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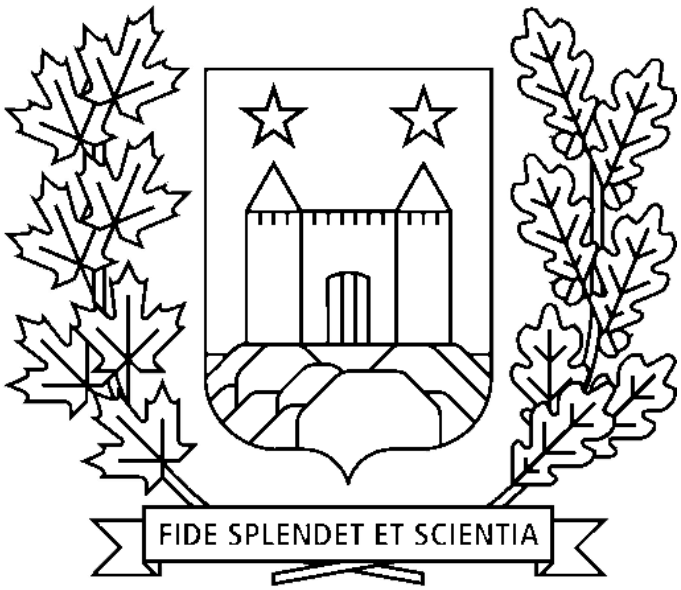

The Role of Astrocytes in the Effects of Early-Life Stress on Lateral Amygdala-Dependent Behaviour

By Ifeoluwa Adedipe

Department of Neurosciences

Faculty of Medicine

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This brief entitled

The Role of Astrocytes in the Effects of Early-Life Stress on Lateral Amygdala-Dependent Behaviour

Presented by

Ifeoluwa Adedipe

Was evaluated by a jury composed of the following persons:

Dr Ravi Rungta

Chairperson-Rapporteur

Dr Caroline Menard

Jury Member

Dr Ciaran Murphy-Royal

Research Director

Summary

Early Life Stress (ELS) is associated with an enhanced susceptibility to the development of stress-related disorders, such as major depressive disorder (MDD). The lateral amygdala (LA), a brain region important for the regulation of emotive and cognitive behaviours is vulnerable to the effects of ELS. However, the mechanisms by which ELS impairs behaviour are poorly defined. Previously, research has focused on the neuronal mechanisms underlying stress-induced behavioural impairments, however the role of glial cells in this circuitry remains undetermined. Astrocytes, a type of glial cell, are key determinants of behaviour. Hence, we aimed to identify the role of astrocytes in the effects of ELS on LA-dependent behaviour. To accomplish this, we used a rodent model of maternal separation and limited bedding and nesting to replicate the effects of ELS on the developing brain by assessing its long-term effects on astrocytes and lateral-amygdala dependent behaviour. Although ELS did not influence anxiety-like behaviour in mice, ELS significantly impaired threat-detection, a cognitive process involving the ability to accurately distinguish between a previously learned threatening tone (the conditioned stimulus) and a non-threatening tone in a novel context. Additionally, decreasing astrocyte stress sensitivity by deleting astrocyte glucocorticoid receptors significantly enhanced cognitive function in both ELS and naïve mice. Overall, our results suggest that astrocytes are pivotal in the regulation of the effects of ELS on cognitive impairment. This data highlights the importance of astrocytes as potential therapeutic targets for mitigating cognitive dysfunction, a pervasive symptom of psychopathology.

Key words: ELS, Psychopathology, Astrocytes, Amygdala, Lateral Amygdala, Behaviour, Fear Learning and Memory, Cognition, Auditory Discriminative Fear Conditioning, Glucocorticoids, Glucocorticoid receptors

Résumé

Le stress en début de vie (ELS) est associé à une susceptibilité accrue au développement de troubles liés au stress, tels que le trouble dépressif majeur (TDM). L'amygdale latérale (AL), une région du cerveau importante pour la régulation des comportements émotionnels et cognitifs, est vulnérable aux effets du ELS. Cependant, les mécanismes par lesquels l'ELS altère le comportement ne sont pas très bien définis. Auparavant, de nombreuses études se sont concentrées sur les mécanismes neuronaux qui sous-tendent les troubles comportementaux induits par le stress, mais le rôle des cellules gliales dans ce circuit reste indéterminé. Pourtant, les astrocytes, un type de cellule gliale, sont des déterminants clés du comportement. Nous avons donc cherché à identifier le rôle des astrocytes dans les effets de l'ELS sur le comportement dépendant de l'AL. Pour ce faire, nous avons utilisé un modèle de rongeur avec séparation maternelle, limitation de la litière et de la nidification pour reproduire les effets de l'ELS sur le cerveau en développement afin d'évaluer ses effets à long terme sur les astrocytes et le comportement dépendant de l'amygdale latérale. Bien que l'ELS n'ait pas eu d'influence sur le comportement anxieux des souris, ce dernier a altéré de manière significative la détection des menaces, un processus cognitif qui implique la capacité de distinguer avec précision un son menaçant précédemment appris (le stimulus conditionné) d'un son non menaçant dans un contexte nouveau. De plus, la diminution de la sensibilité au stress des astrocytes par la suppression des récepteurs glucocorticoïdes astrocytaires a amélioré de manière significative la fonction cognitive chez les souris ELS et naïves. Globalement, nos résultats suggèrent que les astrocytes jouent un rôle central dans la régulation des effets de l'ELS sur les troubles cognitifs. Ces données soulignent l'importance des astrocytes comme cibles thérapeutiques potentielles pour atténuer le dysfonctionnement cognitif, un symptôme omniprésent de la psychopathologie.

Mots clés : ELS, Psychopathologie, Astrocytes, Amygdale, Amygdale latérale, Comportement, Apprentissage et mémoire de la peur, Cognition, Conditionnement discriminatif de la peur auditive, Glucocorticoïdes, Récepteurs des glucocorticoïdes

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List of Abbreviations

ACE	Adverse Childhood Experience
ADFC	Auditory Discriminative Fear Conditioning
BA	Basal Amygdala
BLA	Basolateral Amygdala
CA	Childhood Adversity
CE	Central Amygdala
CEl	Central lateral Amygdala
CORT	Corticosterone/Cortisol
CRF	Corticotropin Releasing Factor
CS+	Conditioned Stimulus
CS-	Neutral tone
DI	Discrimination Index
ELISA	Enzyme-linked immunosorbent assay
ELS	Early Life Stress
EPM	Elevated Plus Maze
GABA	Gamma Aminobutyric Acid
GFAP	Glial Fibrillary Acidic Protein
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Responsive Element
HPA	Hypothalamic Pituitary Adrenal
IN	Interneurons

LA	Lateral Amygdala
LTM	Long-Term Memory
LTP	Long-term Potentiation
MDD	Major Depressive Disorder
MFF	Maladaptive Family Functioning
MR	Mineralocorticoid Receptor
OFT	Open Field Task
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFC	Prefrontal Cortex
PND	Post Natal Day
RM	Repeated-Measure
SDR	Steroid Displacement Reagent
SHRP	Stress Hyporesponsive Period
US	Unconditioned Stimulus

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"You can spend a lifetime healing from a few moments of your childhood"

- Anonymous

Chapter 1
Introduction

1.1 Early-Life Stress (ELS) has long-term consequences for health outcomes

ELS comprises any instance of extreme or chronic stress during childhood that has detrimental impacts on one's quality of life (Smith & Pollak, 2020). Indeed, a traumatic moment or period experienced early in life can have severe and potentially fatal consequences for future health outcomes. There is an ongoing mental-health pandemic where an unprecedented proportion of the global population is experiencing impaired mental health or suffering from stress-related disorders such as major depression disorder (MDD) which is associated with debilitating symptomology such as cognitive dysfunction (Perini et al., 2019). In Canada, one out of five Canadians experience a form of mental illness (Ahmad et al., 2015). However, how risk factors, such as ELS, contribute to an increased risk and incidence of mental health illness remains unclear.

ELS is a well-established determinant of psychopathology in adulthood (Heim et al., 2000; Syed & Nemeroff, 2017). Globally, experiences of ELS account for 29.8% of all psychiatric disorders based on a World Health Organisation survey (van Duin et al., 2019). Substantial evidence indicates a significant association between early life traumatic experiences and the development of psychopathologies later in life (Gunnar & Vazquez, 2015; Radtke et al., 2015; Sheth et al., 2017; Teicher & Samson, 2013). Evidently, adults who have experienced an early life stressor are more likely to report suffering from a mental health condition, later in life (Gunnar & Vazquez, 2015). Unfortunately, having an occurrence of ELS is uncomfortably common, with two-thirds of the Canadian population having reported at least one adverse childhood experience (ACE) (Ontario Agency for Health Protection and Promotion (Public Health Ontario), 2020). A high incidence of ELS experiences, therefore, has severe consequences for the health of current and future adult populations. However, despite the evidenced association between ELS and mental disorder development, the mechanisms underlying this relation remain unclear. To mitigate the impact of ELS on the mental health of the global community, it is important to better understand the relationship between ELS and adult psychiatric disorder development and the mechanisms that subserve this association.

1.1.1 What is Early-Life Stress?

Until recently, ELS was a risk factor in need of a definition. Although, there had been an increased interest in the relationship between ELS and psychopathology earlier on, there had been inconsistencies in literature regarding how to measure and define ELS as a construct (Mclaughlin, 2018). These inconsistencies are evident even regarding the terminology used for describing stressful experiences earlier in life where terms such as childhood adversity, adverse childhood experiences (ACEs), and household dysfunction, were and are currently used in tandem with ELS (Mclaughlin, 2018). However, there was still ambiguity surrounding what ELS referred to and how it differed from normative childhood stressors.

Normative childhood stress is defined as an ongoing adaptation to changing environmental conditions over time (Monroe, 2008). This definition of stress is comprised of three components: 1) the environmental conditions that bring forth adaptation 2) the organism's stress response, defined as psychological and neurobiological responses underlying these adaptations 3) the adaptation from the continuous interaction between the organism and the changes in its environment (Monroe, 2008). There are several types of stressors. Distinctions between these stressors have been based on 1) temporal characteristics (i.e the age at which ELS was experience), 2) stressor severity, 3) the involvement of the child in the occurrence of their stressor and lastly 4) the source of the stress.

To better define and distinguish ELS from environmentally normative stressors, investigations regarding exposure to stressors during childhood and the development of psychopathology assessed a wide array of stressors specifically concerning their predictive ability of psychiatric disorder development (Mclaughlin, 2018). Previous studies had merely focused on investigating a specific type of stressor such as parental divorce (Amato & Keith, 1991), sexual abuse (Molnar et al., 2001), or poverty (Duncan et al., 1994). The more recent understanding of ELS was birthed from a study of ACEs (Felitti et al., 1998), which was one of the first to investigate several types of adversity to determine the validity of ELS as a crucial determinant of physical and mental health in adulthood (Anda et al., 2006; Felitti et al., 1998). The psychopathological relevance of ELS was established by this study as it found co-occurrences between different types of ELS, including child abuse and neglect, as well as prominent correlations between the exposure to these stressors and adult health outcomes (Felitti

et al., 1998). Despite the findings from this study, an official definition of "ELS" was still not established (Mclaughlin, 2018).

A well-accepted definition by McLaughin (2016) coagulates the characterisation of normal life stress as described by Monroe (2008) with models of experience-dependent brain development (Baumrind, 1993; Fox et al., 2010; McLaughlin, 2016; Monroe, 2008). McLaughin (2016) defines ELS as a set of environmental conditions that are either serious or persistent over time and require substantial adaptation from a child (McLaughlin, 2016). Additionally, these set of environmental conditions, such as sensory, perceptual, and social experiences, reflect deviations from the norm, encompassing either an absence of the expected environment or the presence of unexpected environmental conditions (McLaughlin, 2016). The deviations that ELS represent, can either be chronic or a once-off occurrence that is severe enough to differ from an environment that could be expected. Overall, this characterisation comprises an operational definition of ELS as an exposure to severe and unexpected environmental conditions that necessitates significant social, psychological, and neurobiological adaptations by the afflicted child (McLaughlin, 2016).

1.1.2 ELS and psychopathology

ELS is defined by its predictive ability of psychopathology. Indeed, according to Freud's psychoanalytical theory, having a stressful experience early in life constitutes a key determinant of psychiatric disorder development in adulthood (Nemeroff, 1997). Research supporting the link between ELS and psychopathology is substantive.

There have been several epidemiological studies that have produced correlating and consistent evidence supporting the powerful association between ELS and the risk of psychiatric disorder development later in life (Mclaughlin, 2018). **Epidemiological studies** have provided considerable evidence in support of the meaningful link between ELS and adult psychiatric disorder development and associated cognitive deficits (Perini et al., 2019). An epidemiological study conducted by Green et al. (2010) examined the joint associations between twelve retrospectively reported instances of childhood adversity (CA) and the initial onset of mental disorders in a sample of 9282 adults (Green et al., 2010). Mental disorders included anxiety, mood and substance disorders as described by the fourth edition of the Diagnostic and Statistical

Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 1994). This study utilised a complex multivariate model that accounted for the type of CA; the number of CAs reported that fell under the maladaptive family functioning (MFF) cluster such as: parental mental illness, physical abuse, sexual abuse, and neglect; and the number of non-MFF CAs reported. The results of this study revealed that the CAs have a powerful predictive ability of the onset of mental disorders throughout the lifespan. Additionally, a study conducted by Dunn et al. (2013) assessed whether the age of exposure to maltreatment is related to an increased risk of depression and suicidal ideation in early adulthood (Dunn et al., 2013). Drawing from the National Longitudinal Study of Adolescent Health of 15,701 samples, this study concluded that indeed, an exposure to maltreatment during development influences one's risk of mental disorder development later in life (Dunn et al., 2013).

1.2. Potential physiological systems underlying ELS-induced psychopathology

The impact of ELS has been associated with severe neurobiological consequences that underlie an increased susceptibility to psychiatric disorder development in adults. However, the mechanisms underlying this relation remain ill-determined (Lippard & Nemeroff, 2020). Over the past 20 years, research has begun to investigate the mechanisms that underlie the association between an early-life exposure to traumatic events and the development of psychopathologies in adulthood. (Lippard & Nemeroff, 2020).

Rodent studies have proved to be a powerful and translational tool when investigating the mechanisms that underlie ELS and psychopathology (Walker et al., 2017). Rodent models provide an avenue to examine cellular and molecular mechanisms and test therapeutic treatments that cannot be explored in human studies. A study by Walker et al. (2017) revealed that rodent models of ELS have proved vital to documenting behavioural dysregulation and an increased risk of psychopathology development. Rodent models of stress that have particularly proved useful are those of maternal separation, where rodent neonates are separated from their mothers (Lupien et al., 2009) as well as rodent models of limited bedding material where the amount of nesting material in the home cage of the dam is reduced (Walker et al., 2017). These models have been shown to produce severe behavioural impairments, including cognitive impairments (Huot et al., 2004; Lupien et al., 2009; Walker et al., 2017).

Research has elucidated several potential mechanisms through which ELS may elevate the risk for stress-related disorder development. These mechanisms include disturbances in the inflammatory response system as well as alterations in genetic and epigenetic processes (Hueston & Deak, 2014) (**Figure 1.1**). However, a crucial stress response system that has been persistently implicated in ELS-dependent behavioural impairment, is the HPA axis and, more specifically, its dysregulation (Finsterwald & Alberini, 2014; Lippard & Nemeroff, 2020). Indeed, the drastic influence of ELS on behaviour has been associated with impaired HPA-axis functioning (Tarullo & Gunnar, 2006).

1.2.1 The HPA Axis

The HPA axis is a feedback-regulated neuroendocrine system that forms a key component of the body's response to stress. However, this crucial system been implicated in the pathophysiological development of stress-related disorders (Keller et al., 2016).

Typically**,** the activity of the HPA axis is regulated by the release of corticotropin releasing factor (CRF) from the hypothalamus, following a stressful event, which initiates the secretion of corticotropin from the pituitary gland which lastly causes the release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal cortex (Keller et al., 2016; Pariante & Lightman, 2008). The glucocorticoids then bind to receptors in several target cells and tissues to impact functioning in the peripheral and central nervous system and the rest of the body. Glucocorticoid signalling in the brain is accomplished via the activity of mainly two types of receptors, the higher affinity mineralocorticoid receptors (MRs) which are predominantly expressed in the hippocampus and the lower affinity glucocorticoid receptors (GRs) that are more widely distributed throughout the brain (Keller et al., 2016). At its basal level, cortisol/corticosterone (CORT) enacts its effects via MR, while the feedback activity of CORT on the pituitary and activated areas of the brain, like the amygdala, are navigated by GR activity (de Kloet et al., 1999; de Kloet & Reul, 1987). GRs are ligand-bound transcription factors that bind to specific DNA sequences that activate or suppress gene expression relating to regulating the body's stress response (Saklatvala, 2002). Due to the importance of the HPA axis in the body's stress response, it has been hypothesised that the pathophysiology of mood disorders could reflect a dysregulation of the HPA axis and (Holsboer, 2000).

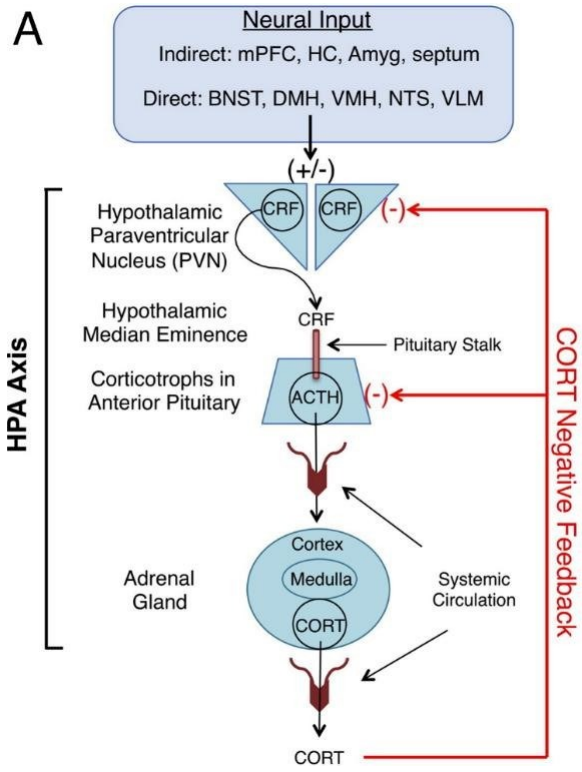

Figure 1.1. The HPA axis. An illustrative diagram of the neural inputs, components, and CORT feedback of the HPA axis. The HPA axis receives direct and indirect neural inputs from distinct regions in the brain which activate or inhibit the HPA axis. Typically, the release of CORT from the HPA axis has an inhibitory effect on the HPA axis through negative feedback where an increase in CORT results in an inhibition of HPA axis activity. Abbreviations: mPFC – medial Prefrontal Cortex; HC – hippocampus; Amyg – amygdala; BNST- bed nucleus of the stria terminalis; DMH - dorsomedial hypothalamic nucleus; VMH - ventromedial hypothalamus; NTS - nucleus tractus solitarius; VLM – ventrolateral medulla; CRF - corticotropin releasing factor; ACTH - adrenocorticotropic hormone; CORT - corticosterone (Spencer & Deak, 2017)

1.3 Childhood Brain Development is sensitive to HPA axis dysregulation

Human have shown that the brain is especially vulnerable to the impact of HPA axis dysregulation during childhood (Radtke et al., 2015; Zajkowska et al., 2022). Chronic elevations in CORT, a consequence of HPA axis dysregulation, modify the way with which the maturing brain circuits interpret external threats and future stress responses, thus contributing to a heightened vulnerability to stress and an elevated risk of stress-related disorder development (Sheth et al., 2017; Tarullo & Gunnar, 2006). The sensitivity of the young brain to HPA axis dysfunction is due to a combination of reasons. Firstly, the HPA axis and its ability to regulate the body's stress response is immature and secondly, the developing brain is highly malleable to experience and particularly, stressful experiences (Tarullo & Gunnar, 2006). Additionally, the immature HPA axis is heavily regulated by social factors such as maternal care and presence, thus maternal neglect, a type of ELS, can have drastic impacts of HPA axis function (Tarullo & Gunnar, 2006). Cumulatively, the response that the immature HPA axis produces in an aversive context, will have long-term consequences on how the brain will respond to stress in the future.

