included auditory cues: upward and downward sweeps

with a two-sided reward paradigm. The animals underwent three phases of training to learn the basics of the paradigm: to use the nose pokes, understand reward locations, and become accustomed to two different sounds. After completing these phases, the animals began the learning task with two distinct assignments: the SameSide (SS) Task and the Random Side (RS)/ Other Side (OS) Task. Figure 14 shows the different tasks and how they were implemented across the cohorts. Over three cohorts, the task was refined based on prior findings to optimise task learning and rule reversal.

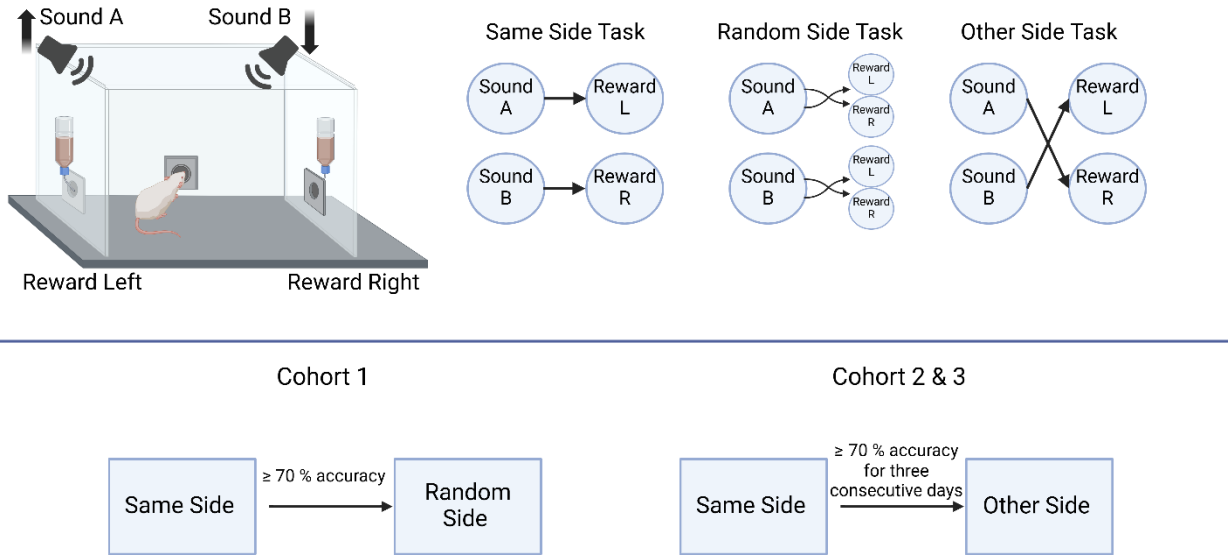

Figure 14. ***Schema of the association task paradigm, tasks, and cohort implementation***. The maze consisted of two auditory cues, an upward sweep and a downward sweep, along with two reward locations: left and right. The three tasks presented are initially distinct. The SameSide task uses Sound A for reward location L, while the Other Side task uses Sound B for the same reward location. The Random Side task switches randomly between which sound corresponds to which reward location. Cohort one used the Random Side task as the rule reversal task, while cohorts two and three used the Other Side task for the same purpose.

In the SS task, a specific sound consistently corresponded to the exact reward location across all sessions. The sound-reward location was consistent within each session in the RS task, but the pairing was randomised and changed between sessions. In the OS task, the sound corresponded to the opposite reward location compared to the SS task, and this association remained consistent across sessions. (Figure 14)

In cohort 1, we initially observed that animals were unable to learn the new rule after the SS task, indicating that modifications to the task were necessary. In Cohort 2, while animals showed some progress, learning on the SS was significantly delayed, preventing further progression to the rule reversal phase. Finally, in cohort 3, we continued with the SS task, seeing fast improvement within the animals and successfully implementing the rule reversal, leading

to the desired learning outcomes. Table 6 summarises the key characteristics and outcomes we saw in each cohort.

Table 6. Summary of Cohort-Specific Learning Task Modifications and Findings.

Cohort	Number of Animals	Task Modifications	Main findings
Cohort 1	4	SameSide task until 70% accuracy is reached → Switch to Random Side task.	3/4 successfully reached >70% accuracy on the SameSide task but failed to adapt to Random Side.
Cohort 2	5	SameSide task until ≥70% accuracy for three consecutive days is reached → Switch to Other Side Task.	Learning progress in the SameSide was slow; only 2/5 of the animals reached 70% for one day after 30 training days.
Cohort 3	3	Same as in Cohort 2.	3/3 of the animals reached 70% accuracy for three consecutive days and adapted to rule reversal successfully.

2.4.2 Cohort 1: Initial Challenges with Adapting to Random Side Task

Four animals in Cohort 1 were successfully trained to use the paradigm and to perform our learning tasks. Animals were trained to achieve 70% accuracy using the SS task. After reaching 70%, the task was switched to an RS, where, for each session, they had to determine which sound corresponded to which reward side. Three out of four rats reached the criteria of 70% (Figure 15A) and proceeded to the RS task (Figure 15B). A noticeable drop in accuracy was observed following the transition to the RS. Higher performance was observed in RS trials where the sound-reward association matched that of the SS task, while lower performance occurred when the association was reversed. This pattern indicates difficulty adapting to the new rule and is reflected in the learning curves, which show a zig-zag accuracy pattern across sessions (Figure 15B-C). One animal (BOE-000038) started to show progress towards the end of the RS task, potentially indicating that it was adapting to the new rule (BOE-000038, Figure 15C).

Figure 15. Performance on SameSide and RandomSide tasks for Cohort 1. A) Accuracy for four animals on the same-side (SS) task. The X-axis represents days since the start of training, and the y-axis represents accuracy calculated with rewarded trials/valid trials (see methods). Black dotted lines indicate the accuracy threshold, where the upper one is 70%. B) Accuracy for the three animals that achieved 70% accuracy on the SameSide and continued with the random side. C) A combined visualisation of performance on both tasks. Solid lines represent the SameSide task, and dashed lines represent the random side task.

2.4.3 Cohort 2: Delayed Learning and Task Adjustments

For cohort 2, the task design was modified following the lack of successful learning after rule reversal in the previous cohort. Minor adjustments were also made to the SS task, extending the learning criterion from achieving 70% accuracy on a single day to maintaining it for three consecutive days to ensure more robust learning. Five animals began training on the SS task. However, difficulties were encountered, as most animals were unable to learn the first task. One animal reached the 0.70 threshold after 20 days of training but failed to stay above it for three consistent days. Only after 30 days did we begin to see progress towards the 70% threshold

(Figure 16). Despite these improvements, training was discontinued due to time limits. It is noted that during this period, some technical inconsistencies in the hardware were present, which may have contributed to the observed difficulties.

