

Emotional memory modulation:

How norepinephrine and glucocorticoids bias memory toward specificity or generalization

Mariana Pais

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Emotional memory modulation: How norepinephrine and glucocorticoids bias memory toward specificity or generalization

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LIST OF ABBREVIATIONS

aca – anterior commissure anterior part	MR – mineralocorticoid receptor
ACTH – adrenocorticotrophic hormone	MRI – magnetic resonance imaging
AP – anterior-posterior	NE - norepinephrine
Au – auditory cortex	PBS – phosphate-buffered saline
BCA - bichinchoninic acid	PBS-Triton – Triton X-100 in PBS solution
BLA - basolateral amygdala	PEG – polyethylene glycol
BSA – bovine serum albumin	PFA – paraformaldehyde
BNST – bed nucleus of the stria terminalis	PFC – prefrontal cortex
CA1 – cornu ammonis 1	PrL – prelimbic cortex
CA2 – cornu ammonis 2	PND – postnatal day
CA3 – cornu ammonis 3	pre-miR - precursor miR
cc – corpus callosum	pri-miR - primary miR
cc+ - corpus callosum and associated subcortical white matter	PTSD – post-traumatic stress disorder
CI – confidence interval	PVN - paraventricular nucleus
Cg – cingulate cortex	qPCR – quantitative polymerase chain reaction
cg - cingulum	ROI – region of interest
CRH – corticotropin-releasing hormone	RSC – retrosplenial cortex
CS – conditioned stimulus	RSD – retrosplenial dysgranular cortex
DI – dysgranular insular cortex	RSga – retrosplenial granular cortex part A
dhc – dorsal hippocampal commissure	RT - room temperature
DG – dentate gyrus	PreS - presubiculum
DMN – default mode network	PPC – posterior parietal cortex
DST – dexamethasone suppression test	pS232 – phosphorylated GR at Serine-232 site
DTI – diffusion tensor imaging	pS246 – phosphorylated GR at Serine-246 site
ec – external capsule	S – subiculum
Ect – ectorhinal cortex	SAT – stress adaptation test
EP – elevated platform under bright light	SAM - sympathetic-adrenal-medullary
fi – fimbria of the hippocampus	sf – septofimbrial nucleus
fmi – forceps minor	SN – substantia nigra
fMRI – functional MRI	TBS – tris-buffered saline
FOV – field of view	TBS-Tween – Tween-20 in TBS solution
GR – glucocorticoid receptor	TE – echo time
HPA – hypothalamic-pituitary-adrenal	TeA – temporal association cortex
ic – internal capsule	TMT – trimethylthiazoline
IL – infralimbic cortex	TR – repetition time
ITI – inter-tone interval	US – unconditioned stimulus
LC-NE – locus coeruleus-noradrenergic	UTR – untranslated region
LEntE – lateral entorhinal cortex external part	
miR – MicroRNA	
miRISC - miR-induced silencing complex	

*"The charm, one might say the genius, of memory
is that it is choosy, chancy, and temperamental"*
– Elizabeth Bowen, 1955



CHAPTER 1

General introduction

Emotionally arousing and stressful events tend to be better remembered than neutral ones. Emotional arousal and stress influence memory through coordinated neuroendocrine and neuromodulatory responses that trigger the release of stress hormones such as norepinephrine and glucocorticoids (Aston-Jones & Cohen, 2005; Herman et al., 2016). While early research emphasized the role of stress hormones in strengthening the consolidation of memory, accumulating evidence indicates that they also modulate more fine-grained aspects, such as memory specificity and generalization (Schwabe et al., 2022). From an adaptive perspective, prioritizing emotionally salient information is essential for survival; however, the use of previously acquired knowledge must remain flexible to be appropriately applied to future novel situations. Such characteristics are particularly relevant in stress-related psychopathologies, including post-traumatic stress disorder (PTSD), where fear overgeneralization and impaired safety learning are thought to contribute to the persistence of symptoms (Jovanovic & Ressler, 2010; Lissek, 2012).

Crucially, individuals differ markedly in their physiological response to emotional arousal and stress, as well as in the underlying noradrenergic and glucocorticoid signaling. Given the influence of these neuromodulators on memory, interindividual variability in endogenous stress hormone signaling may lead to differential memory processing of emotional events. Such differential processing, resulting in either more specific or more generalized memories, may therefore contribute to individual differences in vulnerability and treatment response in PTSD. In this thesis, I examine, in rats, how the stress hormones norepinephrine and glucocorticoids modulate memory specificity and generalization, and associated neural circuitry, with particular attention to how endogenous signaling differences influence these processes.

In the upcoming sections, I first describe the noradrenergic and glucocorticoid signaling systems involved in emotional arousal and the stress response. Then, I summarize research on the effects of these stress hormones on memory consolidation and qualitative aspects of memory, such as memory specificity and generalization. Next, I discuss the importance of these memory processes and neuromodulatory systems in the pathophysiology and treatment of stress-related psychiatric disorders, such as PTSD, and how interindividual variability in stress hormone signaling may contribute to differences in memory processing, vulnerability and treatment outcomes. Finally, I conclude with an outline of my dissertation and the specific aims of my research.

EMOTIONAL AROUSAL, STRESS AND NEUROMODULATORY SYSTEMS

Emotional arousal refers to a state of heightened physiological, neural and psychological activation elicited by salient experiences, independent of emotional valence (Ross & Van Bockstaele, 2021). This state promotes attention and vigilance, and modulates learning and memory in favor of stimuli deemed behaviorally relevant (Lee et al., 2018; McGaugh, 2018; Waterhouse & Navarra, 2019). Stress triggers a related but more complex response that occurs when demands or challenges — often called

stressors — exceed an individual's (perceived) ability to cope (McEwen, 1998). It is characterized not only by high emotional arousal, but also by the cognitive appraisal of threat, unpredictability or uncontrollability (Folkman, 2013). Thus, recruitment of neuroendocrine systems depends on coping style and level of stress (de Boer et al., 2017; Koolhaas et al., 2010).

The acute stress response is a highly adaptive mechanism that prepares the organism to cope with immediate challenges while also promoting longer-term adjustments, including behavioral adaptations based on learning and memory. This response relies primarily on the noradrenergic and glucocorticoid signaling systems, and involves tightly orchestrated actions of hormones, peptides, and neurotransmitters in both the periphery and the brain (Figure 1). Given the central role of norepinephrine and glucocorticoids in modulating learning and memory under emotionally arousing and stressful conditions (Roozendaal & McGaugh, 2011), alterations in these signaling systems are thought to contribute to the development and persistence of stress-related disorders like PTSD (Pitman, 1989).

Sympathetic-adrenal-medullary (SAM) System & Noradrenergic Signaling

Upon stressor presentation, the SAM system is rapidly activated, resulting in the local and systemic release of the catecholamines norepinephrine and epinephrine from sympathetic nerve terminals and the adrenal medulla (Mason, 1968; Smith & Vale, 2006; Ulrich-Lai & Herman, 2009). These catecholamines play a critical role in mediating the peripheral components of the “fight-or-flight” response, including increased heart rate, vasoconstriction and blood pressure. Although circulating catecholamines cannot cross the blood-brain barrier (Weil-Malherbe et al., 1959), they can indirectly modulate central noradrenergic signaling by activating β -adrenoceptors on the ascending branch of the vagus nerve (Schreurs et al., 1986). This pathway engages two noradrenergic nuclei in the brainstem: the nucleus of the solitary tract and the locus coeruleus (LC) (Clayton & Williams, 2000; Introini-Collison & Baratti, 1992; Schreurs et al., 1986; Williams & McGaugh, 1993), thereby promoting immediate behavioral responses to threat.

The locus coeruleus–noradrenergic (LC-NE) system is a key neural substrate mediating the effects of emotional arousal on the brain (Aston-Jones & Cohen, 2005; Berridge, 2008). Located in the dorsal pons, the LC presents highly divergent and widespread noradrenergic projections to limbic, cortical and cerebellar regions, among others (Foote et al., 1983). Noradrenergic receptors, including α - and β -adrenoceptors, are widely distributed throughout the brain, with receptor subtypes showing distinct yet partly overlapping patterns of expression and intracellular effects (Civantos Calzada & Aleixandre de Artiñano, 2001; Molinoff, 1984). These anatomical characteristics enable the LC-NE system to dynamically reconfigure large-scale brain networks, activating those that prioritize processing of salient stimuli while suppressing others (Hermans et al., 2014; Zerbi et al., 2019).

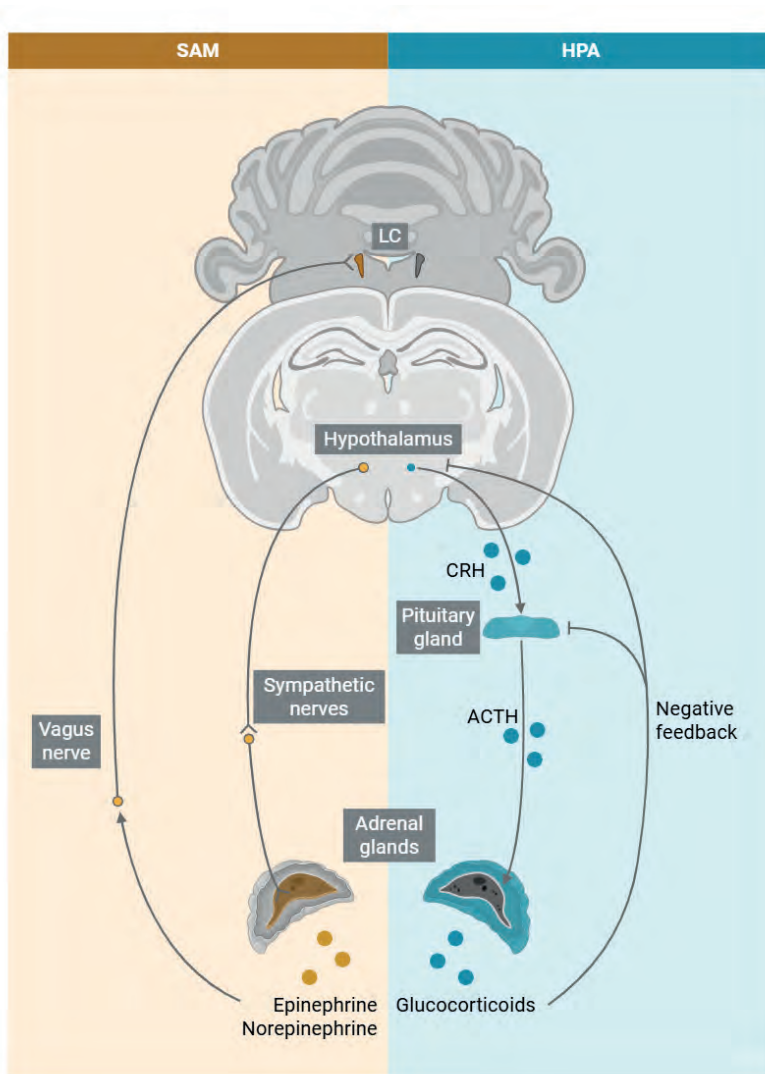


Figure 1 – Stress response. Activation of the sympathetic-adreno-medullary (SAM) system triggers the rapid release of epinephrine and norepinephrine from sympathetic nerve terminals and the adrenal medulla. These catecholamines influence central noradrenergic activity, including the locus coeruleus (LC), via the vagus nerve. Activation of the hypothalamic-pituitary-adrenal (HPA) axis results in the sequential release of corticotropin-releasing hormone (CRH) from the hypothalamus, adrenocorticotrophic hormone (ACTH) from the pituitary gland, and ultimately glucocorticoids from the adrenal cortex, which subsequently engage a negative feedback loop to regulate the stress response. Created in Biorender.com.

Noradrenergic modulation of brain activity by the LC is strongly dependent on its firing patterns, which, together with (re-)uptake mechanisms, determine extracellular norepinephrine levels (Aston-Jones & Cohen, 2005; Poe et al., 2020). Low arousal states, such as drowsiness or disengagement, are characterized by low extracellular norepinephrine levels (Park, 2002). Under these quiet awake conditions, noradrenergic signaling activates high-affinity $\alpha 2$ -adrenoceptors (Atzori et al., 2016; Constantinople & Bruno, 2011), most notably presynaptic autoreceptors, which exert inhibitory feedback on LC neurons (Samuels & Szabadi, 2008; Washburn & Moises, 1989). Activation of $\alpha 2$ -adrenoceptors suppresses cyclic adenosine monophosphate (cAMP)–protein kinase A (PKA) signaling and reduces neuronal excitability (Yi et al., 2013), thereby limiting global and local norepinephrine release (Mather et al., 2016).