1.3.1 ELS-induced HPA axis dysregulation has consequences for neurological functioning and behaviour

Chronic or severe stress is known to cause perturbations in normal HPA axis functioning and regulation, especially in the developing brain, having consequences for neural function and behaviour (Eliwa et al., 2021; Gunasekaran et al., 2021; Tooley et al., 2021). Previous research has suggested that the detrimental impacts of ELS are linked to the impaired integrity of the HPA axis and its associated receptors and hormones. A study on humans by Radtke et. al (2015) confirms this finding (Radtke et al., 2015). In this study, it was found that DNA-methylations in genes of the HPA-axis, specifically GR-related genes, are associated with an increased vulnerability to the development of psychiatric disorders following instances of ELS (Radtke et al., 2015). Additionally, pathological increases in CORT result in the continuous occupation of GRs which is associated with impaired neural functioning such as reduced synaptic plasticity and impairments in neural circuitry underlying behaviours such as emotion and cognition. Hence, HPA axis dysregulation has severe consequences for neural integrity and healthy behavioural expression (Tarullo & Gunnar, 2006).

1.4 The neurobiology underlying emotive and cognitive behaviours are particularly vulnerable to ELS

ELS has been associated with alterations in the neural circuitry underlying the regulation of emotion and cognitive behaviours (Chen & Baram, 2015; de Kloet et al., 1999; Keller et al., 2016; M. D. Mu et al., 2020; Shackman et al., 2015; Wan et al., 2022). During the early postnatal period, the brain experiences significant structural changes, including changes associated with the development of the neural circuits that underlie behaviour (Krugers & Joël, 2014). As such, external factors, including ELS, have substantial consequences for the development of these neural systems, having long-term consequences for behaviour (Krugers & Joël, 2014). During development, particularly in the early postnatal period, human individuals are highly dependent on extrinsic factors (i.e maternal care) for normal behavioural expression, especially cognitive development (Baram et al., 2012; Hackman et al., 2010). Emotional behaviours are also sensitive to experiences occurring during the early postnatal period where sever childhood maltreatment has been shown to have ramifications for behavioural and emotional functioning that are associated with structural alterations in the brain as seen in MRI (Baram et al., 2012; Guadagno et al., 2021; Krugers & Joël, 2014; van Harmelen et al., 2010). Overall, cognitive, and emotional behaviours are vulnerable to traumatic events experienced during early childhood, providing an elevated risk for the development of psychiatric disorders characterised by cognitive and emotive behaviour dysregulation (**Figure 1.2**) (Guadagno et al., 2021).

1.4.1 The Prefrontal Cortex (PFC), Hippocampus and Amygdala are vulnerable to ELS

The PFC, hippocampus, and amygdala are important in the regulation of emotive and cognitive behaviours and evidently, are sensitive to ELS. Structurally, ELS has been correlated with reductions in both PFC and hippocampal volumes (Carballedo et al., 2013; Rao et al., 2010; van Harmelen et al., 2010) and increases in amygdala volume (Lupien et al., 2011). Functionally, ACEs have been correlated with alterations in PFC and amygdala activity particularly during the regulation of negative emotive behaviours (Cohen et al., 2013; Gee et al., 2013; P. Kim et al., 2013). Additionally, ELS has been associated with modifications in hippocampal- and amygdala-related resting state connectivity. As such, the influence of ELS on these brain regions, may provide insight into the development of emotive and cognitive behavioural dysfunction as seen in adult individuals who have experienced ELS.

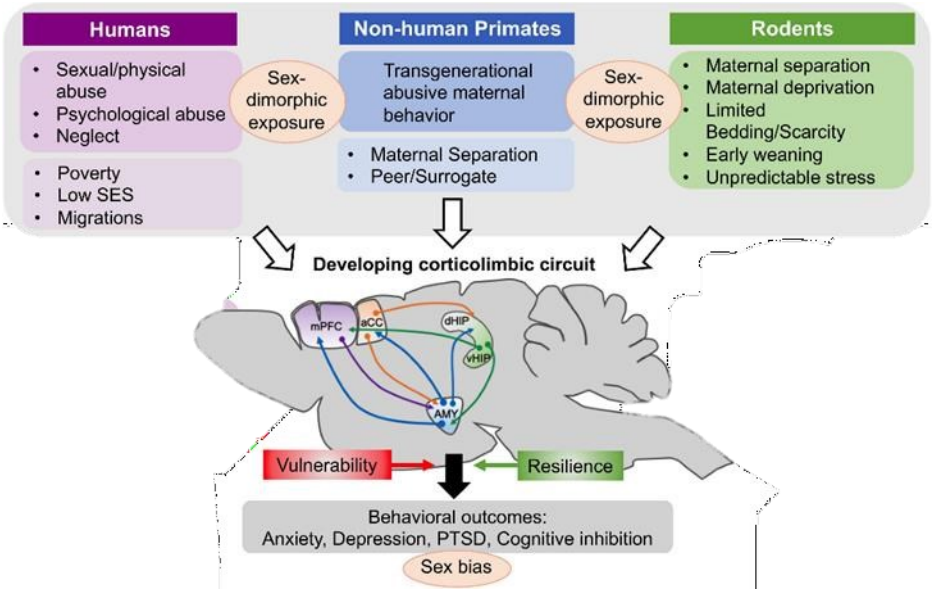

Figure 1.2. ELS, the corticolimbic circuit and behavioural outcomes. An illustrative diagram of the influence of different types of ELS in human and non-human primates and rodents on the developing corticolimbic circuit and corresponding behavioural outcomes (Guadagno et al., 2021)

1.5 The amygdala and its role in fear learning and memory

Central to affective and cognitive processing, is the amygdala (Adolphs, 2013), a complex structure that sits in the temporal lobe (J. LeDoux, 2007). This fascinating region of the brain is most well known for its role in the processing of emotive behaviours such as fear and anxiety. However, the amygdala, in both humans and rodents, also has a role in the regulation of cognitive behaviours such as learning and memory (Pessoa, 2010; Schaefer & Gray, 2007).

The amygdala is particularly important for the regulation of fear learning and memory (Johansen et al., 2011a) and plays a pivotal role in the acquisition and consolidation of fear-associated memories (Chau & Galvez, 2012). The famous case report of patient SM, describes a woman who had bilateral amygdala lesions who was unable to express fear, hence revealing the

necessary role of the amygdala in fear-related behaviours (Feinstein et al., 2011). Studies investigating the relevance of the amygdala in fear learning and memory have found that lesions in the amygdala cause a disruption of the expression of fear responses such as active avoidance, passive avoidance and freezing to conditioned stimuli (Kazama et al., 2012; J. LeDoux, 1998). Additionally, the activation of the amygdala was found to modulate the acquisition and consolidation of affective memories such as fear (J. LeDoux, 2007). Overall, there is substantive evidence that the amygdala is crucial in the regulation of fear learning and memory.

Behavioural paradigms that assess associative learning in rodents have been most beneficial in solidifying the role of the amygdala in associative fear learning. The behavioural protocol in rodents that is mostly utilised for studying the role of the amygdala in fear learning and memory is Pavlovian Fear Conditioning (J. E. LeDoux, 2007; J. E. LeDoux, 2003). This paradigm has been useful for investigating the molecular mechanisms that underlie learning and memory as defensive responses to specific environmental stimuli such as freezing are well-defined and easily assessed (Johansen et al., 2011). In this paradigm, rodents learn to form an association between an initially neutral stimulus (i.e. a tone), which is the conditioned stimulus (CS) and an innately emotionally salient, aversive stimulus (i.e. an electric foot shock), the unconditioned stimulus (US) (Johansen et al., 2011a). Fear Conditioning consists of **acquisition:** the introduction of new information to the brain, **consolidation:** the period following acquisition where the memory becomes stable and **recall:** the ability to access the memory that was previously stored. The assessment of fear conditioning includes the measurement of conditioned responses (i.e time spent freezing) elicited by the CS independent from the US during the testing of memory recall phase.

The amygdala is comprised of several interconnected nuclei. These nuclei include: the basolateral complex of the amygdala (BLA) which comprises of the lateral (LA) and basal (BA) nuclei and the central amygdala (CE) that consists of lateral (CEl) and medial (CEm) divisions (**Figure 1.3**) (Meis et al., 2020). Together, the amygdala nuclei comprise part of the neural circuitry underlying fear (Chau & Galvez, 2012; Janak & Tye, 2015; J. LeDoux, 2007).

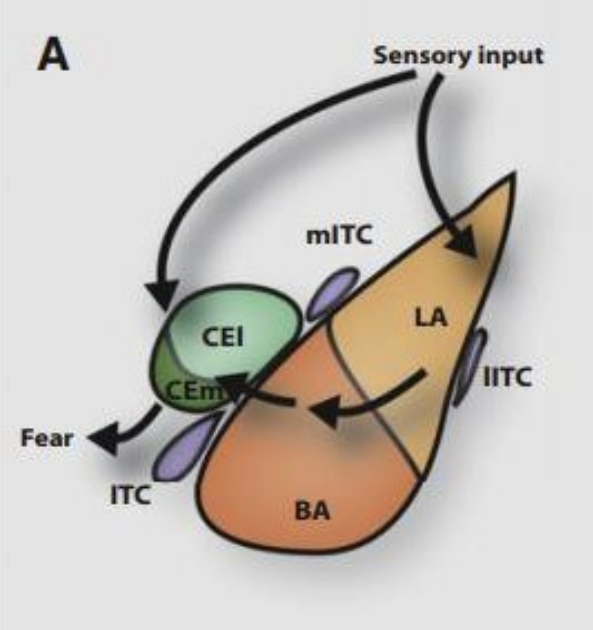

Figure 1.3. The Amygdala nuclei and the fear circuit. An illustrative diagram of the nuclei of the Amygdala and the flow of sensory information between the amygdala nuclei resulting in the expression of fear behaviour (Adolphs, 2013)**.**

1.5.1 The LA is central to fear learning and memory

The LA is an integral component in the neural circuitry underlying fear conditioning. The LA resembles the sensory cortex in that sensory information regarding CS and US from thalamus and cortical afferents converge in the LA (Bordi & LeDoux, 1994). The LA then projects to the rest of the amygdala nuclei and external regions such as the hypothalamus, to regulate the expression of conditioned fear behaviours (Ehrlich et al., 2009). The LA is particularly involved in auditory discriminative fear conditioning (ADFC). A study by Grosso et. al (2018) demonstrates that a subset of excitatory and inhibitory neurons in the LA are essential for the regulation of discriminative ability, a cognitive process enabling individuals to distinguish between threatening and non-threatening stimuli (Erlich et al., 2012; Grosso et al., 2018). Indeed, synaptic plasticity in the LA has been implicated in auditory discriminative fear learning, specifically, the strengthening on synapses in the LA has been linked to the strength of association made between the CS (i.e the tone) and the aversive US (i.e an electric footshock)

(Ehrlich et al., 2009; Erlich et al., 2012; Johansen et al., 2011)**.** Additionally, research has shown that inhibiting the LA via antagonists of NMDA receptors supresses fear conditioning acquisition (Maren et al., 1996; Bauer et al., 2002**)** and preferentially blocking subunits of NMDAR disrupts fear learning without hindering the consolidation of fear-related memories (Ehrlich et al., 2009; Erlich et al., 2012).

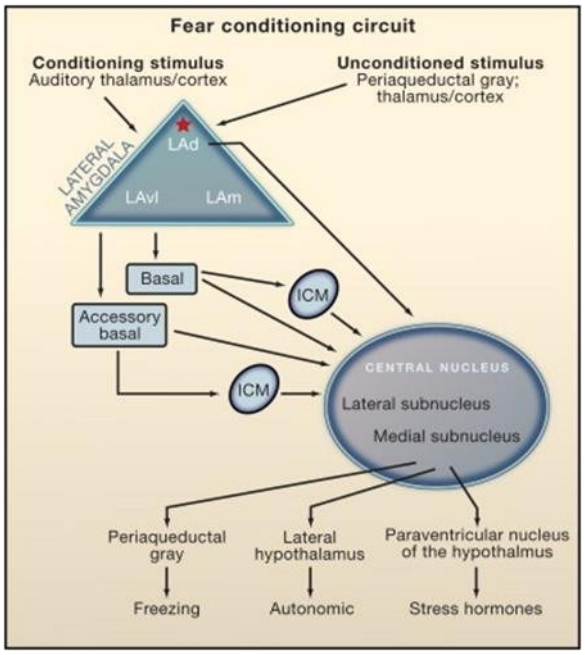

Figure 1.4. The Fear Conditioning Circuit. An illustrative diagram of the fear conditioning circuit depicting the convergence of auditory conditioned stimulus and the aversive conditioned stimulus onto the LA which then projects to the rest of the amygdala nuclei and associated regions. This then produces physiological and behavioural responses relating to the expression of fear (Johansen et al., 2011)

1.5.1.2 GABAergic signaling modulates LA excitability

Long-term potentiation (LTP) at thalamic and cortical afferents onto the LA is tightly regulated by GABA, an inhibitory neurotransmitter (Janak & Tye, 2015). GABAergic interneurons (INs) mediate the excitability of the LA and serve as targets of neuromodulation to regulate LTP at both thalamic and cortical afferents onto the LA as established by glutamatergic activity (Johansen et al., 2011b; Y. Yang & Wang, 2017). Modulation of inhibitory activity of

these interneurons, essentially gates the excitability of the LA and consequently, the expression of fear responses (Ehrlich et al., 2009). However, LA is stress sensitive. Particularly, in situations of ELS, GABAergic modulation of the LA is impaired resulting in LA hyperexcitability (Jie et al., 2018). However, consequences of ELS-induced LA hyperexcitability on LA-dependent behaviours are undetermined.

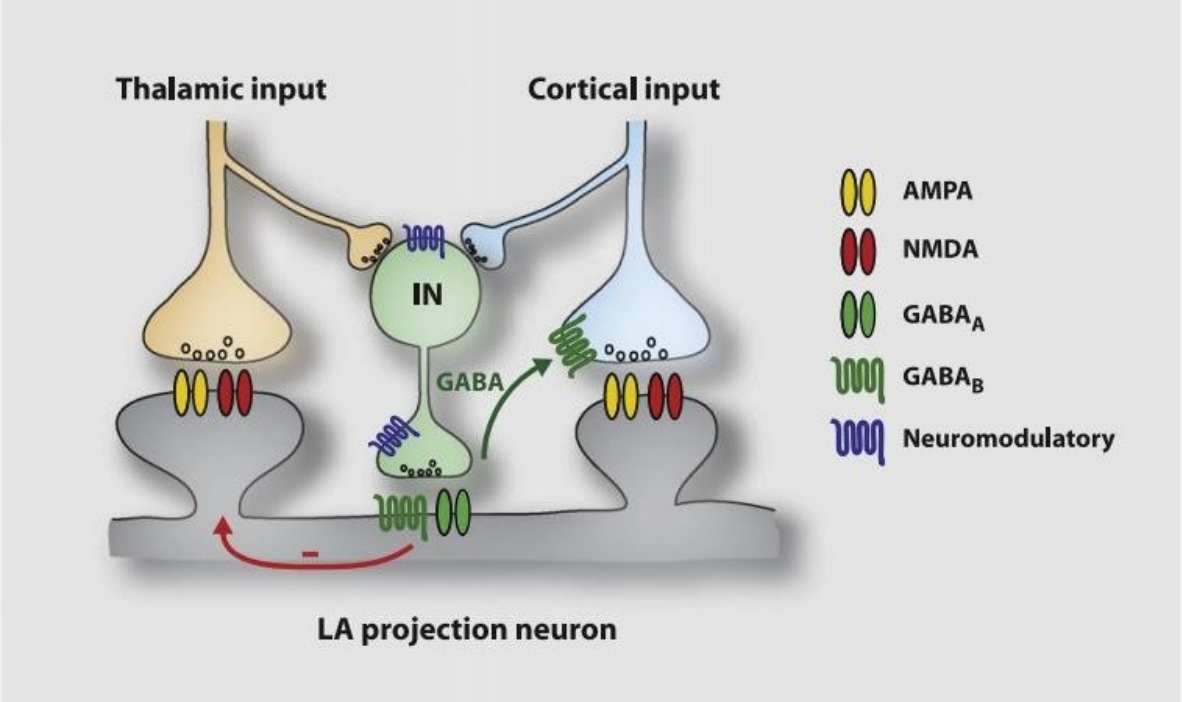

Figure 1.5. GABAergic modulation of Thalamic and Cortical Afferents onto the LA. An illustrative diagram GABAergic activity at a LA projection neuron and its thalamic and cortical afferents (Ehrlich et al., 2009)

1.6 Astrocytes are important regulations of neuronal activity

Neurons are widely recognised as the basis of neural function. As such, research has traditionally focused on neurons when investigating the underlying mechanisms of neural circuits and the development of stress-related pathologies that impact the brain (Lyon & Allen, 2022). However, glial cells (non-neuronal brain cells) comprise more than half of the brain and

yet have received far less attention than neurons (Guadagno et al., 2021). Astrocytes, a type of glial cell, are central regulators of neuronal function (Allen & Barres, 2009). Traditionally, astrocytes have been viewed as support cells that served as an adhesive for the structural components of the nervous system(Allen & Barres, 2009). However, evidence demonstrates that astrocytes have functions beyond adhesive support (Allen & Barres, 2009; Murphy-Royal et al., 2015). In the developing brain, astrocyte development precedes that of synaptic development and play and active role in the structuring and maturation of synapses in the brain. Additionally, astrocytes participate in bidirectional communication with neurons at the synapse. At the synapse, astrocytes respond to neurotransmitters such as glutamate or GABA, which activates these cells, leading to increases in intracellular calcium. In response to the release of neurotransmitters, astrocytes release gliotransmitters such as ATP (Gourine et al., 2010; Guthrie et al., 1999) and D-serine (Wolosker et al., 1999), enabling these cells to modulate neural activity and synaptic transmission (Durkee & Araque, 2019).

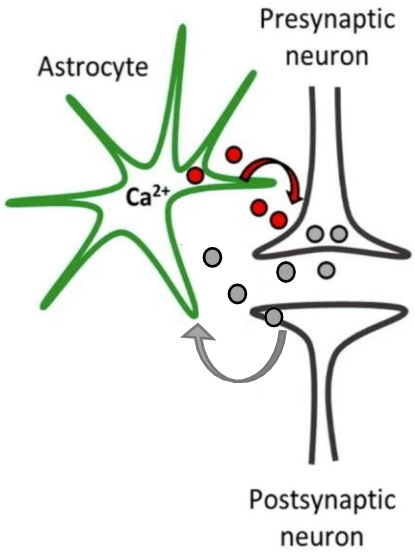

Figure 1.6. Astrocyte-Neuron Bidirectional Communication. An illustration representing Astrocyte-Neuron interaction at the synapse. Astrocytes (in green) sense the release of neurotransmitters (in grey) at the synapse and in turn release gliotransmitters (in red) which modulate activity at the synapse. Adapted from Durkee & Araque (2019)

1.6.1 The role of astrocytes in behaviour

Astrocytes through the modulation of synaptic activity, regulate behaviour (Lyon & Allen, 2022). Astrocytes have been shown to actively participate in the regulation of neural circuits subserving behaviour, this includes regions such as the hypothalamus (L. Yang et al., 2015), the hippocampus (L. Yang et al., 2015) and the amygdala (Fan et al., 2021; Li et al., 2020). Indeed, research has shown that genetic modification of astrocyte function as well as optogenetic activation of astrocytes have implications for the expression of normal behaviours such as fear learning and memory (Fan et al., 2021; Li et al., 2020), thus indicating the importance of astrocytes in cognition and the detrimental consequences if these cells were to be impaired.

1.6.2 The role of astrocytes in ELS-induced behavioural impairments

Astrocytes are sensitive to the effects of stress. Using their end-feet processes to wrap around vasculature in the brain, astrocytes can regulate blood flow and the transport of ions and hormones to and from the brain (Allen & Barres, 2009). This astrocytic function allows astrocytes to transport energy substrates such as glucose, in the form of lactate, to neurons, providing the crucial metabolic support necessary to sustain the high energetic demands of neuronal processes such as LTP (Murphy-Royal et al., 2020). However, this function leaves astrocytes susceptible to changes in CORT concentration in the blood as glucocorticoids are able to cross the blood-brain barrier into the neural parenchyma (Mason et al., 2010). Additionally, astrocytes express GRs significantly more than neurons (Murphy-Royal et al., 2020). Hence, changes in CORT in the blood has consequences for astrocyte signalling and activity (Dolotov et al., 2022; Murphy-Royal et al., 2019). Evidently, astrocytes have been implicated in stress-related disorders such as depression (Leng et al., 2018; Mechawar & Savitz, 2016; Murphy-Royal et al., 2020; Nagy et al., 2014). Post-mortem brain tissue taken from patients that have suffered from depression show alterations in astrocyte structure and proteins (Dolotov et al., 2022; Tanti et al., 2019; Torres-Platas et al., 2016). Furthermore, the expression levels of astrocytic proteins such as aquaporins, cytoskeletal proteins and gap junction channels have been shown to be modified following situations of stress (Murphy-Royal et al., 2020). Due to the importance of astrocytes in synaptic integrity and behaviour such as learning and memory, the impairment of these cells may predict detrimental impacts on neural functioning and behavioural

expression. Hence, astrocytes present as a pertinent component of the mechanisms subserving the relationship between stress and stress-induced behavioural changes. However, despite the clear importance of astrocytes in behaviour, the impacts of stress on astrocyte structure and function and how they influence behaviour are ill-determined.