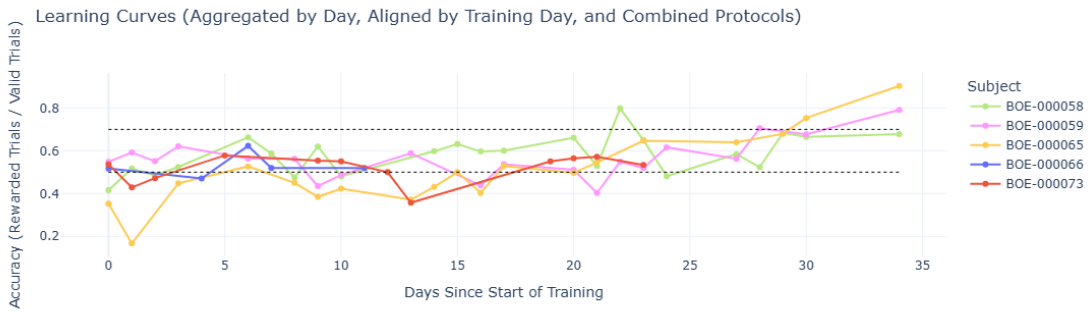

Figure 16. ***Cohort 2 Animals' performance in the SameSide task***. The plot follows the same format as in Figure 2, displaying accuracy over training days. Accuracy is calculated as rewarded trials/valid trials (see Methods), with the black dotted lines indicating accuracy thresholds, including the 70% criterion.

2.4.4 Cohort 3: Success with Modified Tasks and Rule Reversal

Performance meeting the criterion was observed in this cohort. The SS task continued until a 70% threshold was achieved for three consecutive days; at this point, the rule reversal phase commenced with the OS task. Following the rule reversal, a drop in accuracy can be seen in Figure 17, indicating that animals were following the previous rule, as it is consistent with all the animals. Although they performed poorly in the first few sessions, accuracy improved rapidly, surpassing 50% in a few sessions and reaching over 70% in the OS task.

Figure 17. ***Performance on the same-side task and following rule reversal.*** This figure illustrates performance on the same-side task with connected lines. The red line indicates the rule reversal, marking the transition to a different task. The dotted lines represent performance during the other-side task. The black dotted horizontal lines are 0.5 and 0.7 accuracy thresholds. Note: at the time of this analysis, subject 83 completed only one session of the other-side task, although training continued beyond this point after data collection for this thesis.

2.4.5 Impact of Task Modifications on Learning Outcomes

The group composition between LBN and CTRL is not provided; the focus here was to establish a task that can be implemented in the future. Moreover, the group composition, the overall sample size, and training tasks were unbalanced and small; therefore, statistical comparisons between the LBN and CTRL groups are unreliable. The key findings revolved around the overall trends observed during the tasks.

Cohorts 1 and 3 successfully reached the accuracy threshold in the SS task relatively fast, with cohort 1 taking under 10 days and cohort 3 slightly above 10 days (Figure 18A-C). Cohort 2 took over 30 days. However, we observed issues in the maze during cohort 2 that impacted a lot of the animals' motivation.

For each task, average accuracy rates were calculated to observe the trend in performance. Cohort 2 was excluded from these average calculations due to unreliable results caused by issues with the maze/hardware. The time points considered were from each animal's first session within the tasks they participated in, their middle progress, and their last session in that task (Figure 18D). For the SS and OS, the last session corresponds to when the animal reached the performance threshold. In contrast, for RS, the last session refers to the final session completed by the animal, as no clear performance threshold was reached due to the absence of improvement. In the SS task, average accuracy at the first session was just above 0.5, slightly increasing each animal's middle progress (average accuracy 0.55), and finally surpassed the performance threshold by the last session. In the OS task, animals began with lower accuracy (below 0.4) than in the SS first session. For both the OS and SS tasks, accuracy increases; however, in the RS task, there is no improvement, as the line remains flat at around 55% accuracy (Figure 18D). In RS, higher performance was observed when sound and reward location matched those in the SS task, and lower performance when the association was reversed. While the SS task achieved better overall accuracy than the OS, the magnitude of increase from the first to the last session was comparable. The average time to reach the final session across all subjects was 10.17 days.

Figure 18. Time to reach the first accuracy threshold and Accuracy rates throughout the tasks. A) Average time to reach 70% accuracy on the SameSide task grouped by cohorts. B) Number of days each animal spent to reach 70% accuracy on the same-side task. C) Individual learning curves for animals that reached the 70 % threshold in the SameSide task. D) Mean accuracy rates across each task's first, middle, and final sessions (SameSide, N=7; OtherSide, N=2; RandomSide, N=3). Accuracy was averaged across animals for each task and time point to capture the overall progression of each task.

2.5 Body Weight Development Across the Postwean Phase

To evaluate the effects of ELS on physical development, body weight was monitored across key developmental time points in CTRL and LBN animals during the post-weaning period until behavioural testing started and slightly after behavioural testing. Data were analysed at postnatal weeks 3 (P21), 5 (P35), 6 (P42), 7(P49), and 16 (P112), with consideration for both group and sex.

As shown in Figure 19A, mean body weight increased with age in both groups. At week 3, CTRL animals had significantly higher body weight than LBN animals ($p = 0.0001$, *t*-test; ***).

At week 5, no significant difference was observed (*p=0.2958*, *Mann-Whitney U*), although LBN seems to be heavier on average. By week 6, CTRL animals again showed significantly higher weights (*p=0.0033*, *t*-test; **). No significant group differences were found at week 7 (*p=0.4396*) or week 16 (*p=0.6601*), both tested with the *Mann-Whitney U* test due to abnormally distributed data.