In contrast, high arousal states, such as stress or hypervigilance, are associated with increases in extracellular norepinephrine (Berridge, 2008). Under these conditions, moderate-affinity $\alpha 1$ -adrenoceptors are strongly activated, accompanied by recruitment of low-affinity β -adrenoceptors (Arnsten, 2000; Atzori et al., 2016). Activation of $\alpha 1$ - and β -adrenoceptors engages convergent calcium- and cAMP-dependent signaling pathways that can drive widespread phosphorylation of cAMP response element-binding protein (CREB) (Arnsten, 2009). As a transcription factor, phosphorylation of CREB promotes the expression of brain-derived neurotrophic factor (BDNF) and other plasticity-related genes (Lonze & Ginty, 2002). This arousal state and degree of noradrenergic activation is associated with enhanced learning and memory dynamics, particularly when combined with concurrent activation of the stress-induced glucocorticoid response (McGaugh, 2018).

Hypothalamus-Pituitary-Adrenal (HPA) Axis & Glucocorticoid Signaling

Within minutes of stressor presentation, the slower neuroendocrine response is initiated via the activation of the HPA axis. The paraventricular nucleus (PVN) of the hypothalamus releases corticotropin-releasing hormone (CRH), which, via the portal system, stimulates corticotroph cells in the anterior pituitary gland to secrete adrenocorticotrophic hormone (ACTH) (Antoni, 1986; Vale et al., 1981). In turn, circulating ACTH stimulates the synthesis and release of glucocorticoids from the adrenal cortex into the general circulation (Dallman et al., 1987). This slow increase in blood glucocorticoid levels reaches a peak within thirty minutes.

Glucocorticoids (primarily cortisol in humans; corticosterone in rodents) enable an individual to cope with stressful experiences by coordinating immune, metabolic, neuronal and cognitive responses (McEwen, 2007). They exert their neural and cognitive effects by readily crossing the blood-brain barrier and binding directly to corticosteroid receptors in the brain. Glucocorticoids bind to two different corticosteroid receptor types: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (Reul & de Kloet, 1985, 1986). Given their high affinity for glucocorticoids, MRs are highly occupied even at baseline (i.e., non-stressed) conditions. Their abundant distribution in limbic areas support basal HPA-axis tone,

circadian rhythmicity and initial HPA-axis responsiveness. In contrast, the low-affinity GRs are ubiquitously distributed throughout the body and brain, and are further recruited when circulating glucocorticoid levels rise, such as during the circadian peak and in response to stress (Conway-Campbell et al., 2007; Kitchener et al., 2004; Reul et al., 1990; Reul & de Kloet, 1985; Spencer et al., 1993).

Glucocorticoid binding exerts both rapid non-genomic effects within seconds to minutes as well as slow genomic effects over hours to days. Rapid non-genomic actions are mediated by membrane-associated receptors, which trigger intracellular signaling cascades (Dallman, 2005; Groeneweg et al., 2012; Tasker et al., 2006). Slow genomic actions are mediated by conformational changes of cytosolic receptors after glucocorticoid binding, which induces nuclear translocation (Pratt et al., 2006; Robertson et al., 1993; Scheschowitsch et al., 2017). In the nucleus, these complexes act as transcription factors, binding to glucocorticoid response elements in gene promoter regions and thereby regulating gene expression and the synthesis of proteins involved in plasticity and metabolism (Buurstede et al., 2022).

Notably, cellular sensitivity to glucocorticoids is regulated by an ultra-short negative feedback mechanism involving the GR co-chaperone FK506-binding protein 5 (*FKBP5*) gene (Lockett et al., 2024). In the absence of glucocorticoids, the GR resides in a cytosolic chaperone complex containing FKBP51 (encoded by the *FKBP5* gene), which reduces receptor affinity for glucocorticoids and slows GR nuclear translocation. Upon glucocorticoid binding, FKBP51 is replaced by FKBP52, which increases receptor affinity, facilitating receptor activation and nuclear translocation. Glucocorticoid-induced *FKBP5* expression subsequently reinstates FKBP51-mediated inhibition, thereby forming the intracellular negative feedback loop regulating GR sensitivity (Fries et al., 2017). Interestingly, a recent study has shown that, under basal (i.e., non-stressed, non-treated) conditions, FKBP5 expression may be driven by MR (Hartmann et al., 2021).

At the level of an individual, glucocorticoid-mediated negative feedback ensures the termination of the neuroendocrine response, preventing excessive or prolonged activation of the HPA axis. It consists of two temporally and mechanistically distinct phases: a rapid non-genomic and a slow genomic feedback. Within seconds to minutes, activation of membrane-associated receptors in the hypothalamus and pituitary rapidly suppresses the release of CRH and ACTH (Dallman & Yates, 1969; Jones et al., 1974, 1977; Keller-Wood & Dallman, 1984; Osterlund et al., 2016), thereby limiting glucocorticoid output while the stressor may still be present. In addition, glucocorticoid-mediated non-genomic feedback occurs in other brain regions, including the hippocampus and prefrontal cortex, which in turn inhibit PVN output (Groeneweg et al., 2012). In contrast, approximately 30-90 minutes after receptor binding, glucocorticoids promote stable, long-term suppression of HPA-axis activity by inhibiting gene expression of CRH in the PVN and of the ACTH precursor in the pituitary (de Kloet et al., 2005; McEwen, 2007). Although GRs are the dominant mediators of both rapid and delayed feedback (Di et al., 2005), MRs may contribute to rapid suppression, particularly under conditions of mild stressor intensity (Pace &

Spencer, 2005). Together, these negative feedback mechanisms inhibit glucocorticoid release from the adrenal glands and gradually allow circulating concentrations to return to basal levels, thereby restoring homeostasis.

Given the central role of the HPA axis in regulating both circadian and stress-induced glucocorticoid secretion, the temporal dynamics of this system determine the magnitude, timing and effects of glucocorticoid signaling throughout the body and brain. These dynamics are particularly relevant for memory, as glucocorticoids play a critical role in establishing behavioral adaptations to stressful situations.

STRESS HORMONE EFFECTS ON MEMORY CONSOLIDATION

Emotional arousal, stress and stress hormones are well known to modulate memory (McGaugh, 2006). Memory is not a unitary phenomenon but a dynamic process composed of multiple stages: encoding, consolidation, and retrieval, among others (Kandel, 2014). Encoding refers to the initial processing of information, during which environmental stimuli are detected and transformed into a neural representation. Consolidation is an active process by which this encoded memory trace is stabilized over time. Retrieval involves the reactivation of the memory trace. Each of these stages relies on distinct but interacting neural systems and molecular mechanisms.

Memory Consolidation

Consolidation, in particular, has been a major focus in the field of memory research, as it presents a unique window of time — often referred to as the “consolidation window” — during which memories are labile and particularly susceptible to modulation by internal and external factors (McGaugh, 2000; Müller & Pilzecker, 1900). This window spans from minutes to hours after encoding. From a molecular perspective, the consolidation window can be divided into two phases: short-term consolidation, which is protein-synthesis independent; and long-term consolidation, which is protein-synthesis dependent (Kandel, 2001).

Short-term consolidation involves the modification of existing proteins (e.g., CREB phosphorylation) (Kandel & Schwartz, 1982), thereby transiently supporting the stabilization of memory. In contrast, long-term consolidation relies on transcriptional changes that induce *de novo* protein synthesis to increase the levels of existing proteins or express new proteins (e.g., c-Fos and BDNF) (Steward & Schuman, 2001). This process entails intricate molecular pathways whose activity is partly regulated post-transcriptionally by microRNAs (miRs) (Filipowicz et al., 2008), which induce mRNA degradation or translational repression depending on sequence complementarity (see Box 1 and Figure 2). In this manner, long-lasting structural modifications, such as the formation of new dendritic spines, are established at the local network level (Kandel, 2001; Squire & Alvarez, 1995), thereby supporting persistent behavioral changes.

Beyond this window, long-term consolidation continues for days to weeks in rodents

Box 1 – microRNAs regulate gene expression

MicroRNAs (or miRs) are a class of small non-coding RNAs that act as post-transcriptional regulators of gene expression. In the brain, miRs have been shown to regulate neuronal development and synaptic plasticity.

Biogenesis (Bartel, 2004)

Transcription of a miR gene by RNA polymerase II (POLII) produces a large primary miR transcript (pri-miR), which is cleaved in the nucleus by RNase Drosha into a precursor miR (pre-miR). This ~70-nucleotide molecule displays an imperfect hairpin structure with a 2-nucleotide 3' overhang. The pre-miR is exported to the cytoplasm via an exportin 5 (XPO5)-dependent pathway, where it is further cut by RNase Dicer into an intermediate miR duplex, that is about 21-23 nucleotides long. After unwinding by a helicase and degradation of one strand, the remaining mature miR strand is incorporated into the miR-induced silencing complex (miRISC).

Mode of action (Bartel, 2009)

The miRISC is directed to target mRNAs through extensive base pairing to sequences located in the 3' untranslated region (UTR). The fate of the target mRNA depends on the degree of similarity: (near) perfect complementarity with the miR leads to mRNA degradation, whereas imperfect complementarity results in translational repression. For example, miR-134 has been implicated in learning and memory through its role in dendritic spine remodeling by regulation of several key plasticity-related mRNAs (Shi, 2014). Specifically, miR-134 inhibits the translation of *Creb* by binding to partially complementary sites in the 3'UTR of the mRNA strand, thereby also reducing downstream *Bdnf* expression.

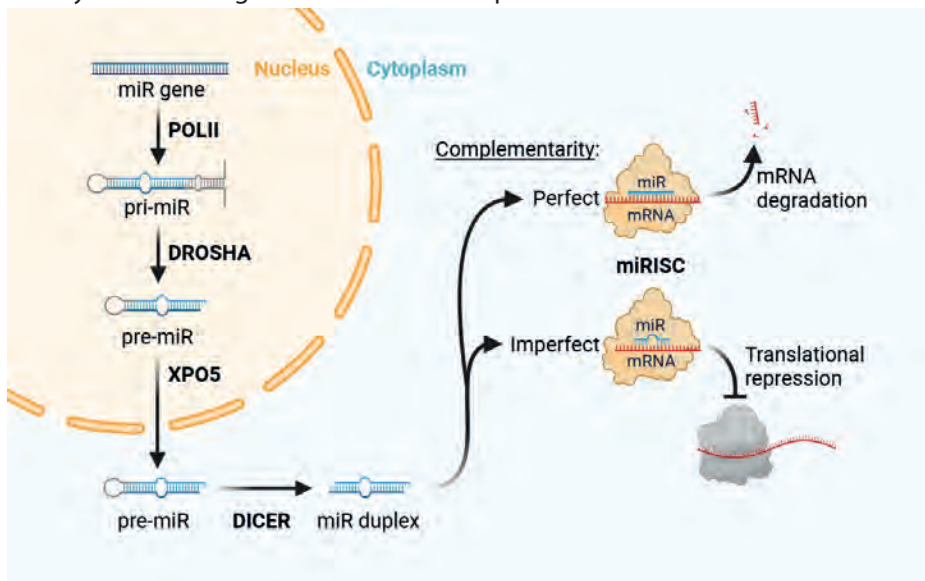


Figure 2 – miR biogenesis and mode of action. Created in Biorender.com.

or even up to years in humans, during which a systems-level reorganization takes place (Frankland & Bontempi, 2005; Nadel & Moscovitch, 1997). During this systems consolidation process, initially detailed hippocampus-dependent episodic(-like) memories become progressively more generalized and semantic as they increasingly rely on cortical networks rather than the hippocampus (Krenz et al., 2023; Wiltgen & Tanaka, 2013). This process supports long-term memory stabilization, integration with existing knowledge and flexible application in future situations. Importantly, it has been shown that stress hormones may enable modulation of systems consolidation dynamics (Atucha et al., 2017; Gulmez Karaca et al., 2025; Krenz et al., 2021).

Synergistic Effects of Stress Hormones on Memory Consolidation

Memories of emotionally arousing and stressful experiences are typically retained better and longer than neutral experiences, which tend to be more easily forgotten (Bradley et al., 1992; McGaugh, 2006). This observation suggests that emotional arousal and stress may engage endogenous mechanisms that regulate memory consolidation. A large body of work has demonstrated that the stress hormones norepinephrine and glucocorticoids are critically involved in mediating the effects of emotional arousal and stress on memory consolidation.

Early evidence for hormonal modulation of memory came from adrenalectomy studies demonstrating that removal of adrenal hormones abolishes arousal- and stress-induced memory enhancement (Borrell et al., 1983, 1984; McCormick et al., 1997). Importantly, these memory deficits could be subsequently rescued by replacement of epinephrine and glucocorticoids. Because epinephrine does not cross the blood–brain barrier, subsequent work demonstrated that its memory-modulating effects depend on the engagement of central noradrenergic mechanisms via activation of β -adrenoceptors on the vagus nerve (Introini-Collison & Baratti, 1992; Liang et al., 1986; Williams et al., 1998).