Chapter 2
Hypothesis and Objectives

The role of astrocytes in behaviour represents a largely unexplored but currently burgeoning field. However, the role of astrocytes in ELS-dependent behavioural impairment remains undetermined. Hence, the objective of this project is to investigate astrocytes in the mechanisms underlying ELS-induced behavioural dysfunction. We hypothesise that ELS induces changes in astrocyte glucocorticoid signalling in the LA, resulting in behavioural deficits.

This hypothesis will be addressed in these four aims:

Aim 1: To characterize the effects of ELS on LA-dependent behaviors particularly relating to anxiety and fear learning and memory

Aim 2: To determine the impact of ELS on astrocyte GR expression and activity

Aim 3: To describe the influence of ELS on the expression of structural astrocyte proteins, GFAP and Cx43, as well as astrocyte density in the amygdala

Aim 4: To investigate the ablation of astrocyte glucocorticoid signalling as a potentially protective measure against ELS-induced behavioural dysfunction

Chapter 3
Methods and Materials

1. Animals

All animal procedures and experiments were approved by the Centre de recherche du Centre hospitalier de l'Université de Montréal / University of Montreal Hospital Research Centre (CRCHUM) Animal Care Committee in accordance with the Institutional Animal Protection Committee (CIPA). Both male and female C57BL6J mice were used throughout this project during post-natal days (PND) 10-70, with ad libitum access to food and water. Mice were housed on a 12 h:12 h light:dark cycle (lights on at 6h30) in whole litters prior to experimentation.

Generation of C57BL/6J litters.

Two C57BL/6J females were mated with one male in the CRCHUM animal facility. The male was removed one week following impregnation and females were separated into individual cages 1-3 days prior to giving birth. Litters were weighed and counted, and cages were cleaned the day of birth. The sex of offspring was determined at PND 17. Offspring were weaned at postnatal PND21 with males and females weaned separately and placed into cages of 2 to 5 mice. Following birth until the day of sacrifice, cages in which the mice were housed were changed once every two weeks.

Generation of Transgenic GRflox litters Mouse lines.

B6.Cg-Nr3c1tm1.1Jda/J (GRflox) mice were obtained from Papouin lab at the Washington University School of Medicine in St. Louis, Missouri. These mice are characterised by 'floxed' mutant mice possessing loxP sites that flank exon 3 of the Nr3c1 gene. In order to maintain this transgenic mouse line in-house, homozygotes (homo x homo) and heterozygotes (homo x B6/J) were bred initially, and offspring were bred continually once matured. The genotype of each mouse was confirmed using polymerase chain reaction (PCR).

Polymerase Chain Reaction. For DNA extraction, an base/acid reaction was used along with heating to 95 C for 15 minutes in between, to extract the DNA from the mouse tails. Once PCR is completed, the replicated DNA samples were stained and loaded on a 2 % agarose gel containing ethidium bromide (EtBr). The migration process is performed using electrophoresis and the gel is exposed to UV lighting using a gel imager (Vilber). To determine genotypes, 100 bp ladders and controls were loaded in to confirm band size. GRflox mutants (homozygotes)

have one higher band at 300 bp, heterozygotes show 2 bands at 247 and 300 bp and wild types (WT or non carriers) show a lower band at 247 bp.

2. Early Life Stress (ELS) Protocol

In order to model maternal neglect and separation, pups were separated from their mother by being placed into different cages for 4 hours/day and were returned to their home cage afterwards. This was done consistently for seven days during PND10-17. Both home cages and the cages used for separation, contained a third of the original bedding to model disorderly maternal care, further inducing postnatal stress. The body temperature of the pups was maintained by placing the cages containing the pups on a heating pad. Nesting material in the home cage was restored on PND 17 (**Figure 4.1**) (Peña et al., 2017)

3. Serum Collection and CORT Analysis

Blood collection.

Blood was collected from mice between 8h00 and 8h30 as glucocorticoids levels in mice are at their peak in the morning (Dickmeis, 2009). blood was collected from mice. To anaesthetise the mice prior to blood collection, mice were placed in an enclosed chamber filled with isoflurane (5% for induction, 2–3% for maintenance, v/v) for 60 – 90 sec. The depth of anesthesia was determined by observing breathing rates and using toe-pinch to ensure the loss of reflexes. Once it was determined that the mice were no longer conscious, mice were decapitated, and blood was collected in EDA-covered tubes, to prevent clot formation. These tubes were then immediately placed in ice. Following blood collection, the tubes were centrifuged (brand, make) at 4°C at 5000RPM for 5min. Once the blood serum was separated from the blood, the serum was collected using a pipette and placed in 0.5ml Eppendorf tubes and stored in a -80°C freezer.

CORT analysis.

An ELISA Kit (ENZO), stored at 4°C, was used to quantitatively determine the amount of CORT (ng/ml) in the blood serum collected from the mice. This kit consists of: DxS IgG Microtiter plate, Assay buffer 15 concentrate, Steroid displacement reagent, Conjugate, Antibody, Wash buffer concentrate, Standard, pNpp Substrate, Stop solution.

Before use, all solutions were left to warm to room temperature for 30 min. 10µL of each serum sample was aliquoted into 0.5mL Eppendorf tubes. Afterwards, a 1ml 1:100 Steroid Displacement Reagent (SDR) solution was made with deionized water or phosphate buffered saline (PBS). Subsequently, 10µl 1:100 SDR was added to each Eppendorf tube containing the serum samples. These mixtures were vortexed and left to stand for less 5 minutes before being diluted with EIA buffer, which dilutes enzyme conjugates, standards, and samples. 380µL EIA assay buffer was then added to each tube containing serum and vortexed. 20 µL of each sample mixture was aliquoted in their sample wells.
(plating of solutions) The plates were read on a Synergy HTX plate reader (Biotek, Winooski, VT), and its built-in four-parameter logistic regression software (Victor) was used for plotting the standard curve and data extrapolation.

4. Behavioural Testing

Open Field Task (OFT)

The open field task (OFT) is a commonly used behavioural test to assess anxiety-like behaviour (Lee et al., 2012). This task is based on the natural aversion of mice to open and unprotected spaces, where the center of the OFT apparatus represents the open, unprotected space and the periphery, which is enclosed by the apparatus walls, represents the protected space. The OFT apparatus comprises of a white acrylic box with four walls and an open top (H: 29cm× W: 38cm × B: 38cm) (Maze-Engineers, Stoelting). Mouse observations were captured using a webcam (Logitech) mounted to a fixture 1m above the OFT apparatus. The webcam recorded digital videos of ongoing tests to a connected laptop (Lenovo) equipped with a video tracking system (AnyMaze, Stoelting) which measured the total time (s) spent in the centre and the total distance travelled (m).

One day prior to behavioural testing, mice were handled by hand or by tube for approximately 10 minutes. On the day of behavioural testing, mice were rolled into the behavioural room and left for an hour, allowing the mice to habituate to the room. To clean the OFT apparatus, 70% ethanol was applied to the walls and floor of the OFT apparatus before testing and between trials. Following habituation, mice were subjected to behavioural testing. The behavioural task comprised of a total of 5 minutes and during that time the mice were left to

freely explore the OFT apparatus. During the task, the total time spent by the mouse in the center of the apparatus was recorded. The total time spent in the center was used as a quantification of anxiety-like behaviour, where more time in the center represented anti-anxious behaviour and the total distance travelled was measured to quantify locomotor impairments.

Elevated Plus Maze (EPM)

The Elevated Plus Maze is also commonly used for measuring anxiety-related behaviour (Lee et al., 2012). The EPM apparatus is made of white acrylic material and consists of four arms (two open without walls and two enclosed by 30 cm high walls). Each arm is 50 cm long and 10 cm wide (Fig 2) (Maze-Engineers, Stoelting). Each arm of the maze is attached to a metallic platform such that it is elevated by 50 cm off the floor. Like the Open Field Task, the EPM is also based on the natural aversion of mice to open unprotected areas. However, the EPM consists of two open arms which represent the unprotected spaces and two closed arms which represent the protected spaces.

The EPM was performed one day following OFT. On the day of testing, mice were rolled into the behavioural room and were left for 1 hour to habituate. Following habituation, mice were removed from their cages and placed at the centre of the four arms of the maze, facing an open arm. A webcam mounted on a fixture above the EPM apparatus recorded digital videos to a connected laptop (Lenovo) equipped with a video tracking system (AnyMaze, Stoelting) which recorded the number of entries and the time spent (s) in closed arms or open arms during the 5min testing period. Anxiety-like behaviour was quantified by both the number of entries into, and the duration spent in the open arms.

Auditory Discriminative Fear Conditioning

To assess lateral amygdala dependent behaviour, auditory discriminative fear conditioning (ADFC) was performed on the mice (W. bin Kim & Cho, 2017). The ADFC apparatus consists of a USB camera, sound generator, shocker, an acrylic enclosure with electrified grid floor, sound-attenuating chamber with light and fan and a visible light source. Four days prior to ADFC, mice were single housed in individual cages. Two days after, mice were handled for approximately 10 minutes by hand and tube. On the day of the first day of ADFC, the conditioning phase, mice were wheeled into the behavioural room and left for an

hour to habituate prior to the start of the behavioural task.

ADFC comprises of two phases: conditioning and memory recall. During the conditioning mice are first exposed to a neutral stimulus (CS-; white noise, 20s) repeated six times (**Figure 4.3**). After a two-minute, interval mice are then exposed to a beep tone which becomes the conditioned stimulus (CS+;12 kHz tone, 20s) as it co-terminates with a mild foot-shock (unconditioned stimulus (US); 0.5mA, 2s) delivered in the last two seconds of tone presentation, also repeated six times. During the conditioning phase, mice are exposed to both the neutral stimulus (CS-) and the conditioned stimulus paired with foot shock (CS+/US) in a distinct context characterised by checkered walls and an exposed shock grid which is washed down by 70% ethanol. Memory recall is tested exactly 24 hours later by exposing the animals to CS- and CS+ (counterbalanced) in a novel environment characterised by stripped walls and a white floor covering, washed down by 0.5% hydroperoxide. Removing contextual cues eliminated the recruitment of brain regions such as the hippocampus (W. bin Kim & Cho, 2017; Li et al., 2020). Fear learning was quantified by the percentage of time spent freezing to CS- and CS+/US during the conditioning phase. Fear memory was quantified by the percentage of freezing time to CS- or CS+ following their distinct presentation in the memory-recall phase. Affective cognitive function was quantified using the following index:

Discrimination Index (DI) = (CS+ freezing – CS- freezing) / (CS+ freezing + CS- freezing)

Using this index, a value equal to 1 represents freezing only to CS+ reflecting accurate discrimination. A value equal to 0 indicates an absence of the ability to accurately discriminate, with equal freezing to both CS+ and CS-, and a value equal to -1 denotes incorrect discrimination with freezing to CS- only.

5. Stereotaxic surgeries

Mice (P23-P50) were anesthetized via isoflurane (5% for induction, 2–3% for maintenance, v/v). Depth of anesthesia was determined by observing breathing rates and toe-pinch ensured proper loss of reflexes. Following deep anesthesia, mice were head fixed on a stereotaxic apparatus (David Kopf Instruments) with a bite bar and ear bars, with ventilated anesthesia administration. In all, 0.05 µl of buprenorphine was injected subcutaneously (Buprenex, 0.1 mg/mL), and artificial tears were applied to the eyes before beginning surgery. The hair on the scalp was shaved prior to surgery, and the incision was washed with 10%

povidone iodine and 70% ethanol, three times each, alternating. An incision was made on the scalp to expose bregma and the craniotomy site with Lateral Amygdala coordinates: AP=-1.4mm, ML=+/-3.5mm, DV=-5.0mm. A 2–3-mm craniotomy was made at the injection site using a small burr (Fine Science Tools), powered by a drill (K.1070, Foredom). Saline (0.9%) was applied to keep the skull cool, to maintain skin hydration, and to remove bone debris. AAVs were injected via a beveled borosilicate pipette (World Precision Instuments) at an infusion rate of 200 nL/min by a mechanical pump (Pump11 Pico-Plus Elite, Harvard Apparatus). In all, 1 µl of virus was infused into the lateral amygdala, and each virus contained the astrocyte-specific GfaABC1D promoter driving the following constructs at the indicated titer: eGFP, (1e12 gC/mL), GFAP-Cre eGFP (Shigetomi et al. 2013) 1.7e12 gC/mL). Following injection, the needle was left in place for 10 min to allow for fluid pressure normalization. Following needle withdraw, scalp was sutured with silk sutures and mice were closely monitored, kept on a heating pad. Mice were given carprofen 24 hours following surgery (0.05 mL, 0.1 mg/mL) and were monitored 2, 3 and 7 days following surgery. Experiments were performed 2–4 weeks post-injection (Murphy-Royal et al., 2020)

6. Immunohistochemistry

To prepare fixed brain sections, mice were perfused surgically using 4% paraformaldehyde (PFA). Mouse carcases were then kept in bags containing 4% PFA for 48 hours. Mouse brains were then extracted and placed into falcon tubes containing 4% PFA for two days. The fixed brains were then placed in falcon tubes containing 30% sucrose solution for two days. Mice brains were then sectioned into 30 µm thick sections on a cryostat (Leica) and processed for immunohistochemistry.

Free-floating slices were washed three times in 1 x PBS for 15 minutes. Following this, slices were then permeabilized in a block-perm solution (3% Bovine-Serum-Albumin, 0.5% Triton$^{10\%}$ in 1 X PBS) for one hour. Sections were then incubated with primary antibodies for 24 hours at 4°C. Slices underwent three subsequent washes for 15 minutes in 1 x PBS, prior to being incubated with secondary antibody for 1 hour. Brain slices were then washed in 1 x PBS for three times before being mounted onto glass slides using fluorescence mounting medium with DAPI (Vectashield) and cover-slipped. Slides were left at room temperature for 24 hours to dry and then were stored at 4°C (Murphy-Royal et al., 2020).

Antibody pairs used are as follows: Rabbit anti-S100ß (1:1000, Abcam, ab52642) and goat anti-rabbit Alexa 488 (1:1000, Jackson Immuno Research, 111-545-144); Mouse anti-GR Alexa (1:500, ThermoFisher, MA1-510) and goat anti-mouse Alexa 647 (1:1000, ThermoFisher, A32728); Chicken anti-GFAP (1:1000, ThermoFisher, PA1-10004) and goat anti-chicken Alexa 568 (1:1000, ThermoFisher, A11041), mouse anti-Cx43 (1:1000 ThermoFisher, 3D8A5) and goat anti-mouse Alexa 647 (1:1000, ThermoFisher, A32728). Brain sections of the amygdala were imaged on an Confocal Leica TCS_SP5 MP microscope (63x) and analyzed in ImageJ.

For viral validation, brain sections of the amygdala were washed again three times, mounted on glass slides, dried, and were mounted onto glass slides using fluorescence mounting medium with DAPI (Vectashield) and cover-slipped. Brain sections were then images on an Olympus Fluoview 1000 upright confocal microscope and analyzed in ImageJ.(Murphy-Royal et al., 2020)

Statistical Analysis

GraphPad prism 9.3.1 was used to perform all statistical analyses. First, it was determined whether the data was normally or non-normally distributed, establishing whether a parametric or non-parametric was used. Two-group comparisons of normally distributed data with one independent variable were analysed using an Independent t-test. Two-group comparisons of non-normally distributed data with one independent variable were analysed using a Mann-Whitney U test. For data with multiple groups and two-independent variables a two-way ANOVA or repeated measures (RM) Two-Way ANOVA was used with appropriate post-hoc tests for comparison between groups.

Chapter 4
Figures

Figure 4.1 – Early Life Stress Protocol

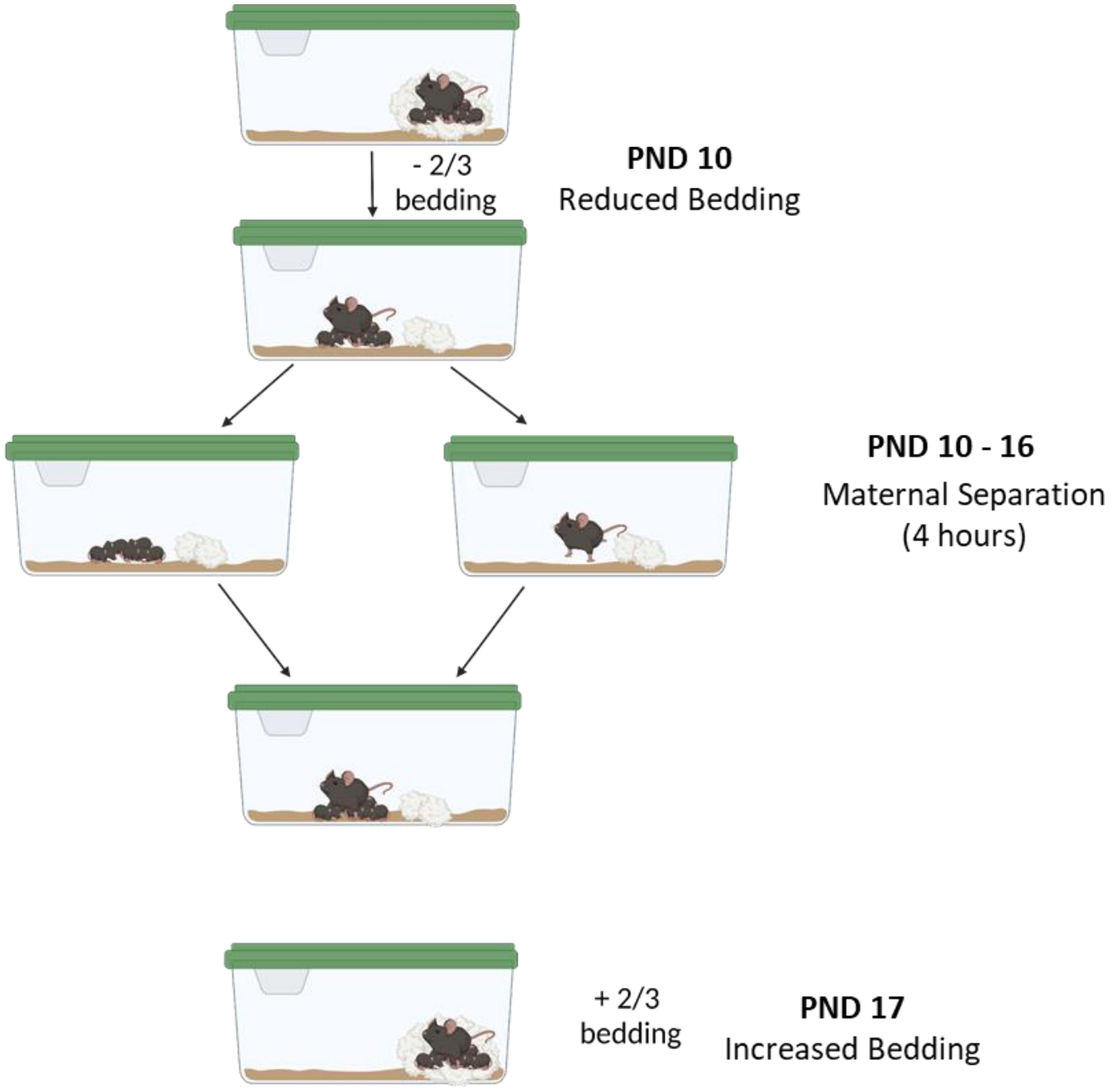

Figure 4.1. A schematic representation of the ELS protocol performed between PND 10 and PND 17 where pups were separated from their mother by being placed in different cages between PND 10 and PND 16. Separation occurred for 4 hours a day with two thirds of bedding removed from both the home cage and the separated cages. At PND 17, bedding was restored to normal levels.