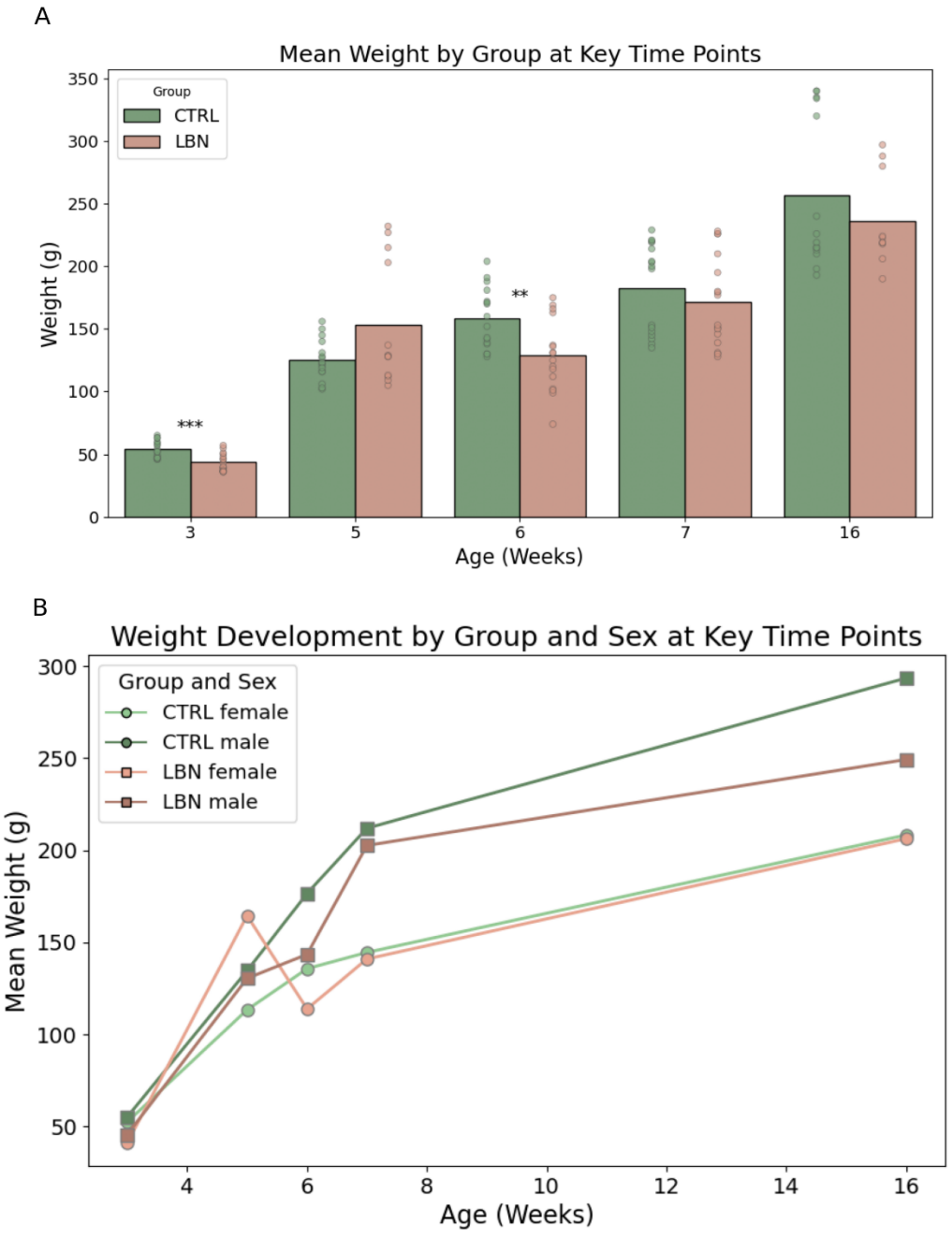

Figure 19. Weight development across Postnatal Ages in CTRL and LBN Groups. A) Bar plots show mean body weight (in grams) at postnatal weeks 3 (P21), 5(P35), 6(P42), 7(P49), and 16 (P112), separated by experimental group (CTRL: green; LBN: red). Individual data points are overlaid on each bar to show variability. **B)** Line plots depict longitudinal weight development across the same time points,

separated by group and sex. Males are represented by squares and darker colours; females by circles and lighter shades. ** 0.01 *** 0.001.

To further investigate developmental trajectories, a longitudinal weight dataset was separated by sex (Figure 19B). Male animals in both groups weighed more than females at all time points, except at week 5, where there was an increase in the weight of LBN females that decreased right after week 6 by about 50 grams. Notably, LBN males exhibited delayed catch-up growth compared to CTRL males, whereas LBN females followed a trajectory more closely aligned with CTRL females after 6 weeks. CTRL males had consistently the highest body weight after 6 weeks.

3 Discussion

3.1 Overview of key findings

This study investigated how ELS induced by the LBN model affects behavioural performance across emotional, cognitive, and social domains in adult rodents. The findings revealed high variability among ELS animals during the OFT, indicating that they may exhibit more distinct coping mechanisms and behavioural patterns compared to controls. In the EPM, the behavioural outcomes were opposite to what was seen in the OFT, suggesting interindividual variability during different anxiety-like situations. Additionally, ELS animals tend to prefer unfamiliar companions compared to CTRLs, with ELS males exhibiting a higher stress state. These patterns may reflect both vulnerability, such as heightened stress reactivity in LBN males, and resilience, such as maintained or enhanced novelty preference in females of ELS.

3.2 Negative valence system: EPM and OFT

Findings from the negative valence systems domain revealed a dissociation between the OFT and EPM, two tasks commonly used to assess anxiety-like behaviour.

In the OFT, animals exposed to ELS spent significantly more time in the arena's centre than controls, especially LBN females, which showed the highest centre times. While increased centre time is typically interpreted as reduced anxiety-like behaviour, this result is unexpected

in the context of ELS, which is generally associated with heightened anxiety[12,62,87,88]. The increased centre time observed in LBN-reared animals, especially in females, may indicate that ELS leads to blunted anxiety responses or altered risk assessment rather than heightened anxiety. Furthermore, a more detailed analysis of speed profiles revealed that LBN females spent significantly more time in high-speed movement than CTRL females, whereas LBN males showed more frequent stillness behaviour. This pattern may reflect a state of high arousal or hyperactivity rather than reduced anxiety-like behaviour in females, while LBN males may exhibit passive coping strategies, highlighting the sex differences. The positive correlation between time in the centre and time spent running supports this interpretation, suggesting that LBN females may enter the centre more due to increased activity levels.

Additional observation was that during the OFT, the LBN group exhibited a broader spread in behaviour, indicating greater individual variability, whereas the control group showed a more consistent behavioural pattern.

In contrast, during the EPM, no significant group differences were observed in commonly used anxiety-like measurements, such as open arm time or the number of entries. However, total movement was higher in the control group. Within the LBN group, females showed an increase in stillness and reduced time spent in running movement. This contrast between the OFT and EPM is notable, as the LBN females spent the most time in running movement and the least time in stillness in the OFT, whereas in the EPM, they spent a greater proportion of time in stillness and showed a lower running time than the other groups.

These contrasting results highlight how the OFT and EPM, while targeting constructs such as potential threat and avoidance behaviour, engage different aspects of the negative valence domain due to structural and procedural differences. The EPM is more etiologically fear-inducing due to elevated and exposed open arms, whereas the OFT allows for a more gradual exploration. The OFT has a longer duration (10 minutes), possibly allowing animals time to habituate, while the EPM is shorter (5 minutes) and assesses the acute fear state through that. Additionally, the structure of the OFT and EPM could influence anxiety-like behaviour and locomotion patterns. Since the OFT is an open field with no proper safe space, it may cause the animal to exhibit hyperactive behaviour. In contrast, the EPM features narrow arms with high walls, which can create a sense of safety, and it is simply harder to run quickly in these maze-like structured arms. A comparative study [89] reported that exploratory activity, measured by distance travelled, decreased over time in both EPM and OFT. However, the decline was abrupt

in the OFT, whereas in the EPM it was more gradual. Interestingly, this study also found that the EPM was less sensitive than the OFT in detecting anxiety-related differences.