Acute systemic and local administration studies in rodents have established that norepinephrine and glucocorticoids modulate memory consolidation in a time- and dose-dependent manner (De Quervain et al., 1998; Gold & Van Buskirk, 1975; McGaugh & Roozendaal, 2002). Specifically, stress hormone action on memory consolidation presents an inverted-U logarithmic dose-response curve, where an optimal dosage enhances memory consolidation while a high dosage is less effective or even impairs it (Joëls et al., 2006). These memory-modulating effects of norepinephrine and glucocorticoids are predominantly mediated by β -adrenoceptors and GRs, respectively (Donley et al., 2005; Ferry et al., 1999a, 1999b; Roozendaal & McGaugh, 1997b). Importantly, dose-dependent memory modulation by stress hormones has been consistently replicated in humans (De Quervain et al., 2017; Newcomer, 1999; Schwabe et al., 2012, 2022; Young et al., 2011).

Notably, glucocorticoids particularly enhance the consolidation of emotionally arousing experiences (Okuda et al., 2004), regardless of valence (Wichmann et al., 2012). Converging evidence from both animal and human studies demonstrates that

emotional arousal induces the release of norepinephrine, and that glucocorticoid effects on memory consolidation critically depend on arousal-induced noradrenergic activation (Buchanan & Lovallo, 2001; De Quervain et al., 2017; Fastenrath et al., 2014; Kuhlmann & Wolf, 2006; Okuda et al., 2004; Roozendaal, 2002; Roozendaal et al., 1999, 2002, 2006; van Stegeren et al., 2010). These findings highlight how glucocorticoid-induced memory enhancement relies on a precisely timed and synergistic interaction with the noradrenergic system (de Kloet et al., 2008; De Quervain et al., 2017; Joëls, 2008; Joëls et al., 2011; Roozendaal, 2002; Roozendaal & McGaugh, 2011).

Basolateral Amygdala (BLA) as a Mediator of Stress Hormone Effects on Memory Consolidation

The BLA is critically involved in modulating the memory of emotionally arousing experiences, a mechanism that has been shown to be mediated by norepinephrine and glucocorticoids. Findings from both animals and humans indicate that damage to the amygdala selectively impairs memory of emotionally arousing, but not neutral memories (Bermudez-Rattoni & McGaugh, 1991; Cahill et al., 1996). Further studies have identified the BLA as a central hub for processing emotional salience and assigning value to environmental stimuli (LeDoux, 2000; Roozendaal et al., 2009). The BLA not only receives strong noradrenergic input from the LC, it also presents relatively moderate to high abundance of MRs and GRs, respectively (Morimoto et al., 1996; Reul & de Kloet, 1985).

Targeted pharmacological manipulations within the BLA have consistently demonstrated that norepinephrine and glucocorticoids dose-dependently enhance memory consolidation across a variety of different training experiences (LaLumiere et al., 2003; Liang et al., 1990; Roozendaal et al., 1996; Roozendaal & McGaugh, 1997a, 2011). Importantly, emotionally arousing training induces release of norepinephrine in the BLA (Galvez et al., 1996; Quirarte et al., 1998). The systemic glucocorticoid-adrenergic interactions have been replicated within the BLA, showing that glucocorticoid-induced memory enhancement depends on intra-BLA noradrenergic activation (Quirarte et al., 1997; Roozendaal, 2002; Roozendaal et al., 2006). Collectively, these findings position the BLA as an important region for mediating stress hormone effects on memory consolidation (Roozendaal et al., 2002).

Extensive research supports the idea that BLA activity modulates memory consolidation by influencing neuroplasticity in other brain regions (McIntyre et al., 2005; Packard et al., 1994; Petrovich et al., 2001; Pitkanen et al., 2000; Price, 2003; Roozendaal & McGaugh, 2011; Sah et al., 2003). Consistent with the widespread expression of receptors for both norepinephrine and glucocorticoids throughout the brain, local post-training administration of these stress hormones into several brain regions has been shown to enhance memory consolidation (Ferry et al., 1999a; Liang et al., 1986, 1990; Roozendaal & McGaugh, 2011). However, while local administration into the BLA indiscriminately enhances memory across different training experiences, the effect of stress hormones in other brain regions seems to depend on the specific

information acquired during the training experience (Nathan et al., 2004; Packard et al., 1994). Moreover, both lesions as well as local administration of a β -blocker in the BLA prevent stress hormone administration effects in other brain regions, showing that BLA activity is necessary for stress hormones to modulate memory consolidation processes in distributed brain systems (Roozendaal et al., 1999; Roozendaal & McGaugh, 1997a). As such, the memory modulation theory posits that the BLA serves as a central amplifier, where noradrenergic activation coupled with glucocorticoid binding signals other brain regions to store memories more robustly (McGaugh, 2000; Roozendaal et al., 2009; Roozendaal & McGaugh, 2011).

STRESS HORMONE EFFECTS ON QUALITATIVE ASPECTS OF MEMORY

Qualitative Aspects of Memory

Although the enhancing effects of emotional arousal, stress and their underlying neuromodulators on memory consolidation are well established, their influence on qualitative aspects of the strengthened memories remains less clear. In particular, it is still debated how emotional arousal and stress may modify aspects such as memory specificity, generalization, fidelity, trustworthiness, and susceptibility to distortion or misinformation. Research addressing these aspects has been mostly performed in humans and has yielded seemingly contradictory findings. Several studies report that emotional arousal or stress exposure enhances memory detailedness (Kensinger, 2007; Ochsner, 2000; Segal et al., 2012; Steidl et al., 2006; Wiemers et al., 2013). In contrast, other work indicates that emotional information is preferentially remembered in a generalized, gist-like manner, centered on the core theme of the episode rather than its details (Levine & Edelstein, 2010; Loftus, 1979; Morgan et al., 2004; Payne et al., 2002; Qin et al., 2012; Richards & Gross, 2006; Rimmele et al., 2011). Such memories have also been associated with reduced context dependency, excessive confidence in later recollection, and even an increased vulnerability to false or distorted information (Schwabe & Wolf, 2009; Sharot et al., 2004; Talarico & Rubin, 2003).

Together, these findings indicate that emotional arousal and stress do not merely influence how strongly events are remembered, but may critically affect the quality of the resulting memories. Importantly, clear mechanistic conclusions of these effects are hindered by substantial differences in arousal or stress manipulations, behavioral tasks and outcome measures across studies. Moreover, many studies lack direct assessment of the underlying hormonal responses, limiting insight into the distinct contributions of noradrenergic and glucocorticoid signaling to qualitative memory aspects (Morgan et al., 2004; Payne et al., 2002; Rimmele et al., 2011; Steidl et al., 2006). Despite this variability, many seemingly opposing findings converge on a common dimension: the balance between memory specificity and generalization. Memories described as vivid and detailed, or alternatively as gist-like, can be understood as reflecting differences in how precisely an event is encoded and later discriminated from similar experiences. The specificity–generalization axis therefore provides a unifying framework for integrating disparate observations on emotional memory

quality.

Stress Hormone Modulation of Memory Specificity and Generalization

By leveraging newly developed behavioral paradigms, recent animal studies have begun to elucidate how emotional arousal, stress and their underlying neuromodulators influence memory specificity and generalization. Our laboratory has been the first to directly compare the effects of norepinephrine and glucocorticoids on the specificity and generalization of episodic-like memory using the dual-event inhibitory avoidance task in rats (Atucha et al., 2017; Atucha & Roozendaal, 2015; Bahtiyar et al., 2020, 2025; Roozendaal & Mirone, 2020). In this task, rats sequentially explore two contextually distinct inhibitory avoidance apparatuses with a brief delay, receiving a footshock only in the latter context (Figure 3A). After 48 h, retention latencies are tested in both training contexts as well as a novel context. This procedure allows to assess whether rats accurately associate the shock experience with the correct training context.

As expected, systemic administration of corticosterone or yohimbine, a noradrenergic stimulant, enhanced memory strength, evidenced by longer retention latencies in the shock box in drug-treated rats compared to vehicle-treated rats (Figure 3B). However, these drugs presented opposite effects in the episodic-like quality of the memory: yohimbine promoted memory specificity, as indicated by shorter retention latencies in the non-shock box compared to vehicle; whereas corticosterone promoted a generalized enhancement of memory, as evidenced by similarly elevated retention latencies in both the shock box and the non-shock box compared to vehicle (Roozendaal & Mirone, 2020). Interestingly, corticosterone administration did not affect retention latencies in the novel box. These findings could partly explain the contradicting effects of emotional arousal and stress on qualitative aspects of memory.

Several other animal studies using systemic administration support opposing roles of noradrenergic and glucocorticoid signaling on memory specificity and generalization. Similarly to the dual-event inhibitory avoidance task, the object-in-context task employed by Guo (2024) also consists of two training events that must be discriminated, in order to enable appropriate object-context associations. Using this task, they replicated the opposing effects of norepinephrine (Barseguyan et al., 2014) and glucocorticoids on memory specificity and generalization. Another recent study by Song (2021) showed that noradrenergic stimulation by yohimbine enhanced memory detailedness of an object recognition task. Glucocorticoid effects have been further supported by multiple fear conditioning studies showing that exogenous administration facilitates fear generalization (dos Santos Corrêa et al., 2019; Kaouane et al., 2012; Lesuis et al., 2021).

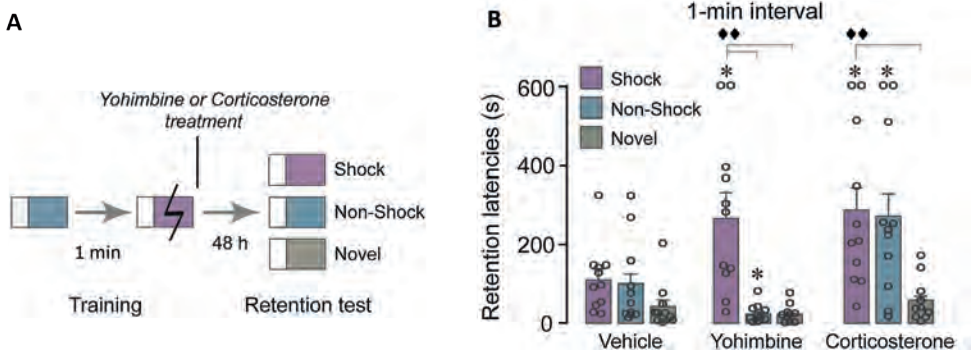


Figure 3 – Opposite effects of norepinephrine and corticosterone on memory specificity and generalization.

A. Experimental design of the dual-event inhibitory avoidance task. During training, rats are exposed to two distinct inhibitory avoidance apparatuses with 1-min interval, but only receive a footshock when entering the dark compartment of the latter context (i.e., shock box). The noradrenergic stimulant yohimbine (1 mg/kg) or corticosterone (3 mg/kg) were subcutaneously administered immediately after training. Forty-eight hours later, retention latencies to enter the dark compartment of these two previously encountered training contexts (shock and non-shock box) as well as of a novel box were tested in a counterbalanced order. **B.** Step-through latencies (mean $\pm$ s.e.m.) in seconds on the 48-h retention test. Vehicle-treated rats did not show accurate shock-context association as indicated by similar retention latencies in the shock box and non-shock box. Yohimbine enhanced both the strength and specificity of memory as shown by higher latencies only in the shock box. In contrast, corticosterone enhanced latencies in both the shock box and non-shock box. * $P < 0.05$, ** $P < 0.01$ vs vehicle, ♦♦ $P < 0.01$ shock box vs non-shock box (Roosendaal & Mirone, 2020).

Neural and Computational Substrate of Memory Specificity and Generalization

Alongside its essential role in the formation of a unitary contextual representation, the (dorsal) hippocampus has been implicated in the discrimination of similar experiences (Rolls, 2013; Yassa & Stark, 2011). The hippocampus is subdivided into three main areas: the dentate gyrus (DG), Ammon's horn – composed of Cornu Ammonis (CA) 1, CA2 and CA3 – and subiculum. Within the hippocampus, information flows through three sequential excitatory synapses, termed the trisynaptic circuit. First, perforant path projections from superficial layers of the medial and lateral entorhinal cortex innervate DG granule cells (Witter, 1993). In turn, DG axons project to CA3 pyramidal neurons via mossy fibers. Finally, CA3 neurons send Schaffer collateral projections to CA1 pyramidal neurons (Amaral & Witter, 1989). Notably, the CA3 presents recurrent axon collaterals that synapse back onto CA3 cells themselves, effectively creating a network by itself (Amaral & Witter, 1989; Witter, 2007). Outside of the canonical pathway, the entorhinal cortex also projects directly to the CA3 and CA1 (Kajiwar et al., 2008; Wu & Leung, 1998).