Figure 4.2 - Effects of Early Life Stress on Anxiety-Related Behaviour

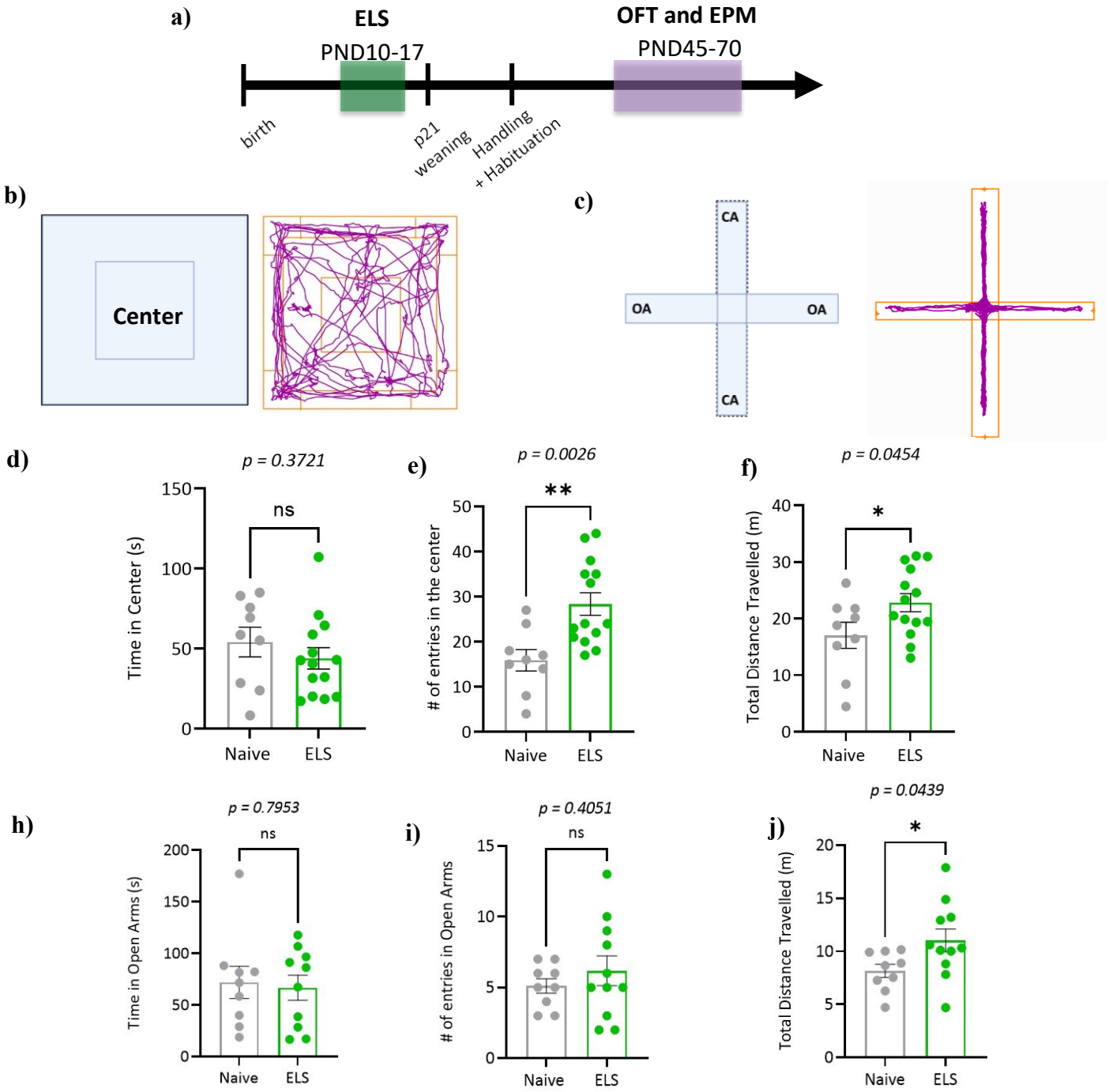

Figure 4.2. (a) A schematic representation of the experimental timeline. **(b)** a schematic representation of the OFT behavioural test as well as a representative trace of a mouse's movement throughout the test. **(c)** a schematic representation of the EPM behavioural test as well as a representative trace of a mouse's movement throughout the test. **(d)** A Scatter dot plot of the time spent in the center (s) during OFT (s) of naïve mice (n=9) and ELS mice (n=14). **(e)** Scatter dot plot of the number (#) of entries in the center during OFT (s) of naïve mice (n=9) and ELS mice (n=14) **(f)** Scatter dot plot of the total distance travelled during OFT (m) during OFT (s) of naïve mice (n=9) and ELS mice (n=14) **(g)** A Scatter dot plot of the time spent in the open arms (s) during EPM of naïve mice (n=9) and ELS mice (n=10) **(h)** Scatter dot plot of the number (#) of entries in the Open Arm during EPM (s) of naïve mice (n=9) and ELS mice (n=10). **(i)** Scatter dot plot representing the total distance travelled (m) during EPM (s) of naïve mice (n =9) and ELS mice (n=10) . Errors bars represent ± SEM.

Figure 4.3 – Auditory Discriminative Fear Conditioning Protocol

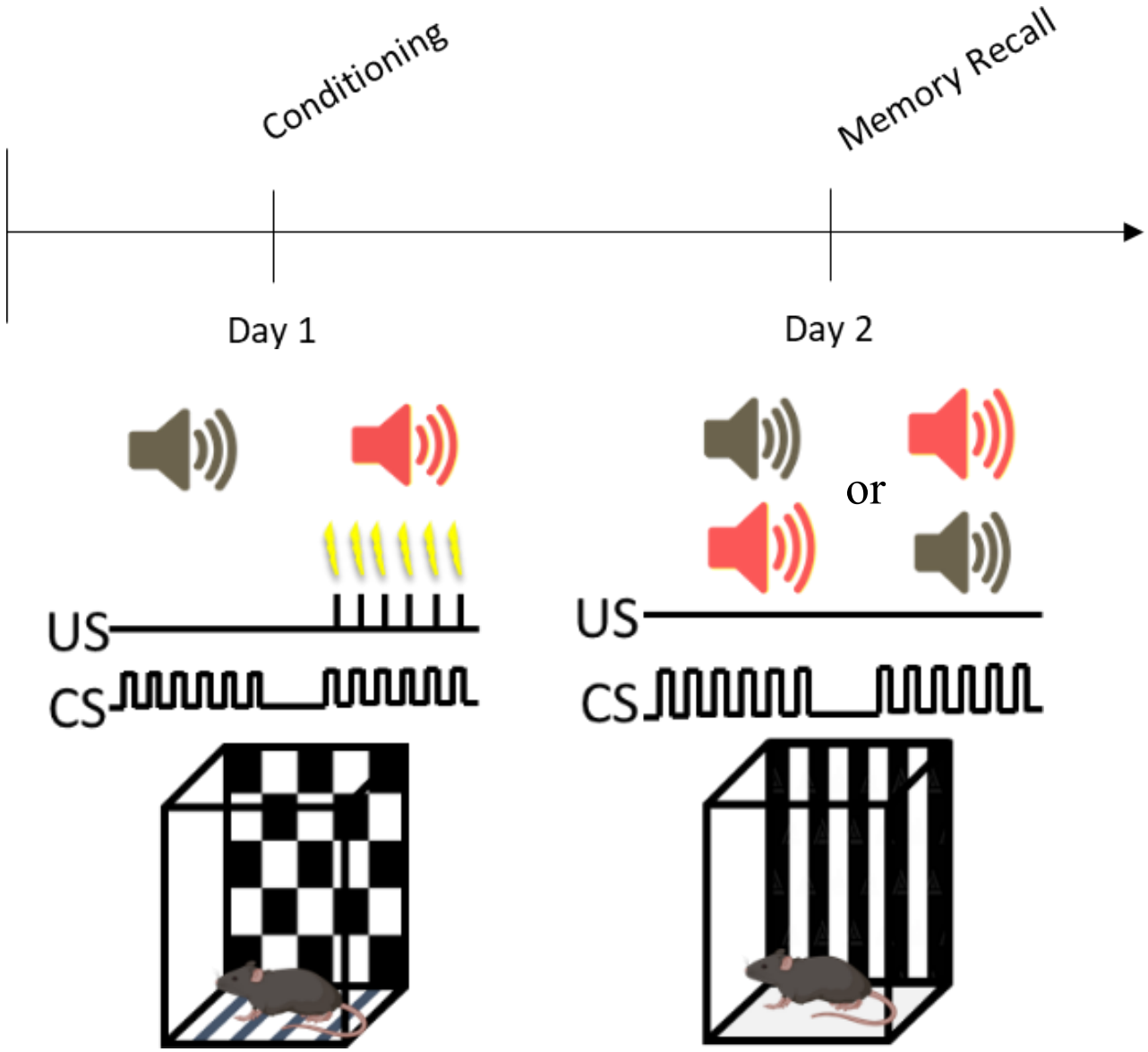

Figure 4.3. A schematic representation of the Auditory Discriminative Fear Conditioning (ADFC) Paradigm. The first day of the protocol represents the conditioning phase where white noise (CS-) is played six times for 20s each without a foot shock and subsequently, a 12 kHz tone is played is 6 times consecutively for 20sec and co-terminated with a 0.5A shock in the last 2 secs (CS+/CS), in a particular context. Day 2 of the protocol represents the Memory Recall phase which is performed in a different context where both CS- and CS+ are presented independently six times for 20s each without a foot shock.

Figure 4.4 – Validation of the Auditory Discrimination Fear Conditioning Protocol

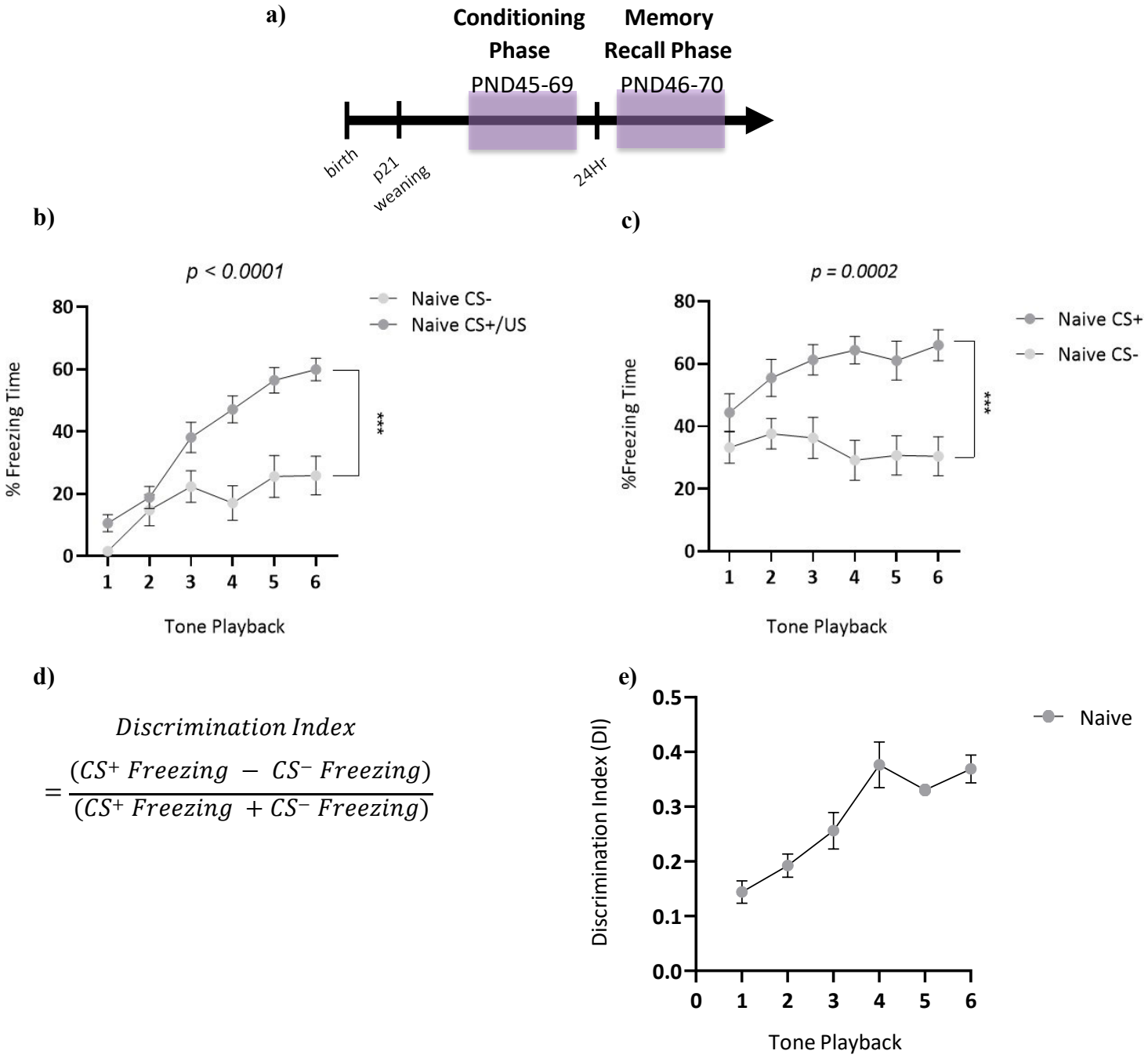

Figure 4.4. (a) A schematic representation of the experimental timeline **(b)** A line graph representing the % freezing time of naïve mice to the aversive association (CS+/US) (n = 20) and the neutral tone (CS-) (n = 13) over six tone presentations during the conditioning phase. **(c)** A line graph representing the % freezing time of naïve mice to the aversive tone (CS) (n = 20) and the neutral tone (CS-) (n = 13) over six tone presentations during the memory recall phase. (d) A mathematical equation representing the discrimination index which is the difference of the % freezing time between the CS+ and CS- divided by the total % freezing time. (d) A line graph representing the discrimination index (DI) of naïve mice (n = 20) over 6 tone presentations. Error Bars represent

Figure 4.5 – Effects of Early-Life Stress on Fear Learning, Memory and Threat Discrimination

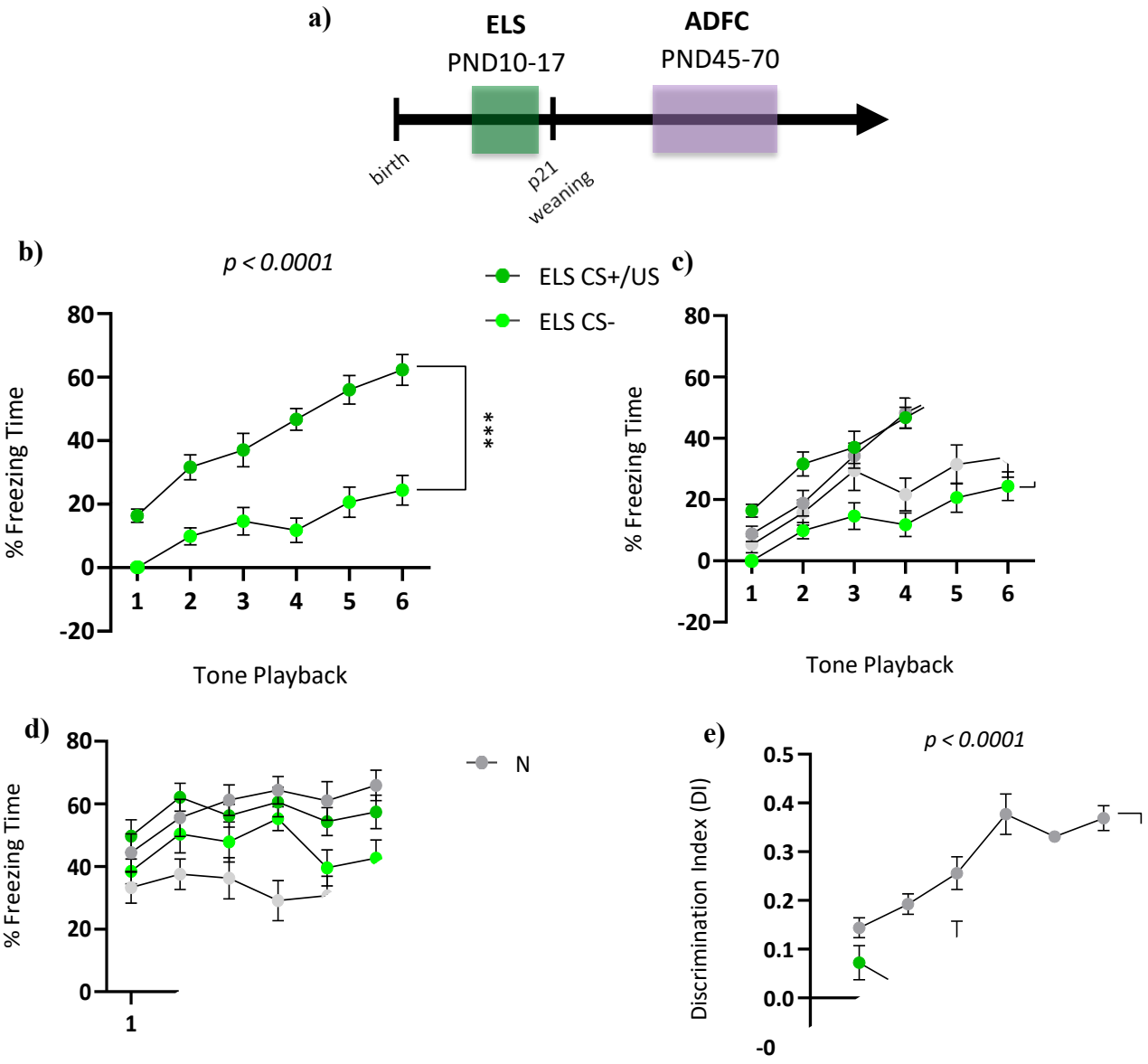

Figure 4.5. (a) A schematic representation of the experimental timeline. **(b)** A line graph representing the % freezing time of ELS mice to the aversive association (CS+/US) (n = 17) and the neutral tone (CS-) (n = 17) over six tone presentations during the conditioning phase. **(c)** A line graph representing a comparison between the % freezing time of naïve mice to the aversive association (CS+/US) (n = 20) and the neutral tone (CS-) (n = 13) and the % freezing time of ELS mice to the aversive association (CS+/US) (n = 17) and the neutral tone (CS-) (n = 17) over six tone presentations during conditioning. **(d)** A line graph representing a comparison between the % freezing time of naïve mice to the aversive tone (CS+) (n = 20) and the neutral tone (CS-) (n = 13) and the % freezing time of ELS mice to the aversive tone (CS+) (n = 17) and the neutral tone (CS-) (n = 17) over six tone presentations during memory recall. **(e)** A line graph representing a comparison between the DIs of naïve mice (n = 20) and ELS mice (n =17) over 6 tone presentations. Error bars represent mean ± SEM.