Furthermore, the timing of the test apparatus may play a role. In our test battery, the OFT was conducted first and may reflect initial novelty-induced activity, while the EPM was conducted last, potentially capturing more fatigue- or stress-related stillness. This could explain the higher locomotion we observed in females during OFT. Female rodents are often reported to show increased baseline activity and exploratory behaviour in novel environments, potentially due to hormonal influences and sex-specific coping strategies[90,91]. However, these findings are not uniform across studies and can vary depending on factors such as strain, lighting, and oestrous cycle[90,92,93]. Importantly, while female rodents in normal conditions may display higher baseline activity, ELS have not been reported to consistently lead to hyperactivity in the OFT and EPM[94,95]. Instead, the effects are more evident in anxiety-like behaviour.

Taken together, these results suggest that LBN rearing alters anxiety-like behaviour in a context-dependent manner, with a possible sex difference. In females, increased activity may mask or complicate the interpretation of anxiety-like behaviour in locomotion-based tests such as the OFT and EPM. In males, more frequent stillness may still indicate a passive coping style. These distinct patterns may reflect different trajectories of adaptation to ELS, with heightened activity in females [potentially representing a resilient or adaptive response in certain contexts, and increased immobility in males pointing to greater vulnerability. Further testing will be necessary to disentangle these interactions and improve the interpretation of behavioural outcomes across sex.

3.3 Novel object task: cognitive functions

This study employed the NOR task to probe recognition memory and broader aspects of cognitive function. NOR primarily targets declarative and episodic-like memory, engaging processes like object discrimination, working memory, and visual attention. However, in this study, animals across both groups showed very low engagement with the objects, with total object exploration times overall summed in both tasks 1 and 2 being only around 20 seconds. Rather than exploring the objects, animals spent most of the trial time immobile in the corners of the arena. This lack of exploratory behaviour limited our ability to assess recognition memory and suggest either insufficient motivation, poor task design, or potentially stress-related

avoidance of the open field. The NOR was conducted after OFT, therefore providing one habituation day in the open field arena.

Based on prior literature, exploration criteria of around 30 - 38 seconds have been used to ensure animals are sufficiently exposed to both objects before memory testing begins [96–99]. Additionally, these studies show a long habituation period, 3-5 days of exploring the empty maze. Given that ELS can reduce exploratory drive, as we observed during both NOR sessions, such alterations are particularly relevant when interpreting cognitive performance in ELS models. Therefore, habituating the animals to the environment could enhance object exploration and alleviate environmental anxiety. Furthermore, our object placement was on one side of the maze, yet still relatively central, whereas in those studies, object placement was typically in the corners, where animals tend to spend more time.

In the future, it should be considered that such adjustments to the task be implemented: (1) implement at least 2-5 days of habituation with 5-minute sessions to ensure environment familiarity; (2) apply a minimum object exploration criterion (e.g., 30-38 seconds within 10 minutes) to qualify inclusion; (3) adapt diagonal object placement to standardise spatial layout.

3.4 Social interaction and corticosteroid responses

Social communication and the complex understanding of others are difficult to model in rodents; however, certain aspects can be reliably assessed using the social interaction task. This task primarily targeted the constructs of affiliation and attachment, as well as some forms of social communication, such as approaching and initiating an interaction, or avoiding contact. This task captures social motivation and preference, which, in rodents, can be measured by quantifying the time spent interacting with a familiar versus a novel conspecific.

In our study, LBN animals, particularly LBN females, showed a stronger preference for interacting with a novel conspecific, suggesting heightened novelty seeking or altered attachment behaviour. This pattern is consistent with prior findings showing that ELS can increase sociability. For instance, Bondar et al. reported that MS females spent more time in the interaction zone when a social partner was present[100]. However, their study assessed sociability in the presence of a single novel partner. In contrast, our results indicate a stronger preference for novelty, as reflected in the ratio of time spent with a novel individual versus a familiar one.

In our findings, while LBN males also prefer the novel animal over familiar conspecifics, they exhibited the highest corticosterone response following social interaction with a stranger. This physiological response may reflect elevated arousal or stress reactivity in unfamiliar social contexts, highlighting a potential sex specific impact of ELS on social affiliative processing and HPA axis regulation. Interestingly, this pattern contrasts with findings from other ELS models, such as MS, where no significant differences in corticosterone levels were observed between MS and control males after acute social exposure, suggesting that the effects may be model-dependent[101,102] . We found that baseline corticosteroid levels were not affected by the LBN paradigm.

The SI task in this thesis lacked additional parameters, which could provide more information about the locomotion, approach attempts, and anxiety-like behaviour. The arena where the SI was conducted provides a spacious and naturalistic environment that allows both the stimulus and experimental rats to move freely. While this setup introduces additional complexity in the analysis, since interactions depend on both animals' behaviour, it also offers the opportunity to capture more nuanced social dynamics, such as approach attempts and mutual engagement, which are more reflective of natural social behaviour. Further analysis could explore these aspects in more detail.

In addition to quantifying approach behaviour, our lab is equipped to track ultrasonic vocalisations, which rodents use to communicate emotional states and social intent. This offers a complementary measure of social communication beyond physical interaction and could help dissect the affective quality of social encounters in future.

3.5 Cue-Association task

We encountered some complications in our association task, particularly in identifying the proper protocol that enables the animals to learn, even after the reversal of rules. Our findings indicate that we have developed a protocol through which animals demonstrate learning and adapt to new rules. Moving forward, we can utilise this protocol to examine possible motivation or reward-based behaviours observed in ELS.

Our results highlight the importance of rule stability for successful learning and behavioural adaptation in the cue-association task. Initially, we implemented a rule reversal protocol in which the sound-reward location pairing changed randomly across sessions (RS-task). Under

this condition, animals showed poor performance and appeared unable to form a new strategy. This suggests that while the task engages a mechanism of cognitive flexibility, excessive unpredictability may exceed the animals' capacity for adaptive updating.

In contrast, when the rule reversal was modified to a stable opposite association (OS task), the animals initially showed a bias toward the old rule but adapted over the course of several sessions. This transition reflects their ability to inhibit previously learned responses and update their internal model when a consistent new contingency is provided. These findings support the idea that successful performance in this task depends not only on sensory discrimination and memory but also on the predictability of the rule structure, which facilitates behavioural flexibility and strategy updating.

3.6 Strengths and limitations:

This study is well-aligned with human psychiatric frameworks, as we translate our rodent model findings within emotional and cognitive domains relevant to human disorders. We did not rely on only one test but used different tests to assess various domains. Additionally, we added physiological parameters to link mechanisms beyond behaviour, such as basal and acute corticosteroids.