In a computational process termed pattern separation, the DG is proposed to rapidly form distinct representations from similar sensory input provided by the entorhinal cortex (Marr, 1971; Rolls, 2013; Yassa & Stark, 2011), and to project this signal to the CA3. Fed by strong, but sparse DG input as well as weak entorhinal input, recurrent and highly plastic connections in the CA3 are proposed to attempt to retrieve previously stored representations using these partial cues, in a process termed pattern completion (Rolls, 2013; Yassa & Stark, 2011). In the CA1, there is a convergence of CA3 and entorhinal input (Kajiwara et al., 2008). This theory has steadily gathered empirical support over the years (Leutgeb et al., 2007; McHugh et al., 2007; Nakazawa et al., 2002, 2003). Altogether, it highlights the hippocampus as a critical locus for contextual representation and discrimination, enabling the formation of distinct memory traces from similar experiences.

Critically, systemic administration studies have supported the idea that stress hormone effects on memory specificity and generalization may be mediated by hippocampal activity. For example, Lesuis et al. (2021) showed that glucocorticoid enhancement of fear generalization was associated with greater granule cell recruitment in the DG. In contrast, Kaouane et al. (2012) reported that glucocorticoid-induced generalization was associated with reduced ventral DG and dorsal CA1 activity. In addition, Guo (2024) showed that glucocorticoid administration after training on an object-in-context task increased GABAergic activity in the ventral blade of the DG and CA1, and yohimbine administration increased activity in the CA1. Further, in yohimbine-treated mice there was a positive correlation in neuronal activation between the dorsal blade of the DG and CA3, which was absent in glucocorticoid-treated mice. Guo (2024) findings further supported crucial hippocampal involvement by showing that inactivation of the whole hippocampus during training and subsequent consolidation period prevented yohimbine-induced enhancement of memory specificity.

Together, these findings suggest that stress may bias hippocampal computations in a hormone-specific manner, thereby shifting the balance between detailed, context-specific memories and more generalized representations. This modulation of mnemonic processing by stress hormones is of particular relevance for stress-related psychopathologies like PTSD, in which alterations in endogenous stress hormone regulation as well as in the balance between memory specificity and generalization are thought to play a central role in vulnerability and the pathophysiology (Kalisch et al., 2017).

STRESS-RELATED DISORDERS AND INTERINDIVIDUAL VARIABILITY

Aberrant Mnemonic Processing in Stress-Related Disorders and Trauma-Focused Therapies

As stress-related psychopathologies are increasingly recognized as memory disorders (Brewin, 2011; Rigoli et al., 2016; Rubin et al., 2008), understanding the mnemonic modifications produced by emotional arousal, stress and underlying neuromodulators may provide critical insight into the mechanisms that lead to the development and

maintenance of these disorders (Pitman, 1989). Strong, but generalized, fear memories are not only a hallmark feature of stress-related disorders, but they are also considered to be a major risk factor for their development (De Quervain et al., 2017; Lis et al., 2020; Lissek, 2012). In particular, PTSD patients commonly exhibit a persistent and exaggerated generalization of the traumatic memories (i.e., overgeneralization), such that fear responses are elicited in response to stimuli that only loosely resemble the original traumatic cues. For example, although a war veteran may appropriately fear loud noises associated with bombs or gunfire, they may later experience intense fear or panic in response to fireworks, a car backfiring, or other non-threatening sounds in a safe environment. Such overgeneralized fear responses can severely impair daily functioning and quality of life. In addition to considerable functional impairment, PTSD is also associated with elevated comorbidity (e.g., depression, anxiety disorders, and substance use disorders) and high lifetime prevalence (Atwoli et al., 2015; de Vries & Olff, 2009).

Trauma-focused therapies are considered the first line treatment for PTSD. These interventions aim to modify the traumatic memories and their associated emotional responses (Astill Wright et al., 2021). For example, some therapeutic approaches seek to alter the meaning of the traumatic memory through processes such as cognitive reappraisal (Koetsier et al., 2024; Price et al., 2016). The most widely used approach, however, is exposure therapy, which relies on repeated and controlled exposure to trauma-associated cues in the absence of threat such that they no longer evoke fear, a process termed fear extinction (Bouton et al., 2021). Despite its status as the gold standard treatment, exposure therapy is not effective for all patients (Bandelow et al., 2007; Bradley et al., 2005; Cusack et al., 2016), as many fail to sufficiently learn safety (Wicking et al., 2016). Others experience relapse when encountering trauma reminders outside the therapeutic setting, a phenomenon known as fear renewal (Bouton et al., 2021). These observations reveal further mnemonic aberrations in PTSD: the inability to form, retain and/or to sufficiently generalize extinction memories.

Converging evidence from neuroimaging studies has demonstrated that PTSD is associated with functional and structural alterations in brain regions critical for mnemonic processing and emotion regulation. Task-based functional imaging has revealed that PTSD patients exhibit altered hippocampal, amygdala and prefrontal cortex activity during fear and extinction learning and retrieval (Bryant et al., 2008; Milad et al., 2009; Rauch et al., 2006). In addition, there are consistent reports of reduced hippocampal and prefrontal volume, as well as less consistent reports of increased amygdala volume (Ben-Zion et al., 2024; Harnett et al., 2020; O'Doherty et al., 2015). Beyond this circuit, studies have further described a wide range of functional and structural alterations in regions involved in the default mode (DMN), salience and executive control networks (Akiki et al., 2017; Miller, Hayes, et al., 2017; Pankey et al., 2022; Sripada et al., 2012; Wang et al., 2021). Moreover, diffusion imaging has also shown alterations in white matter tracts involved in mnemonic processing, such as the corpus callosum and cingulum (Dennis et al., 2021; Siehl et al., 2018; Weis et al., 2018). Together, these neural alterations are thought to contribute to the persistent

fear, impaired extinction learning and contextualization deficits that characterize PTSD.

Collectively, the high disease burden, substantial rates of non-response to treatment, and frequent relapse underscore the urgent need for more effective preventative and therapeutic interventions that directly target aberrant mnemonic processing in stress-related disorders.

Experimental Research on Preventative and Therapeutic Interventions for Stress-Related Disorders

In experimental research, fear and extinction memories are commonly studied using fear conditioning and extinction paradigms, which model fear acquisition, extinction, and extinction recall. Since they can be applied in both rodents and humans, these paradigms are highly translational. In particular, extinction recall is clinically relevant because it reflects the long-term retention of extinction learning and may serve as an indicator of treatment success (Forcadell et al., 2017; Scheveneels et al., 2021). Further, the consolidation window after a fear and extinction learning event represents a critical period of memory lability and has therefore been identified as a key target for both preventative and therapeutic interventions (Astill Wright et al., 2021). As such, pharmacological modulation during this window represents a powerful strategy to modulate both traumatic and extinction memories.

Extensive rodent and human research on stress hormones and memory has laid the foundation for the development of pharmacological treatments that are currently under clinical investigation. Given their role in memory consolidation, stress hormones have been proposed both as preventative interventions to reduce the likelihood of PTSD development in predictable high-risk settings (i.e., following invasive surgery or intensive care admission), and as treatment adjuvants to improve the efficacy of exposure therapy of several stress-related disorders, including PTSD (De Quervain et al., 2017, 2019). Specifically, stress hormones may be used to impair fear memory consolidation, and enhance extinction memory consolidation during exposure therapy.

Following this rationale, several preliminary and randomized controlled trials have investigated norepinephrine- and glucocorticoid-based interventions for phobias and PTSD. For example, initial findings from preliminary studies supported that glucocorticoid administration may facilitate extinction consolidation and reduce fear responses (Brueckner et al., 2019; De Quervain et al., 2017, 2019; Meir Drexler et al., 2019; Merz et al., 2018). However, meta-analyses and randomized controlled trials have yet to provide sufficient evidence for a consistent augmentation effect of extinction by stress hormone administration (Argolo et al., 2015; Inslicht et al., 2022; Lehrner et al., 2021; Yehuda, Bierer, et al., 2015). This is likely due to two major factors. First, these interventions were largely developed within a framework emphasizing memory strength, with comparatively little attention given to how stress hormones may influence memory specificity and generalization. Second, some studies point to

heterogeneous, subgroup-dependent outcomes (Yehuda, Bierer, et al., 2015).

Interindividual Differences in Stress Hormone Signaling and Their Effects on Mnemonic Processing

The PTSD patient population is highly heterogeneous, with marked variability in symptom profiles, as well as in the severity, timing, and chronicity of trauma exposure (Galatzer-Levy & Bryant, 2013). Interestingly, only a proportion of trauma-exposed individuals eventually develop PTSD (Ressler et al., 2022; Shalev et al., 2017). Given the role of stress hormones in emotional memory formation, interindividual differences in endogenous noradrenergic and glucocorticoid signaling are likely to contribute to variability in mnemonic processing, vulnerability to stress-related psychopathology, and treatment response. In line with this, considerable variability in both noradrenergic and glucocorticoid systems has been described across healthy and clinical populations.

Genetic variations in the noradrenergic and glucocorticoid system have been shown to influence mnemonic processing, particularly for emotional memories. Within the noradrenergic system, variants in genes encoding α 2- and β -adrenoceptors, and enzymes involved in catecholamine breakdown have been linked to differences in arousal regulation, amygdala activation during encoding, and PTSD risk (Liberzon et al., 2014; Norrholm et al., 2013; Rasch et al., 2009; Ziegler et al., 2018). For example, individuals with the deletion variant of the α 2B-adrenoceptor gene show enhanced amygdala activation and better memory for emotional stimuli compared to those without this variant (Rasch et al., 2009). Within the glucocorticoid system, variants have been described in genes involved in glucocorticoid synthesis, metabolism, transport, and receptor sensitivity. For example, the BclI polymorphism of the GR gene (*NR3C1*) has been associated with increased GR sensitivity, stronger HPA-axis suppression, and enhanced emotional memory formation (Ackermann et al., 2013; Wüst et al., 2004). In PTSD patients, a specific genotype of this polymorphism has additionally been linked to lower cortisol concentrations, vulnerability to PTSD and greater symptom severity (Bachmann et al., 2005; Hauer et al., 2011).

Growing evidence indicates that early life experiences interact with genetic predispositions to modify HPA-axis regulation and memory. For example, a *FKBP5* genetic variant has been shown to interact with childhood trauma to alter glucocorticoid signaling and amygdala responsivity to threat (Klengel & Binder, 2015; Zannas et al., 2016). These findings support the idea that genetic variants may increase susceptibility to PTSD by determining individual biological responses to stressful or traumatic experiences.

Across both healthy and clinical populations, individuals differ markedly in basal glucocorticoid levels, stress-induced glucocorticoid responses, and negative feedback regulation of the HPA axis. Even when exposed to the same stressor, some individuals exhibit robust glucocorticoid responses whereas others show minimal or no response (Henckens et al., 2016; Man et al., 2023; Zänker et al., 2019). Importantly,

these differences appear relatively stable across repeated stress exposures (Cohen & Hamrick, 2003), suggesting trait-like differences in HPA-axis responsivity. Variation in stress reactivity has also been linked to psychological factors such as threat appraisal, perceived controllability, and resilience (Henckens et al., 2016; Schlotz et al., 2011).

Commonly assessed using the dexamethasone suppression test (DST), interindividual differences have also been described in HPA-axis negative feedback regulation. The DST evaluates the extent to which dexamethasone, a synthetic glucocorticoid with high GR affinity, suppresses endogenous glucocorticoid secretion during periods when levels are expected to rise, including the awakening response or following a stress or CRH challenge. In healthy populations, low-dose DST protocols have revealed that stronger HPA-axis suppression is associated with factors such as female sex, lower income, and reduced physical activity (Direk et al., 2016). Given the importance of glucocorticoid signaling for emotional memory formation and extinction, such differences in HPA-axis regulation may have important consequences for mnemonic processing.

PTSD has frequently been associated with stronger HPA-axis negative feedback and lower glucocorticoid levels (Meewisse et al., 2007; Pitman et al., 2012; Yehuda, 2002, 2009). However, these observations are not uniform across individuals with a PTSD diagnosis. Mirroring observations in healthy populations, women with PTSD tend to exhibit lower glucocorticoid levels and enhanced GR sensitivity compared to men (de Kloet et al., 2007). Further, low-glucocorticoid phenotypes appear to be more prevalent in combat veterans with chronic PTSD, individuals with dissociative PTSD subtype, and survivors of early-life trauma (Heim et al., 2001; Lanius et al., 2012; Yehuda et al., 1990). At the same time, a substantial proportion of individuals with PTSD meet criteria for comorbid major depressive disorder (Rytwinski et al., 2013), which is often characterized by impaired negative feedback and elevated glucocorticoid levels (Pariante & Lightman, 2008), further complicating attempts to delineate a single, disorder-specific HPA-axis phenotype. It remains unclear how these neuroendocrine phenotypes map onto specific symptom profiles and treatment outcomes. However, they are likely to alter the degree of emotional arousal and stress during encoding, consolidation, and retrieval. Differential mnemonic processing, resulting in stronger or weaker, more specific or more generalized memories, may therefore contribute to individual differences in stress resilience and vulnerability, symptom manifestation, and response to treatment.