Figure 4.6 - Effects of Early-Life Stress on CORT production at PND 10, 17 and 45

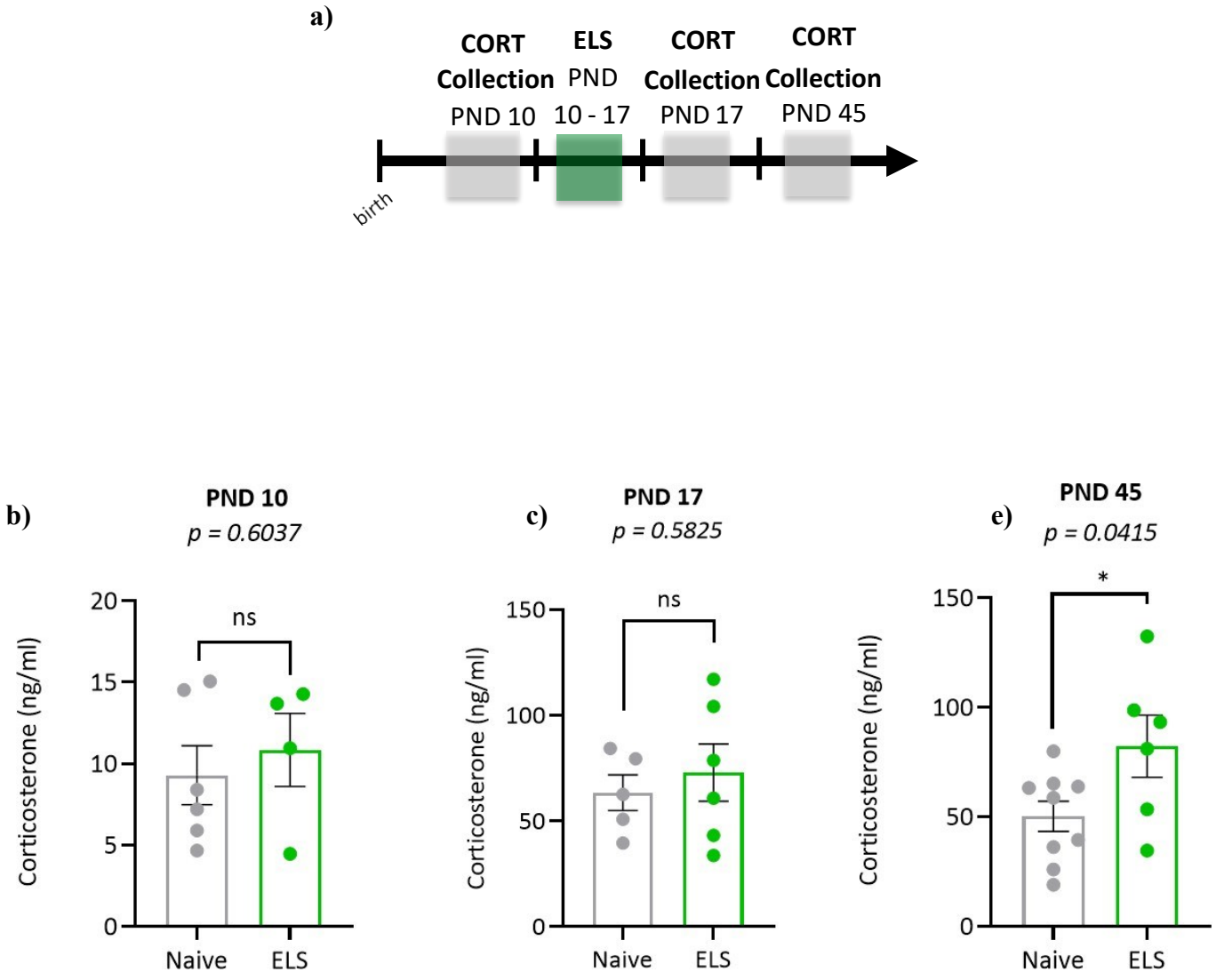

Figure 4.6. (a) A schematic representation of the experimental timeline. **(b)** Scatter dot plot of the amount of corticosterone (ng/ml) in the blood of naïve mice (n=6) compared to ELS mice (n =4) at PND 10. Error Bars represent mean ± SEM **(c)** Scatter dot plot of the amount of corticosterone (ng/ml) in the blood of naïve mice (n=5) compared to ELS mice (n =6) at PND 17. Error Bars represent mean ± SEM **(d)** Scatter dot plot of the amount of corticosterone (ng/ml) in the blood of naïve mice (n=9) compared to ELS mice (n = 6) at PND 45.

Figure 4.7 – Effects of Early Life Stress on Astrocyte Glucocorticoid Receptor Activity

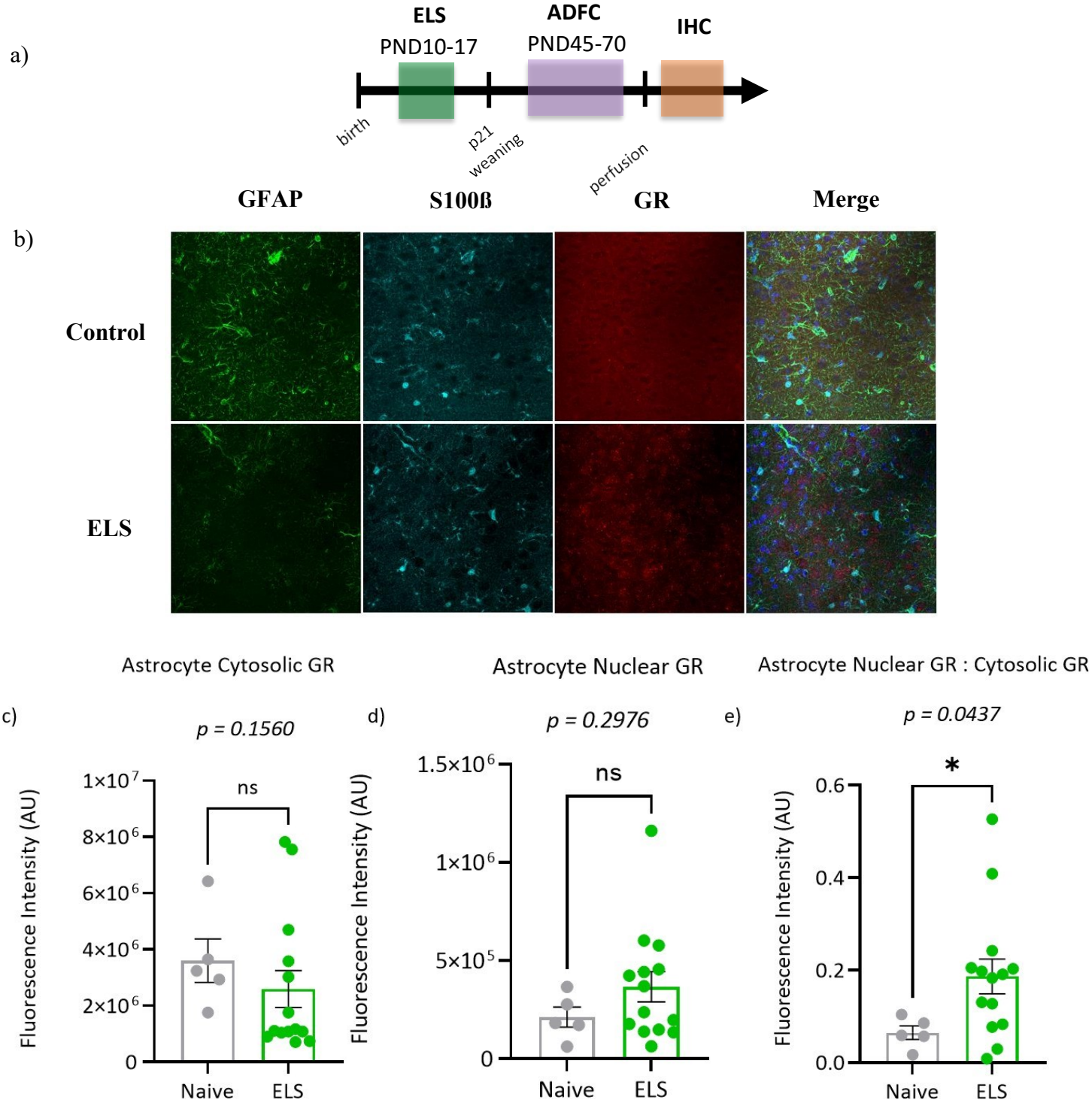

Figure 4.7. (a) A schematic representation of the experimental timeline. **(b)** Representative images of fluorescent sliced scans depicting GFAP, S100ß, GR and all images merged in the LA of both control and ELS groups. **(c)** A scatter plot of the fluorescence intensity (AU) of astrocyte cytosolic GR in the LA of naïve (n = 5) and ELS (n = 14) mice **(d)** A scatter plot of the fluorescence intensity (AU) of astrocyte nuclear GR in the LA of naïve (n = 5) and ELS (n = 14) mice **(e)** A scatter plot of the fluorescence intensity (AU) of astrocyte nuclear GR : cytosolic GR in the LA of naïve (n = 5) and ELS (n = 14) mice. Each dot represents an average of 3 slices. Error bars represent mean ± SEM.

Figure 4.8 – Effects of ELS on Astrocyte Protein Expression and Cell Density in the Amygdala

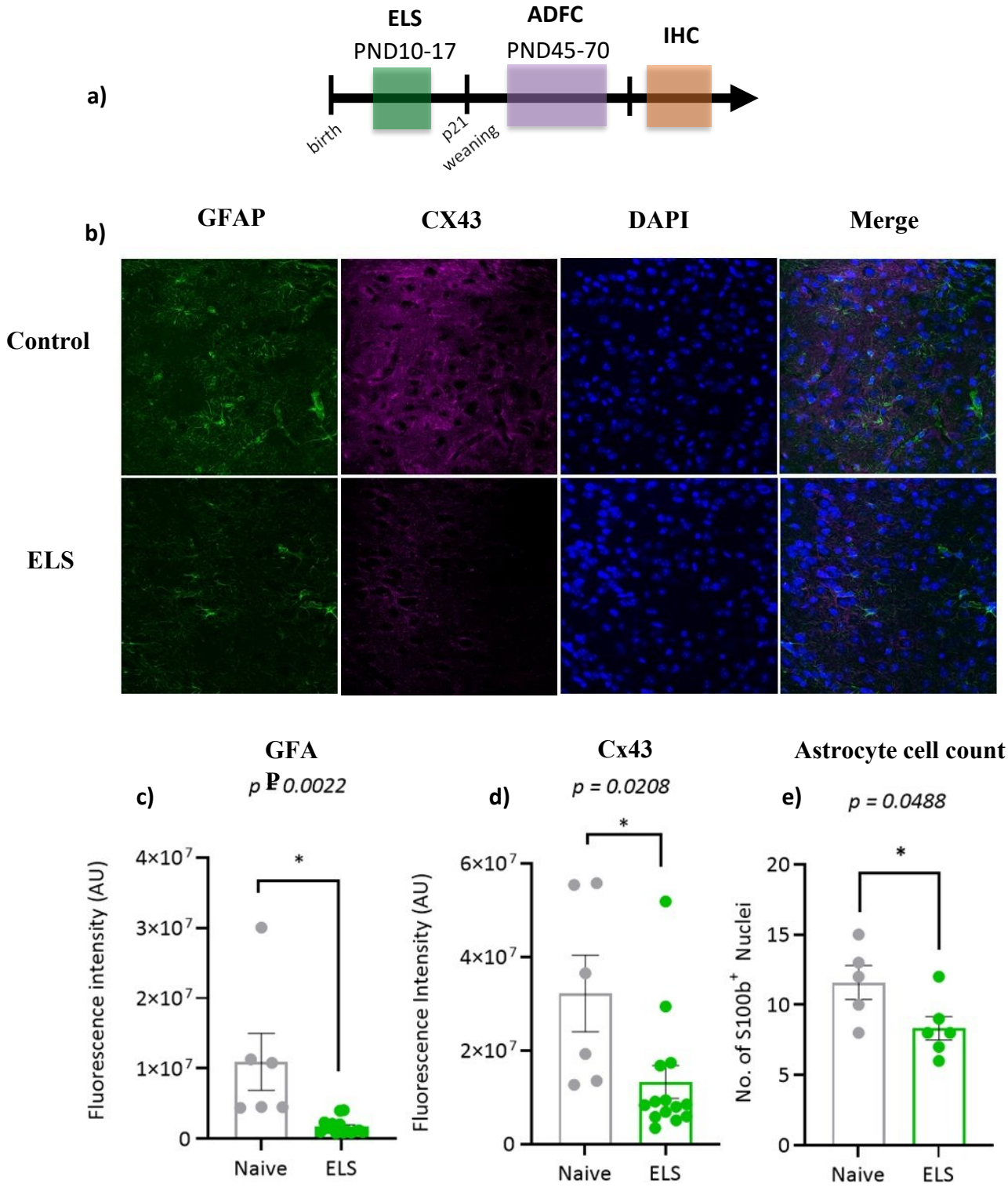

Figure 4.8 (a) A schematic representation of the experimental timeline. **(b)** Representative images of fluorescent sliced scans depicting GFAP, CX43 and DAPI and all images merged in the LA of both control and ELS groups. **(c)** A scatter plot of the fluorescence intensity (AU) of GFAP in the LA of naïve (n = 6) and ELS (n = 14) mice. Each dot represents an average of 3 replicates **(d)** A scatter plot of the fluorescence intensity (AU) of Cx43 in the LA of naïve (n = 6) and ELS (n = 14) mice. Each dot represents an average of 3 replicates **(e)** A scatter plot of the number of S100ß nuclei in the LA of naïve (n = 5) and ELS (n = 6) mice. Each dot represents an average of 3 replicates. Error bars represent mean ± SEM.

Figure 4.9 – Validation of Astrocyte Specific Glucocorticoid Receptor knockout in the Amygdala

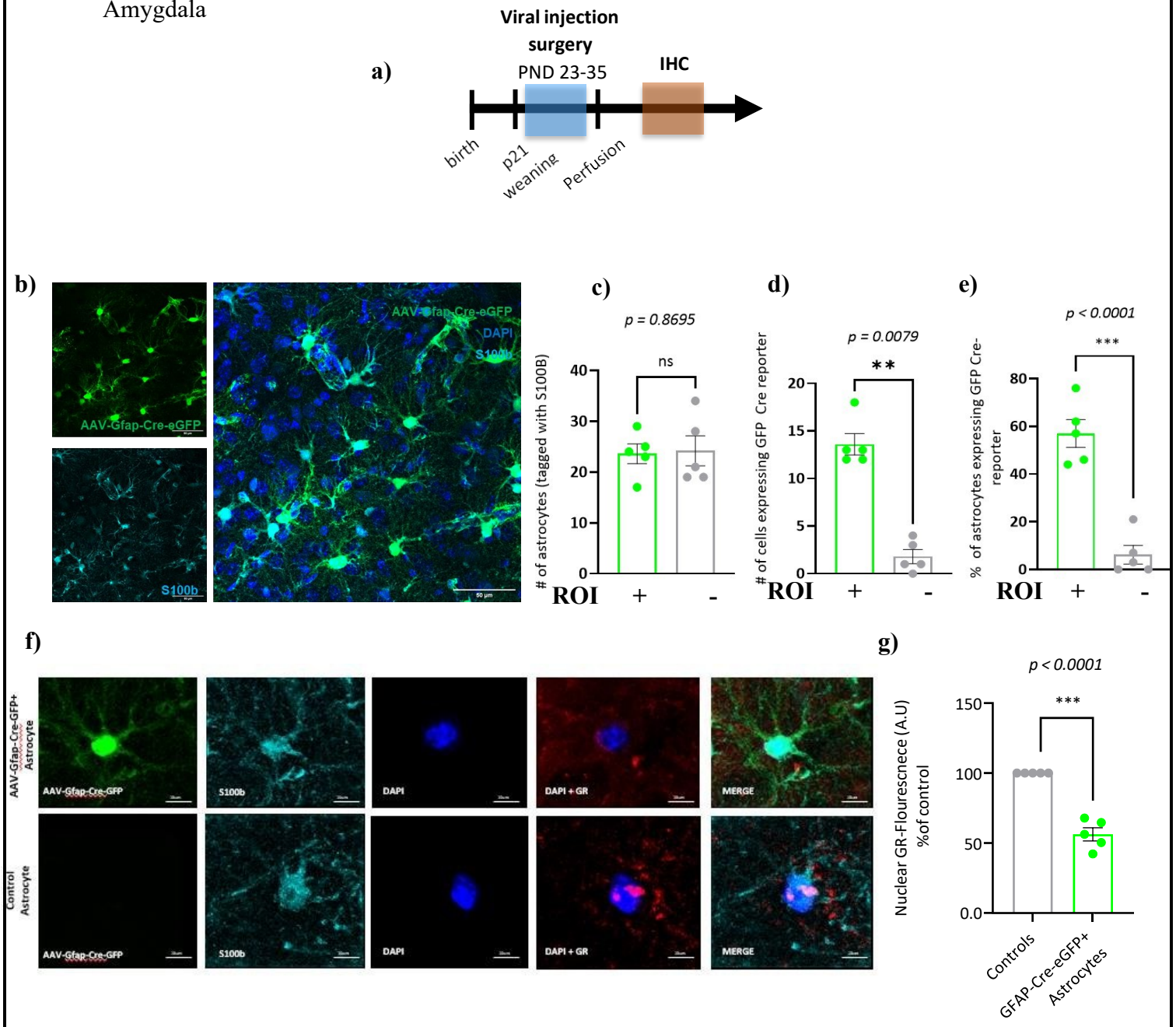

Figure 4.9 (a) A schematic representation of the experimental timeline. **(b)** Representative images of fluorescent sliced scans depicting AAV GFAP-Cre-eGFP, S100ß and all images merged, including DAPI staining in the amygdala of representative mouse. **(c)** A scatter plot of the number of astrocytes tagged with S100ß in the region of interest (ROI) (n = 5) and outside the ROI (n = 5) in the same slice of the amygdala **(d)** A scatter plot of the number of cells expressing the GFAP-Cre-eGFP reporter in the ROI (n =5) and outside the ROI (n = 5) within the same slice of the amygdala **(e)** A scatter plot of the % of astrocytes in the ROI (+) (n = 5) and outside the ROI (-) (n =5) expressing the GFAP-Cre-eGFP reporter within the same slice in the amygdala. **(f)** Representative images of fluorescent sliced scans depicting AAV GFAP-Cre-eGFP, S100ß, DAPI, DAPI and GR and all images merged, including DAPI staining in the amygdala of mice injected with GFAP-Cre-GFP in astrocytes expressing the Cre-eGFP reporter and astrocytes not expressing it (control astrocyte) **(g)** A scatter plot of the Nuclear GR fluorescence (AU) compared to the control (%) of astrocytes (n = 5) and astrocytes expressing the Cre-eGFP reporter (n =5). Each dot represents an average of 3 slices. Error bars represent mean ± SEM

Figure 4.10 – Effects of Astrocyte Glucocorticoid Receptor Knockout in the Lateral Amygdala on Fear Learning, Memory and Threat Discrimination

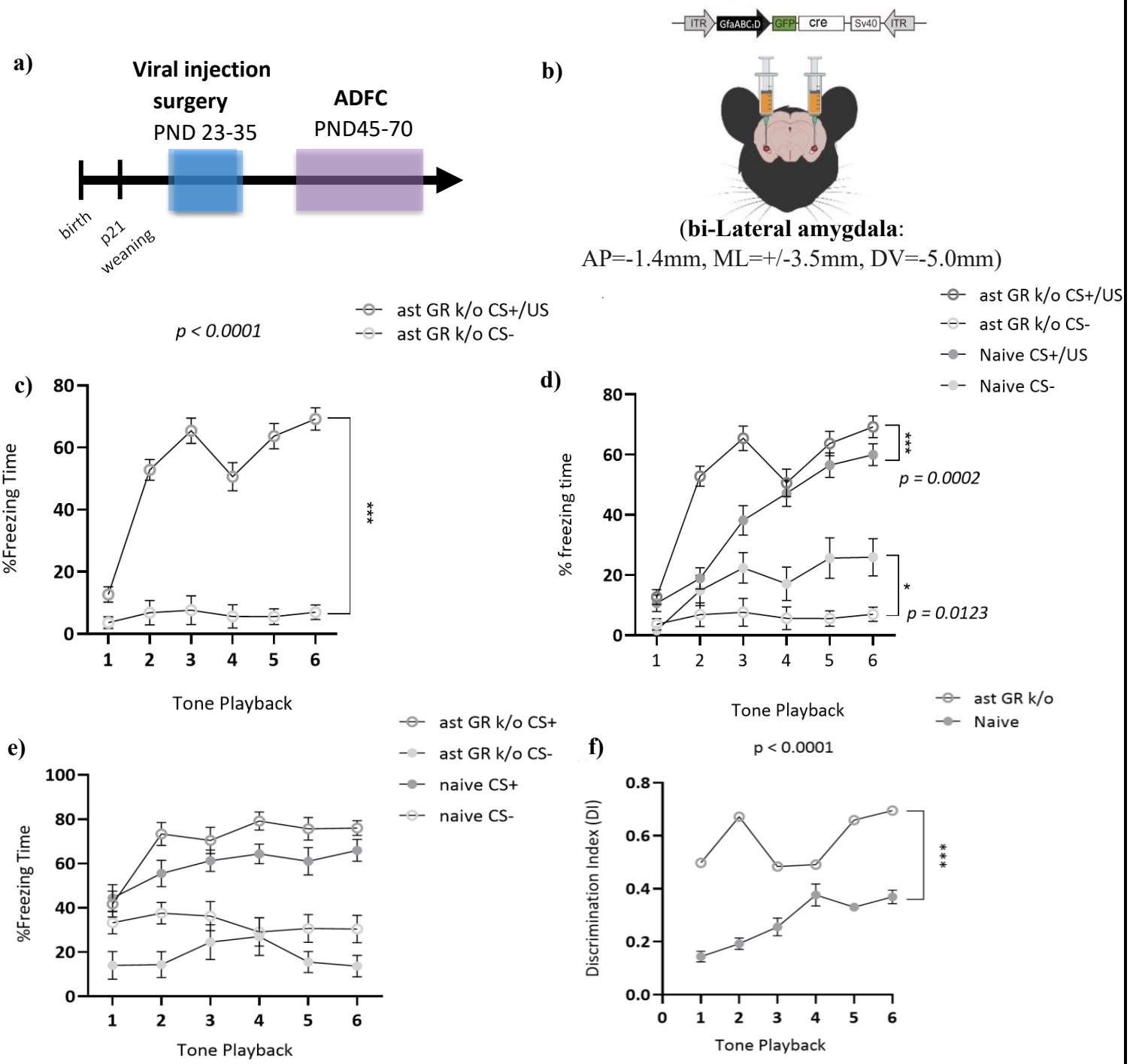

Figure 4.10. (a) A schematic representation of the experimental timeline. **(b)** A schematic representation of the viral injection of astrocyte-specific GFAP-Cre eGFP in the LA in a transgenic mouse **(c)** A line graph of the % freezing time of astrocyte GR k/o mice to the aversive association (CS+/US) (n = 14) and the neutral tone (CS-) (n = 14) over six tone presentations during conditioning **(d)** A line graph of a comparison between the % freezing time of naïve mice to the aversive association (CS+/US) (n = 20) and the neutral tone (CS-) (n = 13) and of astrocyte GR k/o mice to the aversive association (CS+/US) (n = 14) and the neutral tone (CS-) (n = 14) over six tone presentations during conditioning. **(e)** A line graph of a comparison between the % freezing time of naïve mice to the aversive tone (CS+) (n = 20) and the neutral tone (CS-) (n = 13) and of astrocyte GR k/o mice to the aversive tone (CS+) (n = 14) and the neutral tone (CS-) (n = 14) over six tone presentations during memory recall **(f)** A line graph of a comparison between the DIs of naïve mice (n = 20) and astrocyte GR k/o mice (n =20) over 6 tone presentations. Error bars represent mean ± SEM.