One of the methodological strengths of this study lies in the use of the LBN model, which offers several advantages over traditional maternal separation protocols. First, maintaining continuous dam-pup contact avoids confounds associated with physical separation, such as hypothermia and nutritional deprivation [87,103]. This setup allows the pups to remain in contact with the dam, simulating scenarios of suboptimal but present caregiving, akin to certain human ELS conditions. Moreover, limiting experimenter intervention during the stress period reduces the risk of additional acute stress, enhancing the ecological validity of the model.

A key strength of the LBN paradigm lies in its ability to model chronic and unpredictable stress through fragmented maternal care, induced by a persistently impoverished environment. This mimics the unstable caregiving observed in neglectful or dysfunctional family settings more accurately than models involving discrete, scheduled stress episodes. Importantly, while dams in the MS model often compensate for their absence through increased nurturing behaviour upon reunion, the dams' behaviour in LBN remains altered throughout the stress period[103,104]. These features make LBN a suitable model for studying the long-term effects of ELS.

Completing this biologically relevant stress model, we additionally employed SLEAP, a deep learning-based tool for precise movement tracking. This approach enabled a more detailed and objective analysis of anxiety-related behaviour compared to traditional manual scoring methods. This allowed us to capture subtle patterns such as locomotion and varying speeds, and it also provides the foundation for analysing more complex behavioural and interaction patterns in these datasets in future work.

While this thesis lacked home-cage recording analysis to reveal more information about the manipulation and dam-pup interactions, we collected weights during development, which confirmed that LBN-reared pups had lower weights during development and before starting the behavioural testing. These findings were consistent with previous findings[102,105,106].

However, several limitations must be acknowledged. First, while group-level differences were observed, stratifying the data by group and sex reduced the sample size considerably. For example, the total number of LBN females across two cohorts was only eight, which may limit the statistical power to detect more subtle effects. More cohorts are currently being analysed; however, they are beyond the scope of this thesis. Additionally, some technical issues, such as disrupted video recordings, required repeating the social interaction task, potentially influencing the results due to increased habituation.

As discussed before, the novel object task failed to capture memory performance differences reliably and will need to be modified in future studies to improve its sensitivity.

Corticosterone levels were obtained under anaesthesia, which may influence hormone concentrations and affect the reliability of the results [107]. Additionally, the oestrous cycle in females was not monitored, which could have influenced outcomes, particularly in corticosteroid levels or in behavioural responses[108].

Finally, while we collected a rich dataset, not all behavioural parameters could be analysed within the scope of this thesis. Choices had to be made regarding which metrics and approaches to prioritise, leaving additional avenues (e.g., more detailed movement patterns during task and different zones) to be explored in future work.

3.7 Future directions

To add to the behavioural outcomes observed in ELS, we aim to incorporate electrophysiological recordings using Neuropixel probes[109]. These recordings facilitate simultaneous high-resolution monitoring of hundreds of neurons across the targeted brain

regions, such as the PFC and hippocampus. This approach will enable us to investigate how activity patterns in key areas are altered during tasks assessing anxiety-like behaviour, cognitive flexibility, or social interaction. Coupling behaviour with real-time neuronal activity will provide valuable insights into how specific neuronal ensembles encode different aspects of behaviour, and whether ELS shifts the balance between emotion-regulation and cognitive-control networks during task performance.

We have collected brain tissues from all animals and plan to conduct immunohistochemical analyses to explore cellular-level alterations. Specifically, we aim to assess microglia and parvalbumin-expressing interneurons, both of which are implicated in neurodevelopmental and stress-related pathology. This will provide insights into structural and neuroinflammatory changes.

In addition to task-based data, we have collected extensive home-cage video recordings, which offer an opportunity to examine maternal and pup behaviours under LBN versus standard cage conditions. Planned analyses include quantifying huddle size, the amount of time the dam spends with pups, and the spatial distribution of the pups within the cage. We hypothesise that LBN conditions may lead to more fragmented maternal care and increased dispersion of pups across the cage. Although these home-cage observations are not included in the current thesis, they provide important contextual information about early caregiving environments that may influence later-life outcomes, and may even allow predictions at the level of the individual.

Another promising direction for future research is the inclusion of ultrasonic vocalisation analysis during a social interaction task. Vocalisations, particularly in the 50 kHz range, are associated with positive social communications and affective states, while 22 kHz calls are typically linked to distress or aversive stimuli [110]. Integrating vocalisation tracking could provide an additional layer of information on the affective and motivational state of the animals beyond observable behaviour. This would be relevant in the context of altered social engagement observed in LBN animals, as it could reveal whether increased approach behaviour toward stranger animals is accompanied by prosocial communication or heightened arousal. Incorporating this modality would enhance the assessment, better aligning it with affiliation, attachment, and social communication as a form of vocalisation construct within the domain of social processes within the RDoC framework.

3.8 Final remarks and significance of the study

This study established a validated rodent model of ELS in our laboratory, providing a foundation for advancing our behavioural assessment approaches. Notably, animals in the stress group exhibited reduced body weight during early development, a finding consistent with previous studies and supporting the effectiveness of the LBN manipulation[105,111,112]. By refining the behavioural test battery to include tasks that engage animals in more complex cognitive and memory-related processes, we enhance the translational relevance of our findings. Importantly, this model enables the identification of behavioural and physiological markers that may serve as potential biomarkers of ELS-related vulnerability. Given that the underlying mechanisms of ELS are still poorly understood and that TRD continues to pose as a major clinical challenge, particularly among individuals with a history of ELS, our work contributes to bridging this gap. Ultimately, this research lays the groundwork for more targeted interventions and better identification of at-risk populations.

4 Materials and methods

4.1 Animals

Long-Evans rats sourced from Janvier Labs (Route du Genest, France) were used in this study. The animals were housed in the animal facility, where litters for cohorts 1 and 2 were bred. Healthy rats were chosen for breeding, with one male paired with two females in a single cage. Regular checks were conducted to determine if the females were pregnant. Once pregnancy was confirmed, the females were moved to individual cages to continue their gestation.

4.1.1 Ethical considerations

All animal procedures were approved by the State Office for Health and Social Affairs (Landesamt für Gesundheit und Soziales, LaGeSo) in Berlin (G0115- 23) and conducted in accordance with the German Animal Welfare Act and the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

4.2 Experimental Design

4.2.1 Limited Bedding and Nesting Paradigm

The limited bedding and nesting paradigm serves as a validated rodent model for studying early-life stress in rodents [65]. In this model, the availability of bedding and nesting materials is intentionally reduced during the preweaning phase.

4.2.2 Group Allocation and Cohorts

The study was divided into two cohorts. Cohort 1 consisted of n=16 rats (8 females, 8 males), and Cohort 2 consisted of n=16 (9 males, 7 females), as seen in Table 7. Cohorts 1 and 2 were initiated at different times. Cohort 3 was subsequently included and contributed exclusively to the data collection from the association task.