Stratification and Personalized Interventions for PTSD

Currently, the medical community relies on a “one-size-fits-all” approach to the treatment of psychiatric disorders, where treatment selection is determined mostly by provider discretion rather than scientific evidence. This often results in a protracted process of trial-and-error, where the delay in effective care, compounded by the emotional and physiological burden of side effects, exacerbates the disease burden. Identifying the biological variations behind differential mnemonic processing offers

a way forward, allowing to predict treatment response in PTSD patients, as well as susceptibility in healthy populations at risk of trauma exposure.

Low glucocorticoid levels and enhanced HPA-axis negative feedback regulation have been especially highlighted in a subset of PTSD patients (Yang et al., 2020; Yehuda, Bierer, et al., 2015; Yehuda, Hoge, et al., 2015). Given the importance of glucocorticoids for memory consolidation and generalization, it has been proposed that individuals with stronger HPA-axis negative feedback regulation and lower post-stressor glucocorticoid levels may show diminished responsiveness to extinction-based treatments. Consequently, this patient subgroup may particularly benefit from glucocorticoid augmentation during exposure therapy (de Nooij et al., 2024).

THESIS SCOPE AND OUTLINE

Elucidating how interindividual differences in noradrenergic and glucocorticoid signaling contribute to memory specificity and generalization is essential for identifying vulnerability markers, improving patient stratification, and advancing personalized interventions. Research into these questions in humans is complicated by the fact that trauma itself can alter endogenous signaling. Because genetic background, environmental exposure, and physiological state can be more tightly controlled and manipulated in rodents, these models are uniquely positioned to explore the mechanistic basis of differential mnemonic processing. In this thesis, I examine, in rats, how norepinephrine and glucocorticoids modulate memory specificity and generalization, as well as neural circuitry, with particular attention to how endogenous differences in stress hormone signaling influence these processes.

In **Chapter 2**, I examined the neural and molecular mechanisms through which norepinephrine enhances memory specificity. As described, extensive research has shown that noradrenergic activity in the BLA plays a critical role in mediating the effects of stress hormones on memory consolidation by modulating neural plasticity in other brain regions, each of which may contribute to distinct aspects of memory processing. Recent work in rats has demonstrated that systemic norepinephrine enhances episodic-like memory specificity in the dual-event inhibitory avoidance task. However, it remains unclear whether this specificity-enhancing effect is mediated by noradrenergic signaling within the BLA and its downstream influence on plasticity in other brain regions. To address this question, I first tested whether local administration of norepinephrine into the BLA is sufficient to promote memory specificity in rats trained in the dual-event inhibitory avoidance task. Given that the hippocampus—particularly the DG—has been implicated in discriminating between similar inputs, subsequent experiments assessed norepinephrine-induced changes in hippocampal plasticity and its subfields using a range of molecular approaches. Finally, by combining behavioral and pharmacological manipulations, I tested whether a miR-dependent mechanism in the DG mediates the exogenous norepinephrine-induced enhancement of memory specificity, and whether this mechanism is physiologically relevant and engaged when memory specificity is naturally occurring.

In **Chapter 3**, I investigated whether stronger HPA-axis feedback regulation and low post-stressor glucocorticoid levels influence the strength and generalization of extinction memory, as well as its interaction with glucocorticoid treatment. As mentioned, glucocorticoids are known to enhance memory consolidation and may promote memory generalization. As such, glucocorticoids have been explored as pharmacological adjuvants to extinction-based therapies in PTSD patients, although findings have been mixed. Notably, stronger HPA-axis regulation and reduced post-stressor glucocorticoid levels have been reported in a subset of PTSD patients. However, it remains unclear whether individual differences in endogenous glucocorticoid regulation influence responsiveness to extinction-based treatment and glucocorticoid augmentation. To address this question, HPA-axis negative feedback regulation was first characterized in selectively-bred rat lines, where a Low line presents lower post-stressor glucocorticoid levels compared to a Control line. Rats then underwent extinction training in a context distinct from the conditioning context, enabling assessment of both strength and generalization of extinction memory during recall. To determine how endogenous glucocorticoid regulation modulates responsiveness to pharmacological intervention, corticosterone was administered immediately after extinction training. Finally, I examined neural and molecular correlates of the behavioral effects, focusing on glucocorticoid-related modulation of hippocampal activity and connectivity, as well as markers of GR activation.

In **Chapter 4**, I used magnetic resonance imaging (MRI) to assess whether stronger HPA-axis negative feedback regulation influences brain structure and connectivity. Stress and glucocorticoids are known to influence neural plasticity through mechanisms such as dendritic remodeling, neurogenesis and myelination (Fuchs & Gould, 2000; Liston & Gan, 2011; Miguel-Hidalgo, 2023), and endogenous glucocorticoid levels are regulated by the HPA axis. Both HPA-axis regulation and brain structure and resting-state connectivity show substantial interindividual variability in healthy and clinical populations. In particular, altered DMN activity has been implicated as a marker of vulnerability toward psychiatric disorders (van Oort et al., 2020). However, it is unclear to what extent stable interindividual differences in HPA-axis regulation may influence brain connectivity and structure. This chapter examined differences in brain connectivity and structure in the same rat lines with differential HPA-axis negative feedback regulation. To investigate this, a multimodal MRI approach was employed, combining targeted investigation of the DMN with exploratory whole-brain analyses. Rats first underwent resting-state functional MRI under light sedation to assess functional connectivity. This was followed by *ex vivo* structural and diffusion tensor imaging (DTI), allowing assessment of regional brain volumes and white matter microstructure.

Finally, **Chapter 5** summarizes and integrates the main findings of this thesis. In this chapter, I also discuss the translational implications towards the treatment of PTSD and provide my perspective on future directions for research.



CHAPTER 2

Noradrenergic activation of the basolateral amygdala facilitates memory specificity for similar events experienced close in time

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ABSTRACT

Noradrenergic activation of the basolateral amygdala (BLA) promotes strong and lasting memories of emotionally arousing experiences. However, in our lives, we often encounter similar events that may be confused and result in emotional strengthening of incorrect associations. Here we provide evidence, in rats, that noradrenergic activation of the BLA promotes the formation of discrete memories of similar events that were experienced close in time, via a miR-134-regulated consolidation process within the dentate gyrus of the hippocampus. Targeted downregulation of miR-134 in the hippocampus was sufficient to induce memory specificity, without affecting the strength of the memory. Notably, noradrenergic activation of the BLA did not recruit this hippocampal miR-134-mediated mechanism in enhancing memory of a single event. These findings indicate that the BLA engages a qualitatively different neural mechanism on an 'as-needed' basis to facilitate the separation of similar memory representations, enabling the selective strengthening of correct associations into long-term memory.

INTRODUCTION

The capacity to create strong and lasting memories of emotionally arousing experiences is an evolutionarily conserved adaptive survival mechanism (McGaugh, 2000; Roozendaal & McGaugh, 2011). Extensive evidence from both animal and human studies indicates a critical role for noradrenergic activation of the basolateral amygdala (BLA) in this process (McGaugh, 2000; McIntyre et al., 2002; Phelps & LeDoux, 2005; Roozendaal & McGaugh, 2011). Specifically, studies have shown that emotionally arousing training induces the release of norepinephrine (NE) within the BLA (McIntyre et al., 2002) and noradrenergic stimulation of BLA activity then strengthens the memory of that experience by facilitating neural plasticity and information storage processes in other brain regions (McGaugh, 2000; McIntyre et al., 2002; Phelps & LeDoux, 2005; Roozendaal & McGaugh, 2011). However, this modulatory influence has been mainly studied within the context of single-event memories. In real life, our experiences are often composed of highly similar events that can be confused and thus prone to incorrect associations being formed (Cai et al., 2016; Liu et al., 2022). Although linking memories of events that occur close in time—by directing storage into overlapping neuronal ensembles (Rogerson et al., 2014; Silva et al., 2009)—might be helpful in organizing large amounts of similar information (Cai et al., 2016), emotional strengthening of such concurrent memory representations entails the risk that incorrect associations are also reinforced (Bahtiyar et al., 2020; Roozendaal & Mirone, 2020). It remains to be determined whether noradrenergic activation of the BLA assists in an adaptive regulation of emotional arousal effects on cognition, namely by facilitating the formation of discrete memories of similar events experienced close in time, enabling the selective strengthening of correct associations into long-term memory (Dunsmoor & Paz, 2015).

To determine whether noradrenergic activation of the BLA facilitates the separation of similar memory representations into discrete memories, we trained male Sprague–Dawley rats on a dual-event inhibitory avoidance task in which they could explore two similar, but contextually distinct, inhibitory avoidance apparatuses separated by a 1-min interval (Atucha et al., 2017; Atucha & Roozendaal, 2015). A single inescapable footshock was delivered only after the rat entered the dark compartment of the second of these two contexts (Fig. 1A). A memory-enhancing dose of NE (1.0 μg in 0.2 μl) (LaLumiere et al., 2003; Roozendaal et al., 2008) or saline was microinfused bilaterally into the BLA immediately after the training session. After 48 h, the rats were presented the two previously encountered contexts (shock box and non-shock box) and a new box in random order and we assessed the retention latencies to enter the dark compartment of each box, allowing us to determine both the specificity and the strength of memory. Memory strength was defined by retention latencies in the shock box, whereas memory specificity was defined by longer latencies in the shock box relative to the non-shock box.

RESULTS

Posttraining NE in the BLA induces memory specificity

Rats administered saline into the BLA immediately after the training session displayed similar 48-h retention latencies in the shock box and the non-shock box (paired Student's t -test: $t_{12} = 1.42$, $P = 0.18$; Fig. 1B-C), which were both significantly longer than their entrance latencies during training (shock box: $t_{12} = 2.97$, $P < 0.01$; non-shock box: $t_{12} = 2.90$, $P < 0.01$; Supplementary Table 1). Moreover, retention latencies in both the shock box and the non-shock box were significantly longer than retention latencies in the new box ($t_{12} \geq 3.24$, $P < 0.01$). Hence, consistent with a linking of memory (Cai et al., 2016; Liu et al., 2022), these findings indicate that in saline control animals the footshock experience became associated with both training contexts.

If NE administration into the BLA merely strengthened such memory, retention latencies in both the shock box and the non-shock box would be expected to become longer. However, we found that the NE administration induced significantly longer retention latencies in the shock box (unpaired Student's t -test: $t_{23} = 2.82$, $P < 0.01$ versus saline) and shorter retention latencies in the non-shock box ($t_{23} = 2.93$, $P < 0.01$; Fig. 1B-1C and Supplementary Table 1). As a consequence, retention latencies in the shock box were significantly longer than those in both the non-shock box ($t_{11} = 3.95$, $P < 0.01$) and the new box ($t_{11} = 3.93$, $P < 0.01$), whereas retention latencies in the non-shock box and new box did not differ from each other ($t_{11} = 0.41$, $P = 0.69$). Thus, the NE administration not only strengthened the memory but also facilitated the separation of memory of the two training events into discrete memories such that the trained associations, that is, danger with the shock box, safety with the non-shock box, became selectively strengthened. This NE effect, in enhancing both the specificity and the strength of memory, was also found when the order of exposure to the shock box and the non-shock box was reversed during the training session (Fig. 1D and Supplementary Table 1), indicating that the NE administration had not selectively enhanced retention of the training event closer to the timepoint of drug delivery. Yet, the effect was restricted to the BLA because NE administration into the adjacent central amygdala did not affect 48-h retention performance (Fig. 1E). Moreover, we found that NE administration into the dorsal hippocampus (dHPC) did not induce memory specificity but rather indiscriminately increased 48-h retention latencies in both the shock box ($t_{17} = 3.00$, $P < 0.01$) and the non-shock box ($t_{17} = 2.60$, $P < 0.05$; Fig. 1F). Thus, whereas noradrenergic activation of both the BLA and the dHPC can make memories stronger, only NE administration into the BLA can also make them more distinct and specific.