Figure 4.11 – Effect of Astrocyte Glucocorticoid Receptor Knockout in the Lateral Amygdala on ELS-induced impairments on Threat Discrimination

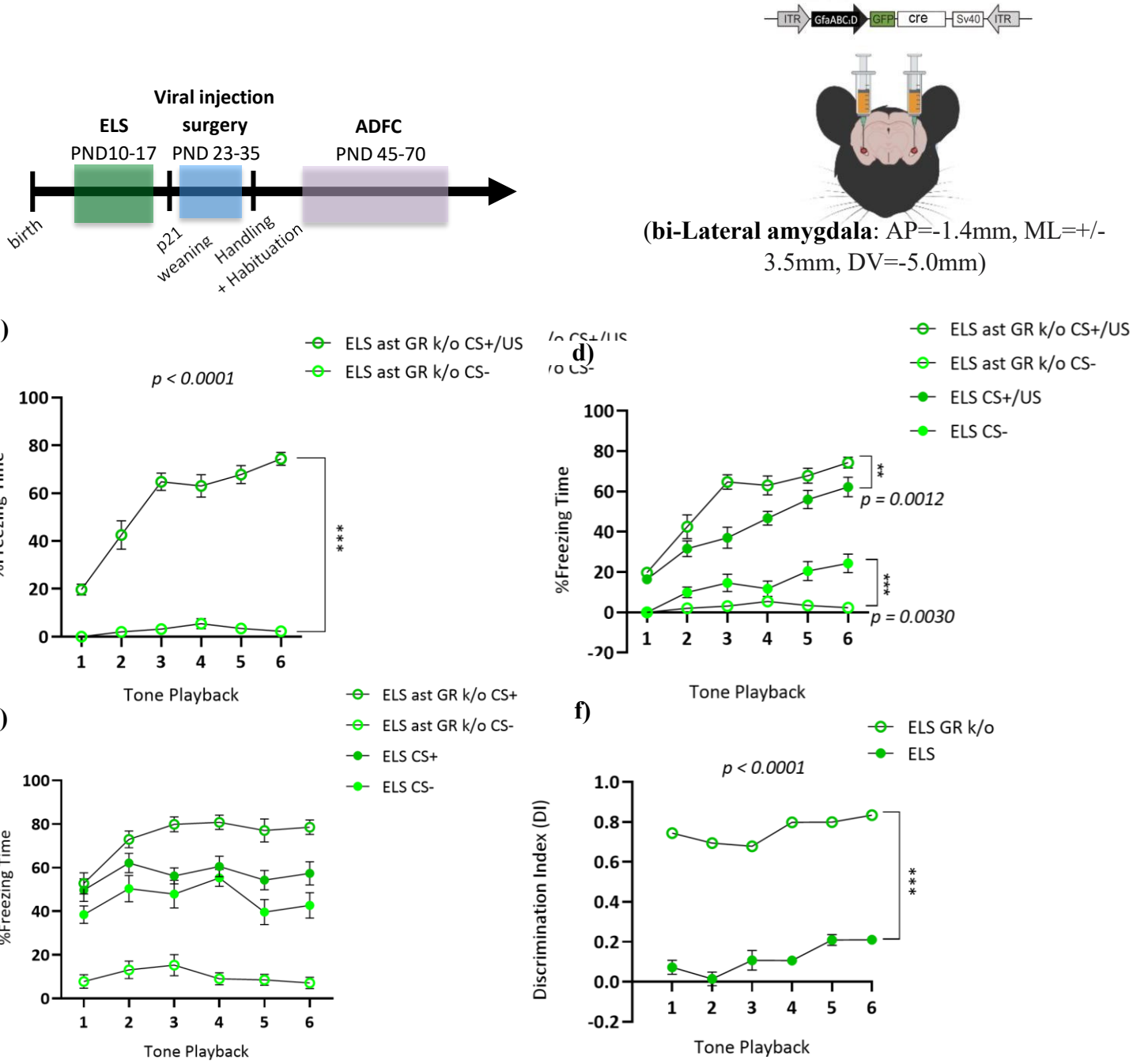

Figure 4.11. (a) A schematic of the experimental timeline. **(b)** A schematic of the viral injection of astrocyte-specific GFAP-Cre eGFP in the LA in a transgenic mouse **(c)** A line graph of the % freezing time of ELS astrocyte GR k/o mice to the aversive association (CS+/US) (n = 20) and the neutral tone (CS-) (n = 20) over six tone presentations during the conditioning phase **(d)** A line graph of a comparison between the % freezing time of ELS astrocyte GR k/o mice to the aversive association (CS+/US) (n = 20) and the neutral tone (CS-) (n = 20) and of astrocyte GR k/o mice to the aversive association (CS+/US) (n = 14) and the neutral tone (CS-) (n = 14) over six tone presentations during conditioning **(e)** A line graph of a comparison between the % freezing time of ELS astrocyte GR k/o mice to the aversive tone (CS+) (n = 20) and the neutral tone (CS-) (n = 20) and of astrocyte GR k/o mice to the aversive tone (CS+) (n = 14) and the neutral tone (CS-) (n = 14) over six tone presentations during memory recall **(f)** A line graph of a comparison between the DIs of naïve mice (n = 20) and astrocyte GR k/o mice (n =20) over 6 tone presentations. Error bars represent mean ± SEM.

Chapter 5
Results

ELS does not change anxiety-like behaviour, but it does influence exploration behaviours

To characterize the effects of ELS anxiety-like behaviour, OFT and EPM were used. OFT is a commonly used tool to assess anxiety-related behaviour in mice by exploiting the innate fear of mice to avoid open spaces, such as the center of the OFT apparatus (Ennaceur, 2012). Hence, a comparison was made between the time spent (s) and the number of entries made in the center of the OFT apparatus. Using an independent t-test, it was revealed that there was no significant difference between the time spent in the center (s) by ELS mice (n = 14) and the naïve mice (n = 9) (Independent t-test; $t = 0.9121$; $p = 0.3721$; ELS: 44.00 ± 6.714s ;Naïve: 54.20±9.268s; mean±s.e.m. ; **Figure 4.2c**) This suggests that ELS does not influence the amount of time spent in the Center of the OFT apparatus. However, it was revealed that there is a significant difference between the number the entries in the center (s) made by ELS mice (n = 14) and the naïve mice (n = 9) where ELS mice made significantly more entries into the center than naïve mice (Independent t-test; $t = 3.417$; $p = 0.0026$; ELS: 28.36 ± 2.491s ;Naïve: 15.89 ± 2.366s; mean±s.e.m.; **Figure 4.2d**). Additionally, an independent t-test was used to analyse the effect of ELS on the total distance travelled between the ELS (n = 14) and naïve (n = 9) mice. The analysis revealed that ELS mice had a significantly greater total distance travelled compared to naïve mice (Independent t-test; $t = 2.128$; $p = 0.0454$; ELS: 22.82 ± 1.602s ; Naïve: 17.04 ± 2.300s; mean±s.e.m.; **Figure 4.2e**). This suggests that although ELS does not seem to influence anxiety behaviour, it does have an impact on exploratory behaviour, indicated by the number of entries into the center and the total distance travelled

To corroborate what was find using the OFT, the EPM was used to evaluate the impact of ELS on anxiety-related behaviour (Lee et al., 2012). EPM also serves as a behavioural task that measures anxiety-related behaviours (Lee et al., 2012). However, the EPM consists of open arms and close arms where mice innately tend to avoid the open arms as these arms are not enclosed by walls and are hence, unprotected spaces. Hence, a comparison was made between the time spent (s) and the number of entries made in the open arms of the EPM apparatus. Statistical analysis by an Independent t-test revealed that there was no significant difference between the time spent in the open arms (s) by ELS mice (n = 10) and the naïve mice (n = 9) (Independent t-test; $t = 0.2635$; $p = 0.7953$; ELS: 66.64 ± 12.17s; Naïve: 71.79 ± 15.55s; mean±s.e.m.; **Figure 4.2h**). Additionally, it was revealed that there was no significant difference between the number the entries in the open arm made by ELS mice (n = 11) and the naïve mice (n = 9) (Independent

t-test; $t = 0.8526$; $p = 0.4051$; ELS: 6.182 ± 1.052 ;Naïve: 5.111 ± 0.5122; mean±s.e.m. ; **Figure 4.2i**). Lastly, an independent t-test was used to analyse the effect of ELS on the total distance travelled between the ELS (n = 11) and naïve (n = 9) mice. The analysis revealed that ELS mice had a significantly greater total distance travelled compared to naïve mice (Independent t-test; $t = 2.167$; $p = 0.0439$; ELS: 11.03 ± 1.709m; Naïve: 4.730 ± 0.6223m; mean±s.e.m.; **Figure 4.2j**). Overall, this suggests that ELS does not influence anxiety-related behaviours as assessed using the EPM however, it does have an impact on the exploratory behaviour of the mice.

Validation of the Auditory Discriminative Fear Conditioning (ADFC) Protocol

To validate the ADFC protocol developed by the Murphy-Royal lab as an appropriate test to measure fear learning and memory, a comparison was made between the percentage of time spent freezing of naïve mice to the aversive association (CS+/US) and the neutral tone (CS-) in the conditioning phase and the memory recall phase (**Figure 4.4**). ADFC is a paradigm adapted from Pavlovian Fear Conditioning, where mice learn to associate an auditory conditioned stimulus (CS+) with an aversive unconditioned stimulus (US) (Kim & Cho, 2017). To gauge if this protocol was successful, mice should display higher freezing to the CS+/US pairing than to the CS- . After successful conditioning, Naïve mice should also display a higher fear response to the CS+ compared to the CS- in the memory recall phase which is quantified by the threat discrimination index (DI).

A Repeated Measure (RM) Two-Way ANOVA was used to analyse successful learning of the aversive association over consecutive tone presentations. The analysis showed there was a significant difference between the percentage of time spent freezing to the CS+/US compared to the CS- during the conditioning phase where the naïve mice spent significantly more time freezing to the CS+/US (n = 20) than the CS- (n = 13), (RM Two-Way ANOVA: $F (1, 31) = 25.63$, $p<0.0001$; CS+/US: 38.55 ± 8.204 %; CS-: 17.92 ± 3.734 % ; mean± s.e.m.; **Figure 4.4b**). This suggests that naïve mice where able to successfully learn to associate the CS+ with an aversive context.

A RM Two-Way ANOVA was also used to analyse the ability of mice to accurately recall the conditioned stimulus in a different context over consecutive tone presentations during ADFC. The analysis showed that there was a significant difference in the percentage of time spent freezing to the CS+ and the CS- during the memory recall phase, the type of tone had a

statistically significant effect on the percentage freezing time where the naïve (tone playbacks) mice spent more time freezing to the CS+ (n = 20) than the CS- (n = 13), (RM Two-Way ANOVA: F (1, 38) = 16.33; p=0.0002; CS+: 58.77 ± 3.220 %; CS-: 32.90 ± 1.400 %; mean±s.e.m;; Figure 4.4c). This suggests that naive mice were able to accurately recall the aversive association learned one day earlier.

ELS Impairs Threat Discrimination

To assess whether ELS mice were able to be successfully learn the aversive association during ADFC, a RM Two-Way ANOVA was used to make a comparison between the percentage of time spent freezing over consecutive tone presentations of ELS mice to the CS+/US and the CS- in the conditioning phase (**Figure 4.5c**). The analysis showed that the type of tone association had a statistically significant effect on the freezing time (%) where the ELS mice spent more time freezing to the CS+/US (n = 17) pairing than the CS- (n = 17), (RM Two-Way ANOVA: F (1, 32) = 47.54; p<0.0001; CS+/US: 41.67 ± 6.869%; CS-: 13.55 ± 3.491 %; mean±s.e.m; **Figure 4.5c**). This suggests that ELS does not impair the ability of the mice to successfully learn to associate the CS+ to an aversive context.

To assess the impact of ELS on fear learning, a Repeated Measure (RM) Two-Way ANOVA was used to make a comparison of the percentage of time spent freezing to the CS+/US and to the CS- over consecutive tone presentations between ELS (n = 17) and naïve mice (n = 20) (**Figure 4.4d**). The analysis showed that there was no significant difference between ELS and naïve mice to the time spent freezing to CS+/US (RM Two-Way ANOVA: F(1, 32) = 1.116; p=0.2986; ELS CS+/US: 41.67 ± 6.869 %; Naïve CS+/US: 37.51 ± 8.363 %; mean±s.e.m.; Figure 4.5d). A RM Two-Way ANOVA was used to analyse the effect of ELS on the percentage of time spent freezing to CS- over consecutive tone presentations in the conditioning phase of both ELS (n = 17) and naïve mice (n = 13) . The analysis showed that there was no significant difference between ELS and naïve mice to the time spent freezing to CS- (F (1, 31) = 3.738; p=0.0624; ELS CS-: 13.55 ± 3.491%; Naïve CS-: 22.90 ± 4.476 %; mean±s.e.m.; **Figure 4.5d**). Overall, this suggests that ELS does not influence the ability to learn to associate the CS+ with an aversive context.

To represent threat discriminative ability, a type of cognitive function, we used a discrimination index (DI) to quantify the difference between the percentage of time spent

freezing to CS+ and CS- during the memory recall phase of the ADFC paradigm. To determine the impact of ELS on threat discriminative ability, a Two-Way ANOVA was used to analyse the difference between the DI of ELS and Naïve mice. The analysis showed that there was a significant effect of ELS on the DI where ELS mice (n = 17) had a significantly lower DI compared to Naïve mice (n= 20) (Two-Way ANOVA: $F(1, 210) = 89.84$; $p<0.0001$; ELS: 0.1202 ± 0.03162 ; Naïve: 0.2781 ± 0.03938 ; mean ± s.e.m.; **Figure 4.5f**).

ELS induces changes in CORT production during development

To characterize the effects of ELS on CORT production during the ELS protocol and during adulthood, an ELISA was used to analyse the amount of CORT in the blood of naïve and ELS mice at PND 10, before the ELS protocol; PND 17, the end of the ELS protocol and PND 45, during young adulthood. An independent t-test was used to make comparisons between the amount of CORT produced by naïve (n = 6) and ELS (n = 6) mice at PND 10 and it was found that there was no significant difference between the two groups (Independent t-test: $t = 0.5403$; $p = 0.6037$; Naïve: 9.298 ± 1.811 ng/ml; ELS: 10.85 ± 2.244 ng/ml; mean±s.e.m ; **Figure 4.6b**). This suggests that there was no difference between the amount of CORT produced prior to the ELS protocol.

A comparison was also made of the amount of CORT produced at PND 17 between naïve (n = 5) and ELS (n = 6) mice. Although the amount of CORT produced was higher in ELS mice than naïve mice there was no significant difference between the two groups. (Independent t-test: $t = 0.5701$, $p = 0.5825$; Naïve: 63.33 ± 8.435 ng/ml; ELS: 72.92 ± 13.57 ng/ml; mean±s.e.m. ; **Figure 4.6c**) This suggests that following the ELS protocol there was no difference between the amount of CORT produced between the two groups.

Lastly, a comparison was made of the amount of CORT produced at PND 45 between naïve (n = 9) and ELS (n = 6) mice. A significant difference was found between the two groups where ELS mice produced a significantly higher amount of CORT than naïve mice. (Independent t-test: $t =2.262$, $p = 0.0415$; Naïve: 50.14 ± 6.886 ng/ml; ELS: 82.24 ±14.16 ng/ml; mean±s.e.m; **Figure 4.6d**) This suggests that although the ELS protocol did not produce immediate differences during the protocol, there were long-term consequences for increased CORT

production during adulthood at PND 45 there was no difference between the amount of CORT produced between the two groups.

ELS increases in astrocyte Glucocorticoid Receptor (GR) activity in adulthood

When GR is bound to GCs, it moves from the cytoplasm to the nucleus to affect gene expression. Thus, ELS-induced increases in CORT could induce changes in the expression of GR receptors in the nucleus and/or cytoplasm or GR activity where the ratio of GR nucleus : GR cytoplasm is altered. To determine the impacts that ELS has on astrocyte glucocorticoid receptor activity via elevations in CORT productions, the relative expression of astrocyte glucocorticoid receptors in both the cytosol and nucleus of astrocytes in the lateral amygdala was quantified via fluorescence intensity (au). Using a Mann Whitney test, the levels of GR expression in the cytosol of astrocytes in the lateral amygdala was compared between naïve (n = 5) and ELS (n = 14) mice. The analysis revealed that there was no significant difference in the expression of GR in the astrocyte cytosol in the lateral amygdala between the two groups (Mann Whitney test: Mann-Whitney $U = 6$; $p = 0.1560$; Naïve: $3.598 \times 10^6 \pm 7.730 \times 10^5$ au; ELS: $2.588 \times 10^6 \pm 6.605 \times 10^6$ au; mean±s.e.m.; **Figure 4.7c**). This suggests that ELS does not change the expression levels of GR in the cytosol of astrocytes in the lateral amygdala.

Using a Mann Whitney Test, the levels of GR expression in the nucleus of astrocytes in the lateral amygdala was compared between naïve (n = 5) and ELS (n = 14) mice. The analysis revealed that there was not a significant difference in the expression of GR in the nucleus of astrocytes in the lateral amygdala between the two groups (Mann Whitney test: Mann-Whitney $U = 23$; $p = 0.2976$; Naïve: $2.129 \times 10^5 \pm 5.123 \times 10^4$ au; ELS: $3.671 \times 10^5 \pm 7.681 \times 10^4$ au; mean±s.e.m.; **Figure 4.7d**). This suggests that ELS does not affect the expression levels of GR in the nucleus of astrocytes in the lateral amygdala.

Using a Mann Whitney Test, the ratio of levels of GR expression between the nucleus and cytosol was calculated as a measure of GR activity of astrocytes in the lateral amygdala, was compared between naïve (n = 5) and ELS (n = 14) mice. The analysis revealed that there was a significant difference in the ratio expression of GR between the nucleus and cytosol of astrocytes in the lateral amygdala between the two groups where ELS mice expressed higher levels of GR in the nucleus of astrocytes in the lateral amygdala than naïve mice (Mann Whitney Test: Mann Whitney $U = 13$; $p = 0.0437$; Naïve: 0.0648 ± 0.0147 au; ELS: 0.1868 ± 0.03739 au;

mean±s.e.m.; **Figure 4.7e**). This suggests that ELS upregulates astrocyte GR activity in the lateral amygdala.