When the litter of cohort one was born, the pups were randomly assigned either to the CTRL or the LBN group, with an equal number of pups of each sex in each group. In the LBN group, the bedding of the cage was reduced, and a mesh wire platform was put on top of it (Figure 21). Limited nesting material was provided to the dam. The control group pups and the dam were transferred into a new cage with standard bedding and nesting material provided.

In cohort 2, eight animals were assigned to the LBN group, while the second litter was assigned to the CTRL group.

Figure 21. Picture of the experimental cage setup used in the Limited Bedding and Nesting paradigm. The cage contained a wire mesh floor with minimal bedding material, restricting access to standard nesting materials. *Picture taken by Pinja Hillman*.

Table 7. Overview of the experimental groups and cohorts

Cohort	Group	Male	Female	Total of Animals	Prewean Period	Postwean Period	Start of Behaviour testing

1	CTRL	4	4	8	3 weeks	2 weeks	P50
1	LBN	4	4	8	3 weeks	2 weeks	P50
2	CTRL	4	4	8	3 weeks	2 weeks	P56
2	LBN	5	3	8	3 weeks	2 weeks	P74

4.2.3 Preweaning Period

During the preweaning phase (P2-P9), LBN group pups were housed in LBN cages with the dam to induce a more stressful environment. The preweaning period lasted from P0 to P21, during which pups remained in the cages with their dams.

The control group were housed in a standard cage environment with the dam during the preweaning phase.

4.2.4 Postweaning Period

From P22, rats were weaned, and pos*t*-weaning, all animals were housed in standard cages in groups of 4 to 5 sex-matched littermates until behavioural testing commenced at P50 to P70. A few weeks prior to the behavioural testing, the animals were switched to a reversed 12:12 ligh*t*-dark cycle, with water and food provided ad libitum. Before beginning the association task, the rats underwent food restriction (1 to 4 pellets per rat). All behavioural testing occurred during the dark phase of the cycle, with tests conducted when corticosteroid levels were low (early dark phase).

4.3 Weight Monitoring

The pups' weights were tracked and documented in an Excel spreadsheet. They were weighed weekly from P20 to P120. A growth curve was constructed by plotting the pups' weekly weights for each group (CTRL and LBN) against their respective postnatal days to assess the overall growth pattern across the postweaning period.

For statistical analysis, key time points corresponding to weeks 3, 5, 6, 7, and 16 were selected to compare group differences in body weight. Data are presented as means for each group. The normality of the weight data at each time point was assessed using the Shapiro-Wilk test. Depending on the distribution, either an unpaired two-tailed *t*-test (for normally distributed data) or a *Mann-Whitney U* test (for non-normally distributed data) was applied to compare the CTRL and LBN groups. Statistical significance was set at $p < 0.05$. All analyses were performed using Python (version 3.12) via the Spyder IDE, using libraries listed in Appendix Table A2.

4.4 Behavioural testing

4.4.1 Behavioural Battery Setup and Data Acquisition

This study utilised several behavioural tasks to investigate behaviour, including the Open Field Test, Elevated Plus Maze, Novel Object Recognition, and Social Interaction. Each task was conducted on a separate day within the same week, with every animal undergoing one behavioural test per day.

A FLIR camera (see Appendix, Table A1, for model) was mounted for each task, and video recording was conducted using the Bonsai Workflow Editor (Table A1), an open-source visual programming framework [113]. The video data was then trimmed to the relevant minutes for each task, after which it was transferred to the SLEAP software [114] for further analysis. An overview of the workflow is presented in Figure 22 (Table A1).

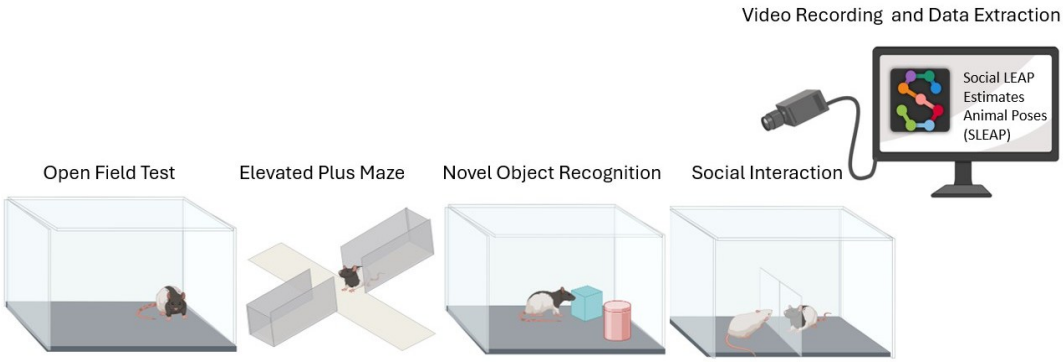

Figure 22. The Behavioural Testing Setup. The behavioural tasks – Open Field Test (OFT), Elevated Plus Maze (EPM), Novel Object Recognition (NOR), and Social Interaction (SI) – are conducted in this sequence. A camera is mounted above the mazes to record video data, which is then analysed using SLEAP [10] for animal pose tracking and maze coordinate extraction. Figure created with BioRender.com

The behavioural tasks were conducted in a dark room with dim red illumination. The tasks were performed during the rats' dark phase (between 9:00 AM and 5:00 PM) to ensure optimal exploration conditions and minimise circadian rhythms' influence on cortisol levels during that phase. Prior to starting the tasks, rats were habituated to the testing room for half an hour, but not to the testing box. The same box was used for the OFT, NOR, and SI, with the addition of a plastic see-through divider equipped with holes during the SI task to allow the rats to smell each other. The behavioural box (100cm x 100cm) consists of light grey flooring with darker grey walls, which stand 50 cm high. During the social interaction, the divider is placed,

providing each rat with a 50 x 100 cm space to explore. The mazes and novel objects were wiped using 70 % ethanol between each animal.

After conducting these behavioural tests, the association task began. This task was carried out in several phases and required months of training for the rats.

4.4.2 Open Field Test (OFT)

In OFT, each animal had a 10-minute session to explore the maze. Tracked data from SLEAP were exported and processed in Python to extract behavioural metrics, including time spent in the centre and the number of entries, locomotion and speed profiles (stillness/sitting, walking pace, and running). These parameters evaluate whether the rat exhibits typical exploratory behaviour or displays fear of an unfamiliar environment by retreating to the corners and avoiding the open centre space.

4.4.3 Elevated Plus Maze (EPM)

In the EPM, each animal had a 5-minute exploration session. The time spent in the open arm compared to the closed arm will be measured, along with the number of entries into each arm. The latency to the first entry was recorded, and the total locomotion and speed profiles. The collected parameters aim to assess the rats' tendencies regarding their drive to explore (curiosity) and fear of open spaces (anxiety).