The use of posttraining NE administration to alter BLA activity shortly after the training session excludes potential influences on, for example, attentional, sensory or motivational processes during the training session that may directly affect task performance or promote the prioritization of the most salient features in memory (Clewett & Murty, 2019; Roozendaal & McGaugh, 2011). Likewise, the NE effect on memory specificity cannot be explained as a result of an improved segmentation of

the two training events into distinct episodes at the time of encoding (Clewett et al., 2020; Dunsmoor et al., 2018). Furthermore, as we found that short-term memory tested 30 min after the posttraining NE administration did not differ from those given saline (Fig. 1G), these findings support the view that noradrenergic activation of the BLA does not directly improve task performance but that the effect likely requires a consolidation process. The cellular consolidation process is a time-restricted process, with neuromodulatory agents being effective only during a restricted time window (McGaugh, 2000). To test whether the NE effect on enhancing memory specificity was limited to the first few hours after the training session, delayed infusions of NE were administered into the BLA 3 h after training. Delayed NE administration also failed to affect 48-h retention performance (Fig. 1H). These findings thus indicate that noradrenergic activation of the BLA enhances both specificity and strength of memory via a consolidation process.

Posttraining NE in the BLA down-regulates miR-134 within the dDG

The ability to separate similar input patterns into discrete memory representations is known as 'pattern separation' and findings suggest that this influence is localized to the dorsal blade of the dentate gyrus (dDG) of the dHPC (Leutgeb et al., 2007; Marr, 1971; Yassa & Stark, 2011). Coordinated regulation of gene expression is essential for the consolidation process of memory (Kandel, 2001). We hypothesized that noradrenergic activation of the BLA may enhance the consolidation of pattern-separated memories within the dDG via posttranscriptional regulation of gene expression by microRNAs (miRNAs) (He & Hannon, 2004). MiRNAs are small noncoding RNAs that are capable of RNA silencing by binding to complementary sequences of their mRNA targets (Schratt, 2009). They are expressed at high levels in the brain and, together with their transient and localized expression in the synaptodendritic compartment and capacity to respond in an activity-dependent manner, make miRNAs ideal candidates for the fine-tuning of gene expression associated with neural plasticity and memory formation (Schratt, 2009). Using an miRNA microarray, we found that NE administration into the BLA immediately after training on the dual-event inhibitory avoidance task markedly altered the miRNA expression pattern within the dDG 30 min later. Consistent with our hypothesis, most miRNAs were found to be decreased (326 miRNAs, $\geq 1 \log_2(\text{fold-change})$ ($\log_2(\text{FC})$) versus saline, pooled data from $n=10$ rats; Fig. 2a and Supplementary Table 2). These effects were largely activity dependent, because NE administration into the BLA of home-cage control rats induced only a slight decrease in miRNAs (48 miRNAs, pooled data from $n=9$ rats).

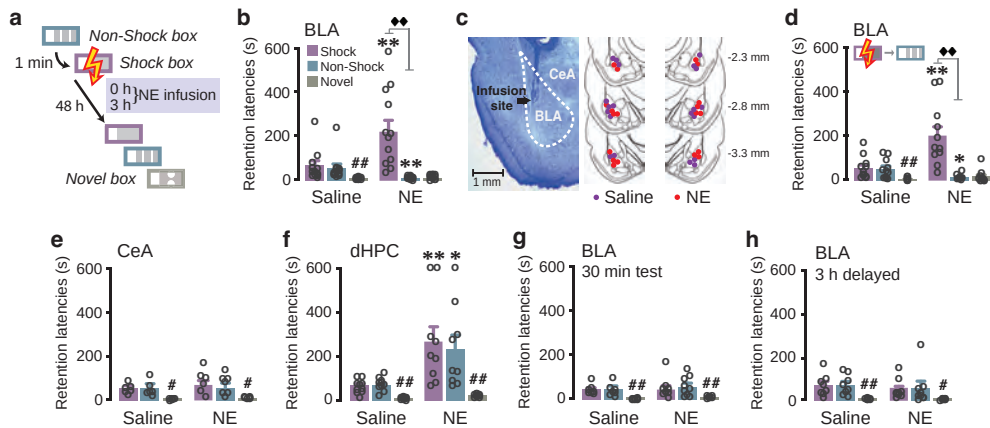


Figure 1 – Posttraining NE in the BLA induces memory specificity.

A. Dual-event inhibitory avoidance task. **B.** Posttraining NE (1.0 µg in 0.2 µl) into the BLA enhancing memory specificity. Step-through latencies in seconds on a 48-h retention test are shown (saline, $n = 13$; NE, $n = 12$, mixed-model ANOVA: NE: $F_{1,23} = 2.90$, $P = 0.10$; context: $F_{2,46} = 20.83$, $P < 0.001$; NE $\times$ context: $F_{2,46} = 11.19$, $P < 0.001$). **C.** Needle-tip placements within the BLA. CeA, central amygdala. **D.** NE administration into the BLA also enhancing memory specificity when rats were trained first in the shock box (saline, $n = 12$; NE, $n = 11$, NE: $F_{1,21} = 7.05$, $P = 0.01$; context: $F_{2,42} = 22.64$, $P < 0.001$; NE $\times$ context: $F_{2,42} = 13.04$, $P < 0.001$). **E.** NE (1.0 µg in 0.2 µl) administration into the central amygdala not affecting 48-h retention (saline, $n = 6$; NE, $n = 8$). **F.** NE (2.5 µg in 0.5 µl) administration into the dHPC inducing a nonspecific memory strengthening on a 48-h retention test (saline, $n = 10$; NE, $n = 9$, NE: $F_{1,17} = 9.72$, $P = 0.002$; context: $F_{2,34} = 15.61$, $P < 0.001$; NE $\times$ context: $F_{2,34} = 5.88$, $P = 0.006$). **G.** NE (1.0 µg in 0.2 µl) administration into the BLA not affecting short-term memory tested 30 min later (saline, $n = 7$; NE, $n = 9$). **H.** Delayed NE (1.0 µg in 0.2 µl) administration into the BLA 3 h posttraining not affecting 48-h retention (saline, $n = 8$; NE, $n = 9$). Data are shown as mean $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.01$ versus saline; # $P < 0.05$, ## $P < 0.01$ versus non-shock box; ♦♦ $P < 0.01$. Small circles indicate individual data points.

Subsequently, we used quantitative PCR (qPCR) to evaluate the expression of some miRNAs previously implicated in synaptic plasticity (see Table 1 in Methods) (Bicker et al., 2014; Gao et al., 2010; Im & Kenny, 2012; Schratt, 2009; Schratt et al., 2006). Specifically, miR-134, a main regulator of the cAMP response element-binding (CREB) and brain-derived neurotrophic factor (BDNF) pathways (Bicker et al., 2014; Gao et al., 2010; Schratt et al., 2006) was significantly downregulated within the dDG ($t_8 = 8.27$, $P < 0.01$, normalized to saline; Fig. 2b). Further analysis revealed that posttraining NE administration into the BLA selectively downregulated miR-134 within the dDG subfield of the dHPC ($t_5 = 3.66$, $P < 0.01$; Fig. 2c) and that this downregulation was transient (30 min: $t_5 = 4.19$, $P < 0.01$; Fig. 2d). As miR-134 was not downregulated at either 15 min ($t_8 = 1.24$, $P = 0.25$) or 60 min posttraining ($t_5 = 1.39$, $P = 0.22$), this finding

suggests that the peak of its reduction lies close to 30 min. Lower expression of miR-134 means that there should be less RNA silencing and posttranscriptional suppression of its target genes (He & Hannon, 2004). Indeed, mRNA expression of *Creb* ($t_7=3.18$, $P<0.05$) and *Bdnf* ($t_7=4.52$, $P<0.01$) were both significantly upregulated (Fig. 2e). NE administration into the BLA did not affect miR-134, *Creb* or *Bdnf* mRNA expression in the dDG of rats that were trained on the task without footshock ($t_8\leq 1.36$, $P\geq 0.21$; Fig. 2f) or of home-cage control rats ($t_8\leq 0.98$, $P\geq 0.36$; Extended Data Fig. 1), indicating that noradrenergic activation of the BLA as such is not sufficient to drive changes in this miR-134 system.

CREB and BDNF are canonical memory mechanisms that play critical roles in memory consolidation (Mizuno et al., 2000; Silva et al., 1998). Moreover, de novo synthesis of BDNF within the dDG has been implicated in the consolidation of overlapping memories (Bekinschtein et al., 2013, 2014). To investigate whether a decreased miR-134 expression could account for the upregulation of CREB and BDNF, a specific locked nucleic-acid-modified oligonucleotide probe against miR-134 (Ant-134, 10 pmol in 0.5 μ l) (Jimenez-Mateos et al., 2012), which achieves specific knockdown of endogenous miR-134, or a scrambled control oligonucleotide (10 pmol in 0.5 μ l) was administered into the dHPC after the training session. As shown in Fig. 2g, selective silencing of miR-134 significantly upregulated *Creb* ($t_6=3.18$, $P<0.05$ normalized to scrambled control) and *Bdnf* ($t_6=2.92$, $P<0.05$) mRNA expression within the dDG 30 min later, which suggests that downregulation of miR-134 by posttraining NE treatment is sufficient to increase *Creb* and *Bdnf* mRNA expression. The mRNA expression of several other plasticity-related genes that are considered nontargets for miR-134 (Campbell et al., 2022) were not significantly affected by the Ant-134 treatment at this timepoint (Extended Data Fig. 2).

To determine whether miR-134 directly interacts with *Creb* and *Bdnf* mRNA, we carried out a dual-luciferase reporter assay (see Table 2 in Methods). As shown in Fig. 2h, compared with the miRNA control group, co-transfection of miR-134 with wild-type CREB 3'-UTR resulted in a large and significant decrease in luciferase activity ($t_4=13.79$, $P<0.01$), indicating that miR-134 binds to CREB (Gao et al., 2010). As a negative control, miR-134 was co-transfected with a mutated CREB containing mutations in the CREB 3'-UTR region. As luciferase activity of miR-134 co-transfected with the mutated CREB 3'-UTR was significantly higher than that of miR-134 co-transfected with wild-type CREB 3'-UTR ($t_4=26.96$, $P<0.01$) and did not differ from the miRNA control group ($t_4=0.78$, $P=0.48$), the mutated site in CREB 3'-UTR was deemed the binding site of miR-134. Co-transfection of miR-134 with wild-type BDNF 3'-UTR also resulted in a significant, albeit smaller, decrease in luciferase activity ($t_4=15.69$, $P<0.01$; Fig. 2i). Luciferase activity of miR-134 co-transfected with a mutated BDNF 3'-UTR region was significantly higher than that of miR-134 co-transfected with wild-type BDNF 3'-UTR ($t_4=3.24$, $P<0.05$). These findings thus indicate that miR-134 also binds to BDNF (Eyileten et al., 2021; Wang et al., 2022), but that it appears unlikely that miR-134 regulates the expression of BDNF solely via this mechanism.

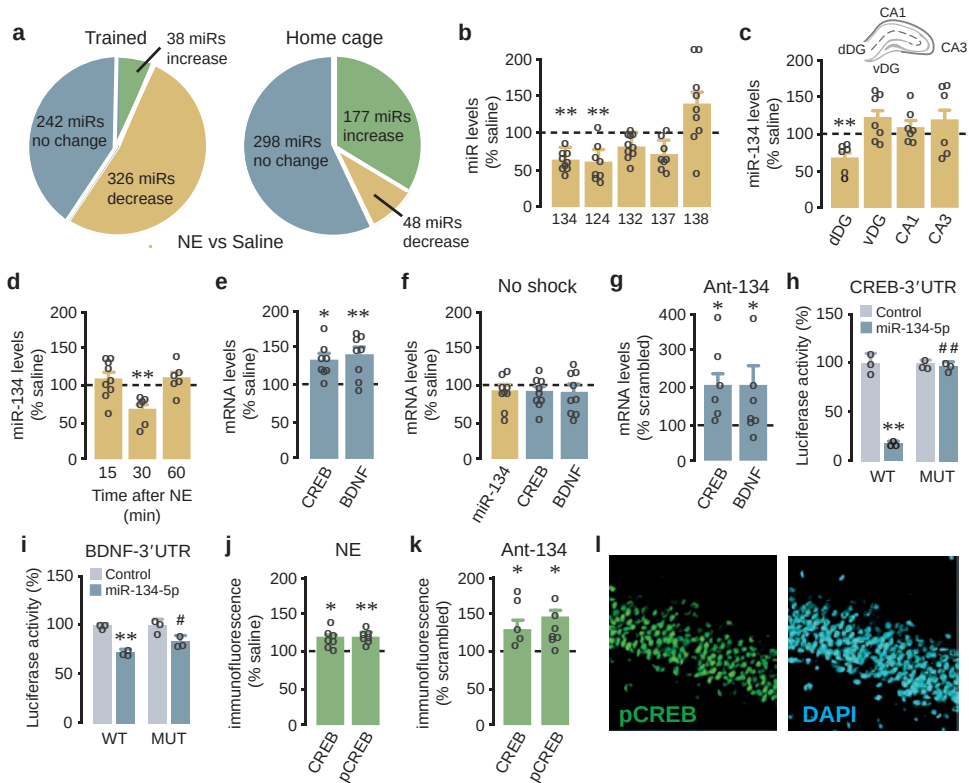


Fig. 2. Posttraining NE in the BLA down-regulates miR-134 within the dDG.