ELS decreases astrocyte protein expression and density

Once activated GR is translocated to the nucleus and influences genetic expression thus having implications for protein expression levels (Crossin et al., 1997; Tertil et al., 2018a). ELS-induced changes in astrocyte GR activity in the LA could correlate with changes in astrocyte-related protein expression and astrocyte cell density (Crossin et al., 1997; O'Callaghan et al., 1989). To determine the impacts of ELS on astrocyte proteins and density, the expression of GFAP, an astrocyte cytoskeletal protein and Cx43, an astrocyte-specific gap junction protein quantified via fluorescence intensity (au). Astrocyte density was quantified by counting the number of S100ß positive cells. Using an Independent t-test, the levels of GFAP expression was compared between naïve (n = 6) and ELS (n = 16) mice. A significant difference in the expression of GFAP in the LA between the two groups was observed where ELS mice had significantly lower astrocyte protein expression compared to naïve mice (Independent t-test: t = 3.498, p = 0.0023 ; Naïve: $1.095 \times 10^7 \pm 4.043 \times 10^6$ au; ELS: $2.263 \times 10^6 \pm 4.462 \times 10^5$ au; mean±s.e.m.; **Figure 4.8c**). This suggests that ELS significantly decreases the astrocyte expression levels of GFAP in the in the lateral amygdala.

Using an Independent t-test, the levels of Cx43 expression were compared between naïve (n = 6) and ELS (n = 14) mice. The analysis revealed that there was a significant difference in the expression of Cx43 in the LA between the two groups where ELS mice expressed significantly lower levels of Cx43 than naïve mice. in the astrocyte cytosol in the lateral amygdala between the two groups (Independent t-test: t = 2.533, p = 0.0208 ; Naïve: $3.219 \times 10^8 \pm 8.189 \times 10^6$ au; ELS: $1.327 \times 10^7 \pm 3.480 \times 10^6$ au; mean±s.e.m.; **Figure 4.8d**). This suggests that ELS significantly decreases the expression levels of Cx43 in the in the lateral amygdala.

Using an Independent t-test, the number of S100B+ cells were calculated as a measure of astrocyte cell density in the LA was compared between naïve (n = 5) and ELS (n = 6) mice. The analysis revealed that there was a significant difference in astrocyte cell density between the two groups where ELS mice have a significantly lower number of S100B cells compared to naïve mice (Independent t-test: t = 2.277, p = 0.0488 ; Naïve: 11.60 ± 1.208; ELS: 8.333 ± 0.8433;

mean±s.e.m.; **Figure 4.8e**). This suggests that ELS results in a decrease in astrocyte cell density in the LA.

Validation of Astrocyte Specific Glucocorticoid Receptor knockout in the Amygdala

To induce the ablation of astrocyte glucocorticoid receptors in the LA, a viral construct containing an astrocyte-specific Cre-eGFP reporter was injected into the LA of transgenic mice expressing a floxed N3RC1 gene. To validate the effectiveness of the expression of the virus in astrocytes in the LA of the mice injected, the number of astrocytes in and outside the ROI was quantified using S100ß fluorescence in the amygdala; the number of cells expressing the GFP-Cre reporter in (ROI +) and outside the ROI (ROI -) was quantified using GFP fluorescence and finally, the number of astrocytes expressing the GFP-Cre reporter was quantified using the GFP fluorescence of $S100ß^{+}$ cells.

To confirm that there were astrocytes in and outside the ROI, an Independent t-test was used to compare the number of ROI+ astrocytes (n = 5) and ROI - (n = 5) and no significant difference was found between the two regions (Independent t-test: t = 0.1697, p = 0.8695 ; ROI +: 23.60 ± 1.939; ROI - : 24.20 ± 2.956; **Figure 4.9b**). This suggests that the number of astrocytes in ROI + and ROI- are comparable.

To confirm that the GFP-Cre reporter was being expressed in the ROI, a Mann-Whitney U test was used to compare the number of cells expressing the GFP-Cre reporter in (ROI +) and outside the ROI (ROI -). It was found that there was a significantly higher number of cells expressing the GFP-Cre reporter in ROI + than in ROI- (Mann Whitney U test: U = 0, p = 0.0079; ROI+:13.60 ± 1.800; ROI-: 1.800 ± 0.7348; mean±s.e.m.; **Figure 4.9c**). This suggests that the cells in the ROI+ expressed a higher level of GFP-Cre reporter than the cells within the ROI-.

To confirm that the cells expressing GFP in the ROI+ were astrocytes, the percentage of S100B+ and GFP + cells in the ROI + and ROI – were compared using an Independent t-test. It was found that there was a significantly higher percentage of astrocytes expressing the GFP-Cre reporter in the ROI + than the ROI- (Independent t-test: t = 7.247, p < 0.0001; ROI +: 57.00 ± 5.814 %; ROI-: 6.200 ± 3.917 %; mean±s.e.m, **Figure 4.9d**). This suggests that the ROI contains astrocytes that express the GFP-Cre reporter.

To confirm the knockout of glucocorticoid receptors in astrocytes, the nuclear GR

expression as % of the controls was quantified in control astrocytes and astrocytes expressing the eGFP reporter in the same brain slice using an Independent t-test. It was found that the percentage of nuclear GR expression in astrocytes expressing the GFP-Cre reporter was significantly lower than the control astrocytes within the same brain slice (Independent t-test: t = 3.904, p = 0.0003; controls: 100 ± 0 %; GFAP eGFP-Cre: 56.26 ± 4.681%; mean±s.e.m, **Figure 4.9g**). This suggests that the astrocytes expressing the eGFP-Cre reporter had lower expression levels of nuclear GR than astrocytes not expressing the eGFP-Cre reporter. Overall, these data suggest that the viral construct with the GFAP astrocyte specific GFP-Cre reporter successfully induced glucocorticoid receptor knockout in astrocytes in the amygdala, the site of injection.

Astrocyte Glucocorticoid Receptor Knockout Improves Fear Learning and Threat Discrimination

To assess whether the knockout of glucocorticoid receptor activity in astrocytes contribute to the expression of fear learning, memory, and threat discrimination, ADFC was used to make a comparison between the percentage of time spent freezing over consecutive tone presentations of naive mice and astrocyte GR k/o mice in the LA, to the CS+/US and the CS- in the conditioning phase (**Figure 4.10**). A Repeated Measure (RM) Two-Way ANOVA was used to analyse the effect of the type of tone association on the percentage of time spent freezing by the astrocyte GR k/o mice in the conditioning phase. The analysis showed that the type of tone association had a statistically significant effect on the freezing time (%) where the astrocyte GR k/o mice spent more time freezing to the CS+/US (n = 14) pairing than the CS- (n = 14), (RM Two Way ANOVA: F (1, 32) = 47.54; p<0.0001; CS+/US: 52.43 ± 8.487%; CS-: 6.054 ± 0.5835 %; mean±s.e.m;; **Figure 4.10c**). This suggests that astrocyte GR k/o mice were able to learn the aversive association successfully.

To assess the impact of ablated astrocyte GR activity in the LA on fear learning, ADFC was used to make a comparison of the percentage of time spent freezing to the aversive association (CS+/US) and to the neutral cue (CS-) over consecutive tone presentations between astrocyte GR k/o mice and Naïve mice in the conditioning phase (**Figure 4.10d,e**). A Repeated Measure (RM) Two-Way ANOVA was used to analyse the effect of ablated astrocyte GR activity in the LA on the percentage of time spent freezing to CS+/US over consecutive tone presentations in the conditioning phase of both astrocyte GR k/o mice (n = 14) and naïve mice (n

= 20) . The analysis showed that there was a significant difference between astrocyte GR k/o mice and naïve mice to the time spent freezing to CS+/US where astrocyte GR k/o mice had a significantly higher freezing time compared to naïve mice (RM Two Way ANOVA: F (1, 32) = 17.77; p = 0.0002; astrocyte GR k/o: 41.67 ± 8.487 %; Naïve: 38.55 ± 8.204%; mean±s.e.m.; **Figure 4.10e**). A Repeated Measure (RM) Two-Way ANOVA was used to analyse the effect of ablated astrocyte GR activity in the LA on the percentage of time spent freezing to CS- over consecutive tone presentations in the conditioning phase of both astrocyte GR k/o mice (n = 14) and naïve mice (n = 13). The analysis showed that there was a significant difference between astrocyte GR k/o mice and naïve mice to the time spent freezing to CS- where astrocyte GR k/o mice had a significantly higher freezing time compared to naïve mice (RM Two Way ANOVA: F (1, 25) = 7.277; p = 0.0123; astrocyte GR k/o CS-: 6.054± 0.5835%; Naïve CS-: 17.92 ± 3.734%; mean±s.e.m.; **Figure 4.10e**).

To represent threat discriminative ability, we used a discrimination index (DI) to quantify the difference between the percentage of time spent freezing to CS+ and CS- during the memory recall phase of the ADFC paradigm. To assess the impact of ablated astrocyte GR activity on threat discrimination, DI was used to make a comparison of difference in the ability to distinguish between the aversive tone (CS+) and the neutral tone (CS-) between astrocyte GR k/o mice and naïve mice in the memory recall phase **(Figure 4.10d)**. A Two-Way ANOVA was used to compare the DI over consecutive tone presentations inf both astrocyte GR k/o (n = 14) and naïve mice (n = 20) . The analysis showed that there was a significant difference between DI of astrocyte GR k/o mice and naïve where mice astrocyte GR k/o mice had a significantly higher DI than naïve mice (Two-Way ANOVA: F (1, 192) = 214.8; p<0.0001; astrocyte GR k/o mice: 0.5831 ± 0.04158; Naive: 0.2781 ± 0.03938; mean±s.e.m.; **Figure 4.10f)**. This suggests that ablated astrocyte GR activity in the LA enhances threat discriminative ability.

Astrocyte Glucocorticoid Receptor Knockout Improves Fear Learning and Recovers ELS-induced Threat Discrimination

Once it was determined that astrocyte GR signalling significantly contributes to the expression of fear learning and memory, it was assessed whether ablating astrocyte GR in the LA of ELS mice would recover ELS-induced deficits in fear learning and memory (**Figure 4.11**). To assess whether ELS with astrocyte GR k/o were able to learn the fear association properly, a

Repeated Measure (RM) Two-Way ANOVA was used to compare the percentage of time spent freezing to the CS+/US and the CS- by the ELS with astrocyte GR k/o mice in the conditioning phase. The analysis showed that the type of tone association had a statistically significant effect on the freezing time (%) where the ELS with astrocyte GR k/o mice spent more time freezing to the CS+/US (n = 20) pairing than the CS- (n = 20), (RM Two Way ANOVA: F (1, 38) = 402.2; p<0.0001; CS+/US: 55.37 ± 8.358%; CS-: 2.738 ± 0.7346%; mean±s.e.m; **Figure 4.11c**). This suggests that ELS with astrocyte GR k/o mice were able to learn the aversive association successfully.

To assess the impact of ablated astrocyte GR activity in ELS-induced deficits on fear learning, ADFC was used to make a comparison of the percentage of time spent freezing to the aversive association (CS+/US) and to the neutral cue (CS-) over consecutive tone presentations between ELS mice and Naïve mice in the conditioning phase (**Figure 4.11d**). A Repeated Measure (RM) Two-Way ANOVA was used to analyse the effect of ablated astrocyte GR signalling in ELS on the percentage of time spent freezing to CS+/US over consecutive tone presentations in the conditioning phase of both ELS with astrocyte GR k/o (n = 20) and ELS mice (n = 17) . The analysis showed that there was a significant difference between ELS with astrocyte GR k/o and ELS mice to the time spent freezing to CS+/US where ELS with astrocyte GR k/o mice spent significantly more time freezing to CS+/US than ELS mice (RM Two-Way ANOVA: F (1, 35) = 12.43, p = 0.0012; ELS with astrocyte GR k/o CS+/US: 55.37 ± 8.358%; ELS CS+/US: 41.67 ± 6.869 % ; **Figure 4.11d**). This suggests that ablated astrocyte GR signalling in the LA enhances fear learning to the CS+/US even after ELS.

A Repeated Measure (RM) Two-Way ANOVA was used to analyse the effect of ablated astrocyte GR signalling in ELS on the percentage of time spent freezing to CS- over consecutive tone presentations in the conditioning phase of both ELS with astrocyte GR k/o (n = 20) and ELS mice (n = 17) . The analysis showed that there was a significant difference between ELS with astrocyte GR k/o and ELS mice to the time spent freezing to CS- where ELS with astrocyte GR k/o mice spent significantly less time freezing to CS- than ELS mice (RM Two-Way ANOVA: F (1, 35) = 16.52, p = 0.0003; ELS with astrocyte GR k/o CS-: 2.738 ± 0.7346%; ELS CS-: 13.55 ± 3.491% ; **Figure 4.11d**). This suggests that ablated astrocyte GR signalling in the LA enhances fear learning to the CS+/US even after ELS.

To represent threat discriminative ability, a type of cognitive function, we used a

discrimination index (DI) to quantify the difference between the percentage of time spent freezing to CS+ and CS- during the memory recall phase of the ADFC paradigm. To determine the impact of ablated astrocyte GR signalling on ELS-induced impairments in threat discriminative ability, a Two-Way ANOVA was used to analyse the difference between the DI of ELS with astrocyte GR k/o and ELS mice. The analysis showed that there was a significant effect of ablated astrocyte signalling on ELS on the DI where ELS with astrocyte GR k/o mice (n = 20) had a significantly higher DI compared to ELS mice (n= 17) (Two-Way ANOVA: $F(1, 210) = 827.6$; $p<0.0001$; ELS with astrocyte GR k/o: 0.7584 ± 0.02563; ELS: 0.1202 ± 0.03162; mean ± s.e.m; **Figure 4.11f**). This suggests that ablated astrocyte GR activity in the LA is able to recover ELS-induce deficits in threat discriminative ability.

Chapter 6
Discussion

Summary of main results

The goal of this project was to investigate the role of astrocytes in ELS-induced behavioural dysfunction. We hypothesised that ELS, through alterations in astrocyte glucocorticoid signalling disrupts amygdala-dependent behaviour. While we observed that ELS did not lead to changes in anxiety-like behaviour, ELS did, however, result in an increase in exploratory behaviour in both OFT and EPM behavioural tasks. To assess LA-dependent behaviour, we established a valid ADFC protocol. While we found that ELS did not impact fear learning it did impact fear memory and cognitive processing as shown by a significant impairment of threat discriminative ability. We also report that ELS induced increases in CORT production during adolescence but not prior to or immediately after ELS. Additionally, ELS produced an increase in astrocytic GR activity. Following this, we found that ELS produced reductions in GFAP and Cx43 protein expression levels in astrocytes as well as a reduction in astrocyte cell density in the amygdala. Once we had established a valid knockdown of astrocytic GR in the LA, we found that targeting astrocyte GR signalling in the LA improved cognitive performance in non-ELS mice. Lastly, we found that ablating GR signalling in ELS mice was able to rescue ELS-induced impairments in cognitive performance. Based on these findings, we were able to successfully implicate astrocyte glucocorticoid signalling in he LA in the mechanism by which ELS produces cognitive dysfunction, suggesting that astrocytes are crucial components in the pathological mechanisms of ELS on behaviour.

ELS does not influence anxiety-like behaviours in mice but does influence exploratory behaviour.

Initially, we set out to characterise the impact of ELS on amygdala-dependent behaviours, including anxiety and fear. However, we report that ELS does not influence anxiety-like behaviour. While we expected that ELS mice would spend less time in the centre than naïve mice, we observed that ELS did not influence the amount of time spent in the center of the OFT apparatus and the amount of time spent in the open arms of the EPM apparatus. The BLA is known for its role in both fear and anxiety (Tovote et al., 2015). However, there exists circuitry in the amygdala that either heighten or decrease anxiety (Tovote et al., 2015). Research indicates that the circuitry within the amygdala subnuclei, including the BLA, have opposing functions in anxiety (Tye et al., 2011). ELS has been associated with BLA hyperexcitability (Sharp, 2017),

hence it is possible that ELS results in the excitation of both opposing neural circuitries underlying anxiety in the BLA at the cellular and molecular level, thus masking any changes in anxiety expressed at the behavioural level. Additionally, literature indicates a heterogeneity in the affects of ELS on anxiety-like behaviours, particularly in rodent models (Wang et al., 2020). A meta-analysis performed by Wang et. al (2020) investigated the effects of ELS on behaviours relevant to psychopathology, such as anxiety, in rodents. Particularly they focused on the impact of maternal separation on anxiety-like behaviours as measured by EPM and OFT. Interestingly they found that although maternal separation resulted in an increase in anxiety-like behaviours in rats, they did not find the same in mice (Wang et al., 2020). These results imply that although mice have been shown to be invaluable tools of translation for ELS on behaviour (Walker et al., 2017), mouse models of ELS may not be useful for investigating the mechanisms of ELS on anxiety-like behaviours.

Interestingly, we report that ELS influences exploratory behaviours in mice. ELS mice made significantly more entries into the center of the OFT apparatus and travelled significantly more in both the OFT and EPM apparatuses compared to naïve mice. This suggests that ELS results in enhanced exploration in mice. Evidently, research has shown that exploration of novelties can be used as a coping mechanism to decrease uncertainty in stressful situations(Aberg et al., 2021; Wilson et al., 2014). Hence mice that have experienced ELS are prone to the utilisation of exploratory behaviour to self-soothe during anxiety-inducing environments.

The validation of ADFC as a behavioural protocol to assess LA-dependent behaviour

In order to assess LA-dependent behaviour we established a protocol that assayed auditory discrimination in mice. A study by Kim & Cho (2017), was one of the first to identify the role of the LA in auditory fear discrimination by inducing a pharmacogenetic deletion of a neuronal ensemble within the LA that resulted in fear generalisation, the inability to accurately distinguish between a previously learned tone and a neutral one (W. bin Kim & Cho, 2017). This was assessed using ADFC (W. bin Kim & Cho, 2017). We report that the ADFC protocol developed by the Murphy-Royal lab is a valid tool for assessing LA-dependent behaviour, particularly fear learning, memory, and threat discrimination, in mice. Indeed, using the ADFC protocol we established, we found that naïve mice were accurately able to associate the conditioned stimulus (CS+) with an aversive context (US) and successfully recall this

association. Using DI, we were then able to quantify cognitive performance by assessing the ability of the mice to accurately differentiate between CS+ and CS-.

ELS impairs cognitive processing without impacting fear learning

Using ADFC to assess the impact of ELS on fear conditioning in mice, we report that while ELS preserves fear learning, it disrupts fear memory and consequently threat discrimination. We observed that ELS produces a significant enhancement in freezing in mice to CS- during memory recall that translated to a diminished threat discriminative ability as quantified by the discrimination index (DI). The LA serves as the neural basis of threat discrimination (Grosso et al., 2018). Sensory information regarding both the CS+ and the CS- converge in the LA which then projects downstream to amygdala nuclei to produce defensive responses (i.e freezing) (Johansen et al., 2011b). An ELS-induced impairment in the ability to distinguish between threatening and neutral contexts suggests that ELS modifies developing LA neural circuitry, producing a hyperexcitability of the LA that causes the overexpression of freezing of mice to a neutral context. It is this hyperexcitability of the LA that may form the basis of stress susceptibility, resulting in behavioural dysregulation.

ELS causes elevations in CORT production

ELS-induced behavioural dysregulation has been associated with disturbances in HPA-axis function (Juruena et al., 2020; Pariante & Lightman, 2008; Tarullo & Gunnar, 2006). Hence, we aimed to determine the impact of ELS on glucocorticoid production. Using an ELISA to assay blood CORT levels in mice, we investigated the impact of ELS on CORT secretion by measuring CORT levels before ELS (PND 10), immediately after ELS (PND 17) and during adolescence (PND 45). We observed that ELS did not alter CORT production immediately after ELS, but we did find that ELS produced a significant increase in CORT during adolescence. Although ELS has been associated with chronic elevations in CORT (Bunea et al., 2017), research also demonstrates that mice experience a stress hyporesponsive period (SHRP) between PND 4 and PND 14 (Cirulli et al., 1994). During this period, there is a decreased responsiveness of the HPA axis to stressors and stress-induced elevations in CORT. SHRP, by resisting changes to basal CORT levels, serves as a protective mechanism against the deleterious effects of elevated CORT on the developing nervous system (Bunea et al., 2017; de Kloet et al., 1999; Keller et al., 2016; Zajkowska et al., 2022). However, following PND 14, stress-induced

elevations in CORT are observed (Tarullo & Gunnar, 2006), which coincides with what we observe in our results. Thus, following PND 14, the developing neural circuitry is left vulnerable to chronic increases in stress-induced CORT levels.