4.4.4 Novel Object Recognition (NOR)

In NOR, each animal had two 10-minute exploration sessions. In the first session, the animal was placed in the maze, where two similar objects were positioned next to each other on one side. During the second session, one of the objects was replaced with a novel object, which was either placed on the right or left to minimise any spatial bias. The rats had one hour of rest in the homecage between the two sessions.

To assess cognitive function and recognition memory, the time spent exploring a novel object was measured. Increased time spent with the novel object indicates intact recognition memory, whereas reduced exploration suggests potential memory impairments. Data were collected and analysed to evaluate memory performance across experimental conditions.

The Discrimination Index (DI) was calculated to quantify object preference, defined as the difference in exploration time between the novel and familiar object divided by the total exploration time.

DI values were correlated with total distance travelled, expressed as Z-scores, to assess potential confounding effects of general locomotor activity on recognition memory. Additional metrics included total object exploration time and total distance travelled. Speed profiles across two test sessions were also extracted to compare general movement patterns over time.

4.4.5 Social Interaction (SI)

In SI, the animals were tested in two sessions. In the initial session, the experimental animal was positioned in the maze, separated by a divider from its cagemate on the opposite side, allowing them to explore for 30 minutes. After an hour of rest, the experimental animal engaged in a second session with an unfamiliar stimulus animal. This session additionally lasted thirty minutes. Following the interaction with the stranger, a blood sample was collected from the experimental rat's tail vein to assess blood cortisol levels.

In social interaction, interaction time with the cagemate and with a stranger was collected. After the second session of social interaction, blood samples were collected to determine corticosteroid levels.

4.4.5.1 Corticosteroid

Basal and acute blood samples are collected from rats to measure corticosteroid levels following social interaction. The basal samples are taken on a day without behavioural testing, following habituation to the room where the blood is drawn to establish baseline levels for each animal and group. Acute samples are collected immediately after social interaction with a stranger.

The blood samples were centrifuged at 2000 rpm for 20 minutes at room temperature, and plasma was collected and stored at -80 °C until ELISA analysis, which was conducted by a technical assistant.

4.4.6 Cue-Association Task

The association cue task is based on a 50 x 50 cm maze consisting of three nose pokes, one for sound initiation and the other two for rewards (Figure 23). The experiment and tasks were controlled using custom-written scripts in MATLAB and Bpod, a behavioural control system that automated the trial presentation, recorded nose-poke responses, and logged task performance data.

The training was conducted in several phases. First, the animals must learn how to drink from the nose pokes (phase 0). In phase 1, the animals learn to go to the middle poke to activate the reward pokes. In phase 2, they must learn to wait in the centre for 1 second to differentiate the sound and determine which reward to receive. The wrong answer is not punished in this phase, and the animal can correct itself. Finally, in phase 3, the rats are presented with two stimuli and must choose the correct one to receive a reward. In phase 4, referred to as rule reversal, the sound and reward locations are switched from phase 3. The sounds are two distinct sweeps, generated by the Bpod. They begin with the same pitch, and the end of the sound was either high-pitched or low-pitched.

The rat's choices were recorded, including which stimulus was selected and whether the choice was correct. Accuracy and learning rates were analysed to assess associative learning and cognitive flexibility.

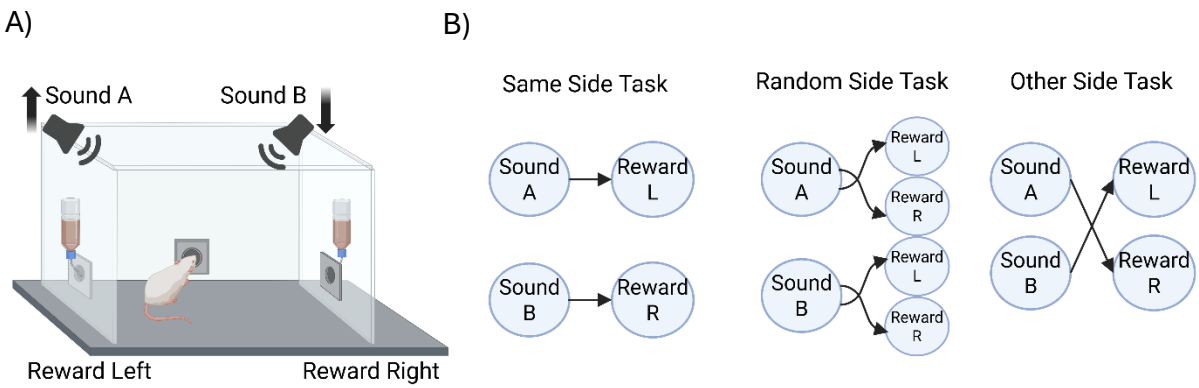

Figure 23. Association Task Maze. A) The maze contains three nose poke ports: one for the sound initiation and two for the reward collection. Speakers are mounted at the top of the maze to deliver two different auditory cues, which guide the animal's response. The task requires the animal to associate specific sounds with the correct reward location, allowing for the assessment of learning and cognitive flexibility. B) Schematic of the three tasks' sound and reward locations. The same-side task consists of one sound corresponding to one location, and the task is carried out until the animal reaches 70% accuracy on three consecutive days. After that, the animal starts with rule reversal, where the sound and reward locations are switched (random-side or other-side).

4.4.7 Video Extraction and SLEAP software

SLEAP was employed as a deep learning-based model for automatic pose estimation to analyse animals' movement during behavioural tasks. This technique tracks body positions without the need for physical markers on the animal. Instead, SLEAP utilises deep learning to identify key

body points based on visual features from the video frames, enabling quantitative analysis of behavioural data.

The behavioural videos were imported into their respective projects (EPM, OFT, NOR and SI) for geometrical and body analysis (Figure 24). First, the videos were manually labelled with frames to define points of interest, including the animal's body (nose, ear left and right, neck, front leg left and right, hind leg left and right, tail start, and tail end) nodes, as well as geometric nodes based on the behavioural task. The geometrical coordinates were necessary to track the maze coordinates to determine the animal's location. These labelled frames were used to train a neural network model within SLEAP, allowing it to predict the positions of these key points across all recorded videos.

Once trained and predictions were accurate, the model was applied to the entire dataset, including all the videos, to extract pose estimations for each frame. The resulting data was extracted (H5 file) and proceeded to statistical analysis.