a, Posttraining NE into the BLA reducing expression of 326 miRNAs ($\geq 1 \log_2(\text{FC})$) within the dDG 30 min later. Pooled data are from $n = 10$. **b**, Posttraining NE into the BLA downregulating miR-134 and miR-124 in the dDG 30 min later. **c**, Posttraining NE into the BLA selectively downregulating miR-134 levels in the dDG. Ventral blade DG (vDG): $t_6 = 1.65$, $P = 0.15$; CA1: $t_6 = 1.13$, $P = 0.30$; CA3: $t_5 = 0.88$, $P = 0.42$. **d**, Temporal dynamics of NE-induced downregulation of miR-134 in the dDG (15 min: $t_8 = 1.24$, $P = 0.25$; 30 min: $t_5 = 4.19$, $P < 0.01$; 60 min: $t_5 = 1.39$, $P = 0.22$). **e**, Posttraining NE into the BLA upregulating *Creb* and *Bdnf* mRNA expression in the dDG 30 min later ($n = 8$). **f**, Posttraining NE into the BLA not affecting miR-134, *Creb* or *Bdnf* mRNA expression in the dDG of rats trained without footshock ($n = 9$). **g**, Posttraining Ant-134 into the dHPC upregulating *Creb* and *Bdnf* mRNA expression in the dDG 30 min later ($n = 7$). **h**, miR-134-5p cotransfected with wild-type CREB 3'-UTR decreasing luciferase activity ($n = 3$ per group). **i**, miR-134-5p cotransfected with wild-type BDNF 3'-UTR decreasing luciferase activity ($n = 3$ per group). **j**, Posttraining NE into the BLA increasing CREB and pCREB protein levels in the dDG 3 h later. Protein levels were normalized to the saline-treated hemisphere ($n = 7$). **k**, Posttraining Ant-134 into the dHPC increasing CREB and pCREB protein levels in the dDG 3 h later ($n = 7$). * $P < 0.05$. **l**, Representative pCREB and DAPI images. * $P < 0.05$; ** $P < 0.01$; # $P < 0.05$; ## $P < 0.01$ versus WT. The bars show mean $\pm$ s.e.m. Individual data points are represented as small circles. MUT,

mutated CREB or BDNF 3'-UTR.

Previous studies have reported that miR-134 also indirectly regulates the expression of BDNF via the CREB pathway (Baby et al., 2020; Gao et al., 2010; Li et al., 2020). CREB acts as a transcription factor, which, on activation, that is, phosphorylation, binds to the cAMP response element of the promoters of its target genes (Kandel, 2012). CREB also binds to several promoters of the *Bdnf* gene (Tao et al., 1998). We found that NE administration into the BLA after the training session significantly increased CREB protein levels ($t_6 = 2.91$, $P < 0.05$) and phosphorylation of CREB (pCREB; $t_6 = 5.12$, $P < 0.01$) within the dDG granule cell layer 3 h later (Fig. 2j), but not in the ventral blade granule cell layer (Extended Data Fig. 3), and that Ant-134 administration into the dHPC induced a similar increase in both CREB ($t_6 = 3.18$, $P < 0.05$) and pCREB protein levels within the dDG granule cell layer ($t_6 = 4.86$, $P < 0.05$; Fig. 2k,l). Thus, an increase in total CREB and pCREB most likely leads to the biosynthesis of various other plasticity-related proteins involved in the cellular consolidation process (Baby et al., 2020).

Hippocampal miR-134 selectively affects memory specificity

Next, we determined whether downregulation of miR-134 within the dDG is an essential step in facilitating memory specificity. Artificial downregulation of miR-134, with bilateral administration of Ant-134 (10 pmol in 0.5 μ l) into the dHPC immediately after training on the dual-event inhibitory avoidance task, was sufficient to induce memory specificity ($t_{11} = 3.07$, $P < 0.05$ shock box versus non-shock box; Fig. 3a,b). Ant-134 treatment induced significantly shorter retention latencies in the non-shock box ($t_{22} = 6.05$, $P < 0.01$ versus scrambled). Importantly, unlike NE administration into the BLA (Fig. 1b), Ant-134 treatment did not increase retention latencies in the shock box ($t_{22} = 0.44$, $P = 0.67$), thereby demonstrating that downregulation of miR-134 regulates memory specificity, but is not required in strengthening the memory. As shown in Fig. 3c, this selective Ant-134 effect on enhancing memory specificity, and not memory strength, was independent of the shock intensity used during the training ($t_7 = 3.88$, $P < 0.01$ shock box versus non-shock box). Delayed administration of Ant-134 into the dHPC given 3 h after the training did not induce memory specificity (Fig. 3d). These findings are thus consistent with those of delayed NE administration into the BLA (Fig. 1h) and highlight that establishment of memory specificity requires a consolidation process.

To further characterize the necessity of downregulation of miR-134 in mediating the effect of NE administration into the BLA on memory specificity, we used a synthetic mimic for miR-134 (Syn-rno-miR-134-5p, 0.25 pmol in 0.5 μ l) to bilaterally overexpress the levels of miR-134 in the dHPC after the training session. Posttraining Mimic-134 administration resulted in an efficient downregulation of *Creb* (paired Student's *t*-test: $t_5 = 3.22$, $P < 0.05$) and *Bdnf* ($t_5 = 3.28$, $P < 0.05$) mRNA within the dDG (Extended Data Fig. 4). Most importantly, hippocampal overexpression of miR-134 completely prevented the effect of posttraining NE administration into the BLA on inducing memory specificity ($t_{13} = 0.28$, $P = 0.78$ shock box versus non-shock box;

Fig. 3e), whereas it left the NE effect on strengthening the memory unaltered. NE administration into the BLA now induced significantly longer retention latencies in both the shock box ($t_{25} = 2.22$; $P < 0.05$ versus saline) and the non-shock box ($t_{25} = 2.11$; $P < 0.05$). As the NE administration did not increase retention latencies in the new box ($t_{25} = 0.06$, $P = 0.95$), these findings suggest that the Mimic-134 treatment prevented the NE effect from separating the memory of the two training events rather than causing a generalization, that is, lack of contextualization, of fear memory across all boxes.

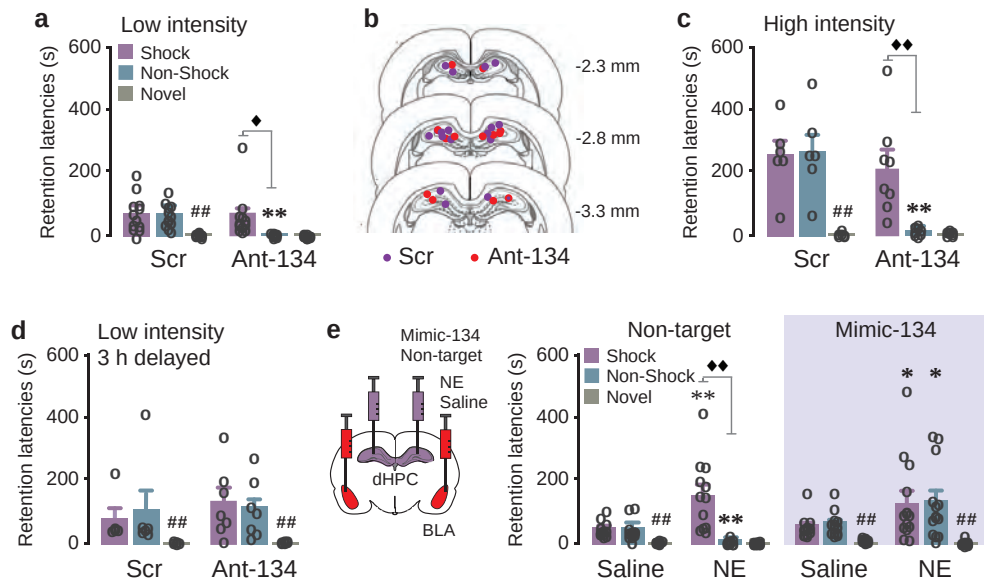


Fig. 3. Hippocampal miR-134 selectively affects memory specificity.

a, Ant-134 (10 pmol in 0.5 μ l) administration into the dHPC after training on the dual-event inhibitory avoidance task (0.38 mA, 1 s) enhancing memory specificity, without affecting memory strength. Step-through latencies in seconds are shown for a 48-h retention test (Scrambled (Scr), $n = 12$; Ant-134, $n = 12$, mixed-model ANOVA: Ant-134: $F_{1,22} = 5.29$, $P = 0.03$; context: $F_{2,44} = 20.39$, $P < 0.001$; Ant-134 $\times$ context: $F_{2,44} = 5.41$, $P = 0.004$). **b**, Needle-tip placements within the dHPC. **c**, Ant-134 administration into the dHPC after high-intensity shock training (0.6 mA, 1 s) also selectively inducing memory specificity on a 48-h retention test (Scr, $n = 6$; Ant-134, $n = 8$; Ant-134: $F_{1,12} = 10.02$, $P = 0.004$; context: $F_{2,24} = 27.21$, $P < 0.001$; Ant-134 $\times$ context: $F_{2,24} = 10.86$, $P < 0.001$). **d**, Delayed Ant-134 administration into the dHPC 3 h posttraining (0.38 mA, 1 s) not inducing memory specificity on a 48-h retention test (Scr, $n = 6$; Ant-134, $n = 7$, Ant-134: $F_{1,11} = 0.20$, $P = 0.66$; context: $F_{2,22} = 8.99$, $P < 0.001$; Ant-134 $\times$ context: $F_{2,22} = 0.58$, $P = 0.57$). **e**, Posttraining Mimic-134 (0.25 pmol in 0.5 μ l) administration into the dHPC blocking the effect of NE (1.0 μ g in 0.2 μ l) into the BLA on memory specificity, but not affecting the NE effect on enhancing memory strength (saline nontarget (NT), $n = 14$; NE NT, $n = 12$; saline Mimic-134, $n = 13$; NE Mimic-134, $n = 14$,

NE $\times$ context: $F_{2,98} = 11.29$, $P < 0.001$; Mimic-134 $\times$ context: $F_{2,98} = 9.74$, $P < 0.001$; NE $\times$ Mimic-134 $\times$ context: $F_{2,98} = 6.92$, $P = 0.002$). * $P < 0.05$; ** $P < 0.01$ versus saline or scrambled; $\blacklozenge P < 0.05$; $\blacklozenge\blacklozenge P < 0.01$; $\#\#\# P < 0.01$ versus non-shock box. The bars show mean $\pm$ s.e.m. Individual data points are represented as small circles.

NE in the BLA naturally engages this miR-134 mechanism

To determine whether this NE-induced downregulation of miR-134 is physiologically relevant and engaged when memory specificity is naturally occurring, we trained rats on a slightly easier version of the dual-event inhibitory avoidance task with a longer interval of 2 min between the two training events (Atucha et al., 2017; Atucha & Roozendaal, 2015) (Fig. 4a). Control rats trained on this version of the task displayed memory specificity on a 48-h retention test (Fig. 4c,e,k and Supplementary Table 1). As shown in Fig. 4b, miR-134 levels in the dDG 30 min after training on the two events with a 2-min interval were significantly reduced compared with those of rats that were trained on these events with a 1-min interval (unpaired Student's t -test: $t_{27} = 2.88$, $P < 0.01$). In addition, NE (1.0 μg in 0.2 μl) administration into the BLA, immediately after training on the task with a 2-min interval, which had no effect on memory specificity (Fig. 4c), did not further decrease miR-134 levels within the dDG (paired Student's t -test: $t_8 = 1.04$, $P = 0.33$, Fig. 4d). As shown in Fig. 4e, Mimic-134 (0.25 pmol in 0.5 μl) administration into the dHPC to bilaterally overexpress miR-134 levels after the training session selectively prevented the memory specificity ($t_9 = 0.93$, $P = 0.38$ shock box versus non-shock box; Supplementary Table 1). These findings thus indicate that increasing the interval between the two training events, that is, reducing the temporal overlap of the two memory traces, induces memory specificity in control animals that is associated with an endogenous downregulation of miR-134 within the dDG. They further provide additional evidence that this decrease in miR-134 levels is essential for establishing memory specificity.