ELS causes elevations in astrocyte GR translocation to the nucleus

Changes in CORT levels have consequences for the activity of their associated receptors. Astrocyte end-feet contact the endothelial cells of the cerebrovascular vessel and express a high level of GRs (Kröll et al., 2009; Murphy-Royal et al., 2020), a receptor known to respond to stress-induced increases in CORT in the blood (Finsterwald & Alberini, 2014), and as such may be affected by ELS-induced elevations in CORT. Hence, by using immunohistochemistry to stain for GRs in the amygdala, we investigated the impacts of ELS and ELS-induced increases in CORT on the expression of and activity of astrocytic glucocorticoid receptors. We report that ELS did not produce a significant change in GR expression levels in astrocytes in the amygdala. However, we found that that mice with ELS had a significantly higher presence of astrocytic GRs in the nucleus compared to the cytoplasm relative to naïve mice. GRs are ligand-bound transcription factors and once bound to CORT, GRs translocate from the cytoplasm to the nucleus to affect gene expression (Carter et al., 2013a; Myers et al., 2014). As such, our data suggests that ELS, through elevations in CORT, results in increased translocation of GRs from the cytoplasm to the nucleus where they potentially influence astrocyte-related gene expression.

ELS decreases the expression of GFAP and Cx43 and reduces astrocyte density

Increased glucocorticoid receptor activity in astrocytes has been shown to induce structural changes within these cells (Carter et al., 2013a; Crossin et al., 1997; O'Callaghan et al., 1989). Hence, we aimed to characterise the impacts of ELS on astrocytes. Using immunohistochemistry, we assessed the impact of ELS on the protein composition of astrocytes as well as astrocyte density in the amygdala. We report that ELS resulted in significant reductions in GFAP, an astrocyte structural protein and in Cx43, an astrocyte specific gap junction channel protein. We also report that ELS resulted in significant reductions in astrocyte density. Overall, our data suggests that ELS compromises the structural integrity of astrocytes as seen by reductions in protein levels and overall cell density. Research has associated glucocorticoid receptor activity in astrocytes with the regulation of the astrocyte transcriptome (Carter et al., 2013). Hence it is likely that ELS, by inducing increases in CORT levels and GR

translocation to the nucleus, reduces the genetic expression of crucial astrocyte proteins such as GFAP and Cx43. Further supporting that ELS induces astrocyte structural defects via astrocyte glucocorticoid signalling, alterations in glucocorticoid receptor pathways have been shown to regulate astrocyte proliferation (Crossin et al., 1997) coinciding with the reductions in astrocyte density in the amygdala. Overall ELS-induced alterations in astrocyte GR signalling has consequences for astrocyte integrity and resultingly astrocyte function, including the regulation of behaviour. Although evidence exists regarding the impact of astrocyte glucocorticoid signalling on behaviour (Skupio et al., 2019; Tertil et al., 2018b), the role of astrocyte glucocorticoid signalling in ELS-induced behavioural deficits are unknown.

Astrocyte GR KO enhances cognitive performance in mice

The role of astrocyte GR signalling in the LA is undetermined. Using a astrocyte GR "floxed" transgenic mouse model and ADFC, we set out to determine the role of astrocyte glucocorticoid signalling in LA-dependent behaviour, particularly auditory discrimination learning and memory. We report that the ablation of astrocyte GR expression in the LA resulted in significant enhancements in fear learning and fear discriminative ability. These results suggest that astrocyte GR signalling is implicated in the expression of fear-related behaviour. ADFC is an innately stressful behavioural paradigm due to the administration of the electric foot shock and the recall of the aversive memory through the representation of the CS+. The improvement of fear learning, memory and threat discriminative ability suggests that the ablation of astrocyte GR expression serves as a protective mechanism against the acute but intense stress associated with the aversive US and CS+. Astrocyte GRs, once stimulated, have been shown to respond via alterations in their transcriptional and metabolic profiles (Carter et al., 2013; Tertil et al., 2018). This suggests that the metabolic support that astrocytes provide to neurons may be compromised in such a way that it contributes to stress-induced hyperexcitability of the LA underlying the overgeneralisation of the fear response that we see in our ELS mice. Hence, by knocking down astrocyte GR expression and disrupting astrocyte GR signalling, we are potentially removing the avenue with which stress induces impairments in LA-dependent behaviour.

The ablation of astrocytic glucocorticoid signaling in the LA rescues ELS-induced impairments in cognitive function

Using a GR "floxed" transgenic mice with an experience of ELS and ADFC, we set out to determine whether ablating astrocyte GR activity would rescue ELS-induced deficits in fear discrimination. We report that the ablation of astrocyte GR expression in the LA enhanced fear learning in ELS mice. We also report that the ablation of astrocyte GR signalling in the LA rescued ELS-induced deficits in cognitive performance as seen by a significant increase in threat discriminative ability. These results suggest that astrocytes via glucocorticoid signalling are implicated in the mechanisms with which ELS produces behavioural impairments. Our data suggests that ELS produces LA hyperexcitability through the exposure of chronic increases in CORT in the developing brain as seen by an overgeneralisation of the fear response to CS- resulting in diminished threat discriminative ability. ELS-induced enhancements in CORT results in increased astrocyte GR activity that is associated with reduced protein expression and cell density, having negative consequences for astrocyte regulation of fear behaviour. However, we see that by ablating astrocyte GR expression in the LA we are able to protect against pathological increases in astrocyte GR expression, rescue and enhance cognitive performance in ELS mice. However, we must consider the implications of a complete ablation of astrocyte GR expression in astrocytes. Research indicates that astrocytes are involved in behavioural flexibility, a form of cognitive processing (Lyon & Allen, 2022) where these cells can sense changes in behavioural states and influence neuronal activity by for example activating inhibitory neurotransmitters such as GABA to regulate behavioural responses (Y. Mu et al., 2019). Astrocyte GRs are sensitive to changes in stress-induced changes in CORT, thereby providing a cue to initiate adaptive behaviours to a stressful situation (Murphy-Royal et al., 2020). Indeed, in stressful situations, astrocytes may contribute to a necessary level of vigilance that enhances defensive responses not only to what is clearly known as a threatening stimulus but also to unknown stimuli that may be potentially threatening. We observe a hypervigilance to threatening stimuli and severely diminished vigilance to unknown stimuli in mice with ablations of astrocyte GR expression which may be maladaptive in alternative contexts. Hence, it is crucial to identify methods or tools to mediate astrocyte glucocorticoid signalling to mitigate impairments in cognition but to preserve behavioural flexibility.

Limitations and Future Directions

Although, HPA axis dysfunction is a well-evidenced component in the mechanism by which ELS produces behavioural dysfunction (Tarullo & Gunnar, 2006), there are additional physiological systems that may be disrupted by ELS, such as immune and inflammatory response systems. It is known that stress can produce inflammation which has implications for the integrity of neural tissues including astrocytes (Andersen, 2022) and for behaviours such as cognition (Farso et al., 2013). Astrocytes are known to enter a state of astrogliosis in response to inflammatory cytokines (Little & O'Callaghan, 2001) which may have negative implications for astrocyte function, particularly for their role in the regulation of behaviour. Additionally, inflammation is not completely distinct from HPA axis disruption. Indeed, inflammation has been shown to compromise the HPA-axis (Hueston & Deak, 2014) that may have implications for behaviour. In the future, it would be useful to measure inflammatory markers in the blood following ELS and correspond these findings with analyses of astrocyte size as size increases during astrogliosis following the exposure to cytokines(Little & O'Callaghan, 2001). A meta-analysis study performed by (Baumeister et al., 2016) demonstrated that proinflammatory markers CRP, Interleukin-6 and tumor necrosis factor 6, have been observed in individuals with adverse childhood experiences(Baumeister et al., 2016). Hence it would be important to target these cytokines, particularly IL-6 which have been associated with astrocyte activity(Choudhary et al., 2021). However, the interaction between these cytokines and astrocytes in the context of ELS is undetermined.

Astrocytes have a host of functions, ranging from homeostasis, involvement in synaptic activity to metabolic support (Durkee & Araque, 2019; Sofroniew & Vinters, 2010)that modulate neuronal function. These astrocyte-to-neuron interactions subserve behavioural expression(Lyon & Allen, 2022). It would be important to investigate how these astrocyte functions are individually impacted by ELS and how they correlate with ELS-induced behavioural dysfunction as we have shown here. For example, it would be beneficial to investigate the role of astrocytes in the modulation of ELS-induced hyperexcitability in the LA. For example, GABAergic interneurons are responsible for modulating the excitation at cortical and thalamic afferents into the LA. Astrocytes have been shown to modulate GABAergic activity (Losi et al., 2014; Lyon & Allen, 2022), may regulate the inhibition of these GABAergic interneurons, consequently modulating the excitability of the LA. This could be investigated using

chemogenetic or optogenetic stimulation of astrocytes in the amygdala and the assessment of GABAergic neuronal activity in the LA using patch-clamping.

The amygdala is a crucial region in the expression of behaviour; however, it does not work in isolation. It would be important to investigate the contributions of both the hippocampus and the PFC in the expression of fear learning and threat discriminative ability via the manipulation of astrocyte GR signalling in both these regions to implicate their role in ELS-induced behavioural impairments. Both the PFC and the hippocampus form are important regulators of emotive and cognitive behaviours and are also sensitive to the effects of ELS (Carballedo et al., 2013; Gee et al., 2013; Rao et al., 2010; van Harmelen et al., 2010). Additionally, the amygdala is involved and responsible for a host of behaviours, including motivation and reward. In this project we focused on anxiety and fear-related behaviours. It would provide a broader scope to this project if future projects included investigations into the role of astrocytes in various amygdala-dependent behaviours by using corresponding behavioural paradigms. For example, it would be pertinent to use tests such as the "Exploration Test" and "Contrast Test" designed by (Spangenberg & Wichman, 2018) that evaluates reward-related behaviours.

Additionally, although the translational value of rodent models for studying ELS has been well-established in literature, there is an unavoidable limit to the translation of the biological mechanisms identified using rodent models and their applicability in humans. Hence, to enhance our findings, it would be important to substantiate these findings with human brain tissue. For example, it would be important to but understandably challenging to use post-mortem tissue of individuals who have had ELS (e.g. child abuse) and a psychiatric disorder in adulthood and investigate the properties of astrocytes and astrocytic GRs in the LA of these individuals,

Conclusion

From this study, we conclude that ELS results in cognitive dysfunction through astrocyte glucocorticoid signalling in the LA. Our results show that ELS impairs threat discriminative ability while sparing anxiety-like behaviour. Additionally, we found that ELS produces significant increases in CORT production and GR activity which has implications for astrocyte integrity and consequently behaviour. Our results implicate astrocyte GR signalling, in the mechanism by which ELS induces behavioural dysfunction. Specifically, the ablation of astrocyte GR activity in the LA was sufficient to rescue cognitive impairments in threat discrimination in ELS mice. Overall, we have established astrocytes as a crucial candidate for the mitigation of ELS-related behavioural deficits in adulthood. This study provides insight into investigating GRs in astrocytes as targets for novel pharmacological and genetic therapeutic interventions for psychiatric disorders such as MDD which currently plagues the modern society,

Chapter 7
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Chapter 8
Appendix

Table 8.1. A table containing detailed statistical analysis tests and results (mean, ±s.e.m, p-value, test values (F, t or U), degrees of freedom (df)) and corresponding figures, groups and group numbers

Figure	Groups	Mean	± s.e.m	Statistical test	p-value	test value	Degrees of freedom
Figure 4.2c	ELS (n = 14) Naïve (n = 9)	ELS (44.00s) Naïve (54.20s)	ELS (6.714) Naïve (9.268)	Independent t-test	*p* = 0.3721	*t* = 0.9121	df = 21
Figure 4.2d	ELS (n = 14) Naïve (n = 9)	ELS (28.36) Naïve (15.89)	ELS (2.491) Naïve (2.366)	Independent t-test	p = 0.0024	t = 3.417	df = 21
Figure 4.2e	ELS (n = 14) Naïve (n = 9)	ELS (22.82m) Naïve (17.04m)	ELS (1.602) Naïve (2.300)	Independent t-test	p = 0.0454	t = 2.128	df = 21
Figure 4.2h	ELS (n = 10) Naïve (n = 9)	ELS (66.64s) Naïve (71.79s)	ELS (12.17) Naïve (15.55)	Independent t-test	p = 0.7953	t = 0.2635	df = 17
Figure 4.2i	ELS (n = 11) Naïve (n = 9)	ELS (6.182) Naïve (5.111)	ELS (1.052) Naïve (0.5122)	Independent t-test	p = 0.4051	t = 0.8526	df = 18
Figure 4.2j	ELS (n = 11)	ELS (11.03m) Naïve	ELS (1.709) Naïve	Independent t-test	p = 0.0439	t = 2.167	df = 18

	Naïve (n = 9)	(4.730m)	(0.6223)				
Figure 4.4b	CS+/US (n = 20) CS- (n = 13)	CS+/US (38.55%) CS- (17.92%)	CS+/US (8.204) CS- (3.734)	RM Two-Way ANOVA	$p<0.0001$	F = 25.63	df = (1,31)
Figure 4.4c	CS+ (n = 20) CS- (n = 13)	CS+ (58.77%) CS- (32.90%)	CS+ (3.220) CS- (1.400)	RM Two-Way ANOVA	$p=0.0002$	F = 16.33	df = (1,38)
Figure 4.5c	CS+/US (n = 17) CS- (n = 17)	CS+/US (41.67%) CS- (13.55%)	CS+/US (6.869) CS- (3.491)	RM Two-Way ANOVA	$p<0.0001$	F = 47.54	df = (1,32)
Figure 4.5d	ELS CS+/US (n = 17) Naïve CS+/US (n = 20)	ELS CS+/US (41.67%) Naïve CS+/US (37.51%)	ELS CS+/US (6.869) Naïve CS+/US (8.363)	RM Two-Way ANOVA	$p=0.2986$	F = 1.116	df = (1,32)
Figure 4.5d	ELS CS- (n = 20) Naïve CS- (n = 13)	ELS CS- (13.55%) Naïve CS- (22.90%)	ELS CS- (3.491) Naïve CS- (4.476)	RM Two-Way ANOVA	$p=0.0624$	F = 3.738	df = (1,31)
Figure 4.5f	ELS (n = 17) Naive (n = 20)	ELS (0.1202) Naïve (0.2781)	ELS (0.03162) Naïve (0.03938)	Two-Way ANOVA	$p<0.0001$	F = 89.84	df = (1,210)

Figure 4.6b	Naïve (n = 6) ELS (n = 6)	Naïve (9.298ng/ml) ELS (10.85ng/ml)	Naïve (1.811) ELS (2.244)	Independent t-test	p = 0.6037	t = 0.5403	df = 10
Figure 4.6c	Naïve (n = 5) ELS (n = 6)	Naïve (63.33 ng/ml) ELS (72.92ng/ml)	Naïve (8.435) ELS (13.57)	Independent t-test	p = 0.5825	t = 0.5701	df = 9
Figure 4.6d	Naïve (n = 9) ELS (n = 6)	Naïve (50.14 ng/ml) ELS (82.24ng/ml)	Naïve (6.886) ELS (14.16)	Independent t-test	*p* = 0.0415	*t* = 2.262	df = 9
Figure 4.7c	Naïve (n = 5) ELS (n = 14)	Naïve (3.598×10^6 au) ELS (3.671×10^5 au)	Naïve (7.730×10^5) ELS (6.605×10^6)	Mann Whitney test	p = 0.1560	U = 6	N/A
Figure 4.7d	Naïve (n = 5) ELS (n = 14)	Naïve (2.129×10^5 au) ELS (3.671×10^5 au)	Naïve (5.123×10^4) ELS (7.681×10^4)	Mann Whitney test	*p* = 0.2976	U = 23	N/A

Figure 4.7e	Naïve (n = 5) ELS (n = 14)	Naïve (0.0648 au) ELS (0.1868 au)	Naïve (0.0147) ELS (0.03739)	Mann Whitney test	*p* = 0.0437	U = 13	N/A
Figure 4.8c	Naïve (n = 6) ELS (n = 16)	Naïve (1.095×10^7 au) ELS (ELS: 2.263×10^6 au)	Naïve (4.043×10^6) ELS (4.462×10^5)	Independent t-test	p = 0.0023	t = 3.498	df =20
Figure 4.8d	Naïve (n = 6) ELS (n = 14)	Naïve (3.219×10^8 au) ELS (1.327×10^7 au)	Naïve (8.189×10^6) ELS (3.480×10^6)	Independent t-test	p = 0.0208	t = 2.533	df = 18
Figure 4.8e	Naïve (n = 5) ELS (n = 6)	Naïve (11.60) ELS (8.333)	Naïve (1.208) ELS (0.8433)	Independent t-test	p = 0.0488	t = 2.277	df = 9
Figure 4.9b	ROI + (n = 5) ROI- (n = 5)	ROI+ (23.60) ROI- (24.20)	ROI+ (1.939) ROI (2.956)	Independent t-test	p = 0.8695	t = 0.1697	df = 8
Figure 4.9c	ROI + (n = 5) ROI- (n = 5)	ROI+ (13.60) ROI- (1.800)	ROI+ (1.800) ROI- (0.7348)	Mann Whitney U-test	p = 0.0079	U= 0	N/A

Figure 4.9d	ROI + (n = 5) ROI- (n = 5)	ROI+ (57.00%) ROI- (6.200%)	ROI+ (5.814) ROI- (3.917)	Independent t-test	p < 0.0001	t = 7.247	df = 8
Figure 4.9g	Controls (n=5) GFAP eGFP-Cre (n = 5)	Controls (100%) GFAP eGFP-Cre (56.26%)	Controls (0) GFAP eGFP-Cre (4.681)	Independent t-test	p = 0.0003	t = 3.904	df = 8
Figure 4.10c	CS+/US (n = 14) CS- (n = 14)	CS+/US (52.43) CS- (6.054)	CS+/US (52.43) CS- (6.054)	RM Two Way ANOVA	p<0.0001	F = 47.54	df = (1,32)
Figure 4.10e	Astrocyte GR k/o (n = 14) Naïve (n = 20)	Astrocyte GR k/o (41.67%) Naïve (38.55%)	Astrocyte GR k/o (8.487) Naïve (8.204)	RM Two Way ANOVA	p = 0.0002	F = 41.67	df = (1,32)
Figure 4.10e	Astrocyte GR k/o (n = 14) Naïve (n = 13)	Astrocyte GR k/o (6.054%) Naïve (17.92%)	Astrocyte GR k/o (0.5835) Naïve (3.734)	RM Two Way ANOVA	p = 0.0123	F = 7.277	df = (1,25)
Figure 4.10f	Astrocyte GR k/o (n = 14) Naïve (n = 20)	Astrocyte GR k/o (0.5831) Naïve (0.2781)	Astrocyte GR k/o (0.04158) Naïve (0.03938)	Two Way ANOVA	p<0.0001	F = 214.8	df = (1,192)

Figure 4.11c	ELS Astrocyte GR k/o CS+/US (n=20) ELS Astrocyte GR k/o CS- (n = 20)	CS+/US (55.37) CS- (2.738)	CS+/US (8.358) CS- (0.7346)	RM Two Way ANOVA	p<0.0001	F = 402.2	df = (1,38)
Figure 4.11d	ELS Astrocyte GR k/o CS+/US (n=20) ELS CS+/US (n = 17)	ELS Astrocyte GR k/o CS+/US (55.37) ELS CS+/US (41.67)	ELS Astrocyte GR k/o CS+/US (8.358) ELS CS+/US (6.869)	RM Two-Way ANOVA	p = 0.0012	F = 12.43	df = (1,35)
Figure 4.11d	ELS Astrocyte GR k/o CS- (n=20) ELS CS- (n = 17)	ELS Astrocyte GR k/o CS- (2.738) ELS CS- (13.55)	ELS Astrocyte GR k/o CS- (0.7346) ELS CS- (3.491)	RM Two-Way ANOVA	p = 0.0003	F = 16.52	df = (1,35)
Figure 4.11f	ELS Astrocyte GR k/o (n=20) ELS (n = 17)	ELS Astrocyte GR k/o (0.7584) ELS	ELS Astrocyte GR k/o (0.02563) ELS	Two-Way ANOVA	p<0.0001	F = 827,6	df = (1,210)

		(0.1202)	(0.03162)				