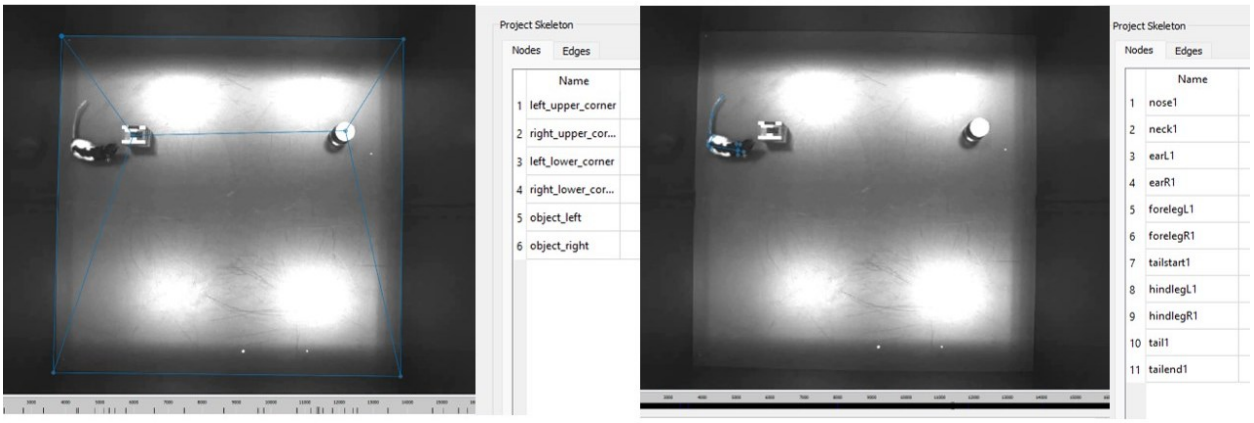

Figure 24. Example of SLEAP project setup for geometric and body key point tracking. The left panel shows the geometrical node configuration used to define relevant coordinates in the behavioural arena (e.g., corners and object locations) in the novel object task. The right panel illustrates the body part tracking nodes placed on the animal (e.g., nose, ears, limbs, tail). These nodes were manually labelled in selected frames and used to train a neural network model in SLEAP, which then predicted the positions of these points across the entire video dataset.

4.5 Statistical Analysis /Data Analysis

4.5.1 Data Analysis Environment and Libraries

All behavioural battery analyses (EPM, OFT, NOR, and SI) were conducted and figures were created using Python (3.12) in the Spyder IDE. The cue association task data was analysed using

Jupyter Notebook (version 7.2.2), an interactive Python environment. Data preprocessing, statistical tests, and visualisations were carried out using the pandas, scipy, matplotlib, seaborn, and statsmodels libraries.

4.5.2 Data Handling and Normalisation

The behavioural data extracted from SLEAP provides separate files for animal and maze coordinates. These files are combined into a single file containing both sets of data, which is then converted into an Excel CSV file using Python, incorporating libraries such as pandas, numpy, h5py, and scipy.interpolate (Appendix, Table A2).

4.5.3 Statistical tests

All the data were tested for normality using the Shapiro-Wilk test. Depending on the distribution, either parametric (independen*t*-samples *t*-test) or non-parametric tests (*Mann-Whitney U*) were applied to compare groups (CTRL vs. LBN). One-way ANOVA was used to evaluate the combined group x sex effects. Where applicable, pos*t*-hoc comparisons (e.g., CTRL males vs. LBN males) were conducted using appropriate two-group comparisons (*t*-test or *Mann-Whitney U*).

Z-Score Standardisation

For correlation and composite index analyses, variables were standardised by calculating Z-scores, using the formula:

$$Z = \frac{(X - \mu)}{\sigma}$$

Where X is the individual value, μ is the group mean, and σ is the standard deviation.

Anxiety Index Calculation

An anxiety Index was created by averaging Z-scores from selected EPM and OFT parameters:

EPM: time in open arms, number of open arm entries, and immobility time

OFT: time in centre, number of centre entries and immobility time.

Before averaging, parameters were reversed if needed (e.g., open arm time was reversed since a lower time suggests more anxiety). This ensured that higher Anxiety Index values always indicated more anxiety-like behaviour.

Cross-correlations between similar EPM and OFT parameters were performed using Spearman's rank correlation.

NOR

Discrimination index (DI) was calculated as:

$$DI = \frac{(T_{novel} - T_{familiar})}{(T_{novel} + T_{familiar})}$$

Where *T* indicates time spent exploring each object.

SI

A social novelty preference index (SNPI) was computed based on a previous study[115], as

$$SNPI = \frac{(T_{stranger} - T_{cagemate})}{(T_{stranger} + T_{cagemate})}$$

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6 Abbreviations list

ACTH	Adrenocorticotropic Hormone
BDNF	Brain-Derived Neurotrophic Factor
CTRL	Control
CRH	Corticotropin-Releasing Hormone
CA	Cornu Ammonis
CAT	Cue Association Task
DG	Dentate Gyrus
DI	Discrimination Index
ELS	Early Life Stress
EPM	Elevated Plus Maze
EU	European Union
GR	Glucocorticoid Receptor
HPA	Hypothalamic-Pituitary-Adrenal Axis
LBN	Limited Bedding and Nesting
LTP	Long-term potential
MR	Mineralocorticoid Receptor
MS	Maternal Separation
MRI	Magnetic Resonance Imaging
NOR	Novel Object Recognition
OFT	Open Field Test
OS	Other Side

PFC	Prefrontal cortex
RDoC	Research Domain Criteria
RS	Random Side
SI	Social Interaction
SHRP	Stress Hyporesponsive Period
SD	Standard Deviation
SNPI	Social Novelty Preference Index
SS	Same Side
SLEAP	Social LEAP
TRD	Treatment-Resistant Depression

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8 Appendices

Appendix Table A1. Materials and software used in the study

Material	Item/ software	Model/Version	Notes
Recording hardware	FLIR Chameleon3	CM3-U3-13Y3M	Firmware: 1.13.3.00
Recording software	Bonsai Workflow	Bonsai.Editor 2.8.1	Used to record behavioural data
Video tracking/ animal pose estimation	SLEAP	1.3.3	
Behavioural control	Bpod State Machine	r2.5	Controlled via MATLAB
	Bpod HiFi Module	HD	
Control interface	MATLAB	R2022a	Used to run Bpod workflows
Data analysis	Spyder	5.5.1	
	Jupyter Notebook	7.2.2	Cue-association task

Appendix Table A2. Libraries

Library	Purpose / Function Used For
pandas	Data handling, merging, and cleaning dataset
Numpy	Numerical operations
Os	File handling and system path navigation
H5py	Reading HDF5 format files from SLEAP
Scipy.interpolate.interp1d	Interpolation of coordinate data during the merging of the maze and body points
Scipy.stats.zscore	Standardisation of behavioural variables (Z-score calculation)
Scipy.stats.mannwhitneyu	Non-parametric Mann-Whitney U tests for group comparisons
Scipy.stats.shapiro	Shapiro-Wilk test for normality testing
Scipy.stats.pearsonr	Pearson correlation coefficient for linear associations between behavioural measures
Matplotlib.pyplot	Basic visualisations

Seaborn	Enhanced statistical visualisations with built-in aesthetics
Statannotations.Annotator	Adding statistical annotations to Seaborn plots
Itertool	Used for handling combinations during grouped comparisons or iteration logic