Our finding that miR-134 was downregulated 30 min after the training session is consistent with other evidence indicating that triggering of long-term potentiation in the DG induces a rapid downregulation of a large number of miRNAs, including miR-134 (Joilin et al., 2014). To understand whether or not the rapid downregulation of miR-134 after the training session is the consequence of an accelerated decay (Chatterjee & Großhans, 2009; Joilin et al., 2014; Krol et al., 2010), we assessed precursor hairpin miR-134 levels. Rapid decay of miRNAs in active neurons was shown to affect only mature, and not hairpin, miRNA levels (Joilin et al., 2014; Krol et al., 2010). As we found that hairpin miR-134 levels in the dDG 30 min after training on the two events with a 2-min interval were not significantly affected (unpaired Student's *t*-test: $t_{27}=0.03$, $P=0.97$; Fig. 4f), these findings thus suggest that the 2-min training session had accelerated the degradation of miR-134. Previous studies have shown that steady-state levels of miRNAs and their activities are regulated by posttranscriptional processes at their 3'-end (Boele et al., 2014; Burroughs et al., 2010; Lee et al., 2019). Therefore, we performed small RNA sequencing (RNA-seq) on a subset of samples to analyze the 3'-sequence variants of both mature miR-134 and hairpin miR-134. We

found one prevalent iso-miRNA form of mature miR-134 and a lower expression of several iso-miRNA forms that were shorter at their 3'-end (Fig. 4g). Training on the task with a 2-min interval selectively reduced expression of the full-length variant of mature miR-134 (22 nt: unpaired Student's t -test: $t_4 = 5.21$, $P < 0.01$), without affecting the expression of truncated variants of miR-134 (20–21 nt: $t_4 = 0.11$, $P = 0.92$), thus providing further support for the view that the longer interval had degraded miR-134. We also found several 3'-sequence variants of hairpin miR-134 (Fig. 4h), with primarily nontemplated additions of one or a few nucleotides, usually A or T, to the 3'-end (Supplementary Table 3). Training on the task with a 2-min interval significantly increased the fraction of hairpin miR-134 with a 3'-terminal A addition ($t_4 = 3.07$, $P < 0.05$; Fig. 4i and Supplementary Table 3) and nonsignificantly decreased the fraction with a 3'-terminal T addition ($t_4 = 2.33$, $P = 0.08$). Such 3'-terminal A additions have been detected on many different miRNAs (Kai & Pasquinelli, 2010) and previous studies have suggested that this represents an important posttranscriptional processing event that affects both the stability and the activity of miRNAs (Katoh et al., 2009). A functional consequence of this modification for miR-134 homeostasis, however, awaits elucidation.

To test whether noradrenergic activity of the BLA, induced by the emotionally arousing training experience itself (McIntyre et al., 2002), regulates this endogenous downregulation of miR-134 in establishing memory specificity, the β -adrenoceptor antagonist propranolol (0.3 μ g in 0.2 μ l) was administered bilaterally into the BLA immediately after training on the dual-event inhibitory avoidance task with a 2-min interval. Opposite to the effect of NE administration into the BLA (Fig. 2b), we found that propranolol treatment significantly upregulated miR-134 levels within the dDG 30 min later ($t_9 = 2.96$, $P < 0.05$; Fig. 4j). Also opposite to the effect of NE administration (Fig. 1b,d), propranolol prevented the memory specificity ($t_{11} = 1.11$, $P = 0.29$ shock box versus non-shock box; Fig. 4k) and impaired the strength of memory on a 48-h retention test. Propranolol administration induced significantly shorter retention latencies in the shock box (unpaired Student's t -test: $t_{21} = 2.60$, $P < 0.05$ versus saline) and longer retention latencies in the non-shock box ($t_{21} = 2.65$, $P < 0.05$), which were both significantly longer than retention latencies in the new box ($t_{11} \geq 4.80$, $P < 0.01$; Supplementary Table 1). These findings thus demonstrate that this NE-induced miR-134 mechanism within the BLA–dDG circuit is naturally involved in regulating emotional arousal effects in facilitating memory specificity for two similar events experienced close in time.

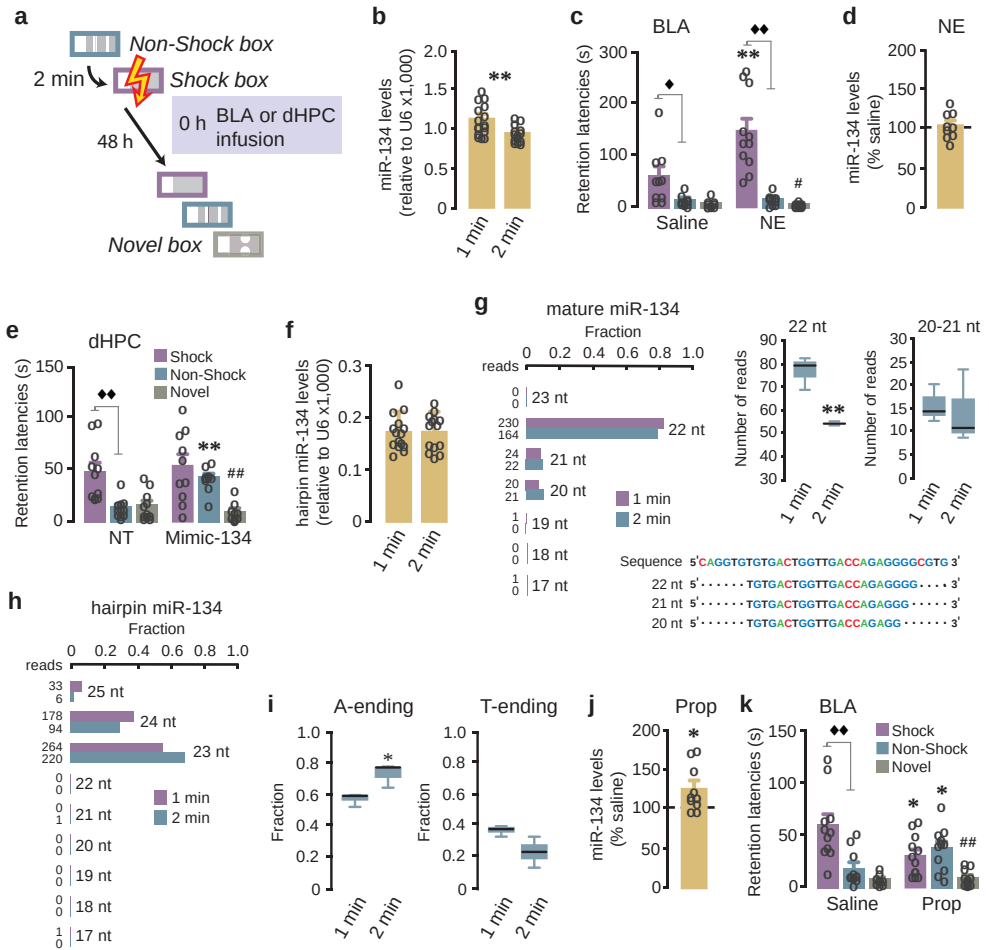


Fig. 4. NE in the BLA naturally engages the miR-134 mechanism in establishing memory specificity.

a, Experimental protocol with a 2-min interval. **b**, MiR-134 levels in the dDG 30 min after training lower with a 2-min interval compared with a 1-min interval (1 min, $n=15$; 2 min, $n=14$). **c**, Posttraining NE into the BLA (2-min interval) not affecting memory specificity (saline, $n=10$; NE, $n=11$). **d**, Posttraining NE into the BLA (2-min interval) not downregulating miR-134 levels within the dDG 30 min later ($n=9$). **e**, Mimic-134 into the dHPC (2-min interval) blocking memory specificity (NT, $n=11$; Mimic-134, $n=10$; Mimic-134: $F_{1,19}=2.65$, $P=0.12$; context: $F_{2,38}=21.99$, $P<0.001$; Mimic-134 x context: $F_{2,38}=4.66$, $P=0.02$). **f**, Training interval comparison of hairpin miR-134 levels in the dDG 30 min later (1 min, $n=15$; 2 min, $n=14$). **g**, The 3'-variants of mature miR-134 after training, 2-min versus 1-min interval. The number of reads is the sum of three samples per experimental condition. **h**, The 3'-variants of hairpin miR-134 after training on the task with a 2-min versus a 1-min interval. **i**, Training (2-min interval) increasing hairpin miR-134 with a nontemplated A addition. **j**, Posttraining

propranolol into the BLA (2-min interval) upregulating miR-134 levels in the dDG 30 min later ($n=10$). **k**, Posttraining propranolol into the BLA (2-min interval) blocking memory specificity (saline, $n=11$; propranolol, $n=12$; propranolol: $F_{1,21}=0.23$, $P=0.64$; context: $F_{2,42}=23.00$, $P<0.001$; propranolol $\times$ context: $F_{2,42}=10.73$, $P<0.001$). * $P<0.05$; ** $P<0.01$ versus saline; $\blacklozenge P<0.05$; $\blacklozenge\blacklozenge P<0.01$; $\#P<0.05$; $\#\#\#P<0.01$ versus non-shock box. The bars show mean $\pm$ s.e.m. The box plots show the interquartile range. Individual data points are represented as small circles.

NE in the BLA does not affect miR-134 after a single event

Our findings indicate that miR-134 within the dDG bidirectionally regulates memory specificity for two similar events experienced close in time: miR-134 loss of function induces, whereas its overexpression blocks memory specificity. By contrast, manipulation of miR-134 levels does not affect memory strength for the aversive experience as such. This raises the question of whether noradrenergic activation of the BLA may selectively downregulate miR-134 levels within the dDG on an 'as needed' basis, that is, after training on a learning task when information of two similar events is present. To examine this possibility, NE (1.0 μg in 0.2 μl) was administered into the BLA after training on a single-event, inhibitory avoidance task (Fig. 5a). Thus, the rat also received an aversive footshock in a particular location of the apparatus, but the training experience comprised a single event and therefore the rat did not have to separate memory representations of multiple events. The NE administration significantly increased 48-h retention latencies in the shock box ($t_{16}=3.28$, $P<0.01$ versus saline; Fig. 5b), but not in a new box ($t_{16}=1.09$, $P=0.29$), indicating a strengthening of the association between footshock and training context. Critically, despite this memory enhancement, the NE treatment did not downregulate miR-134 levels within the dDG 30 min later ($t_7=1.06$, $P=0.32$; Fig. 5c). In addition, Ant-134 (10 pmol in 0.5 μl) administration into the dHPC after training on the single-event inhibitory avoidance task did not affect 48-h retention performance (Fig. 5d), thus confirming that this miR-134-mediated mechanism does not involve single-event memories. To test whether the similarity of the two events critically determines when miR-134 is recruited, we pre-exposed rats to the shock box (without footshock) 1 min before inhibitory avoidance training in that same context. As shown in Fig. 5e, NE administration into the BLA did not downregulate miR-134 in the dDG after two exposures to a single context ($t_8=0.98$, $P=0.36$). These findings thus indicate that noradrenergic activation of the BLA selectively engages this miR-134 mechanism in the dDG after training on an episodic-like task when information of two similar, and conceivably easily confused, events is present.

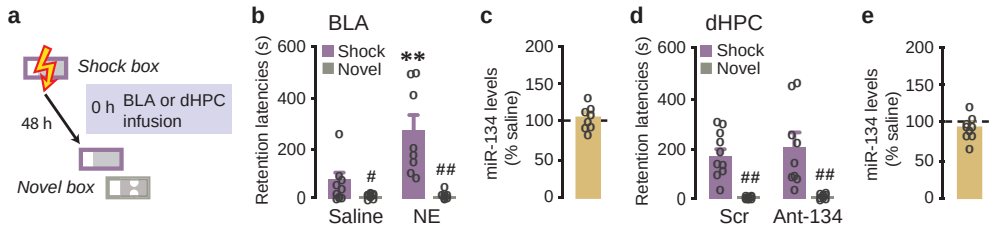


Fig. 5. NE in the BLA does not affect the miR-134 mechanism after a single event.

a, Experimental protocol of the single-event inhibitory avoidance task. **b**, Posttraining NE (1.0 μg in 0.2 μl) administration into the BLA enhancing memory of a single-event, inhibitory avoidance task. Step-through latencies in seconds on a 48-h retention test are shown (saline, $n=10$; NE, $n=8$, mixed-model ANOVA: NE: $F_{1,16}=10.33$, $P=0.005$; context: $F_{1,16}=30.19$, $P<0.001$; NE $\times$ context: $F_{1,16}=11.22$, $P=0.004$). **c**, Posttraining NE administration into the BLA not downregulating miR-134 levels in the dDG 30 min later. The miR-134 levels were normalized to the saline-treated hemisphere (mean $\pm$ s.e.m., $n=8$, paired Student's t -test: $t_7=1.09$, $P=0.20$). **d**, Posttraining Ant-134 (10 pmol in 0.5 μl) administration into the dHPC not affecting 48-h retention (saline, $n=10$; NE, $n=9$, Ant-134: $F_{1,17}=0.14$, $P=0.72$; context: $F_{1,34}=32.62$, $P<0.001$; Ant-134 $\times$ context: $F_{1,34}=0.06$, $P=0.81$). **e**, Posttraining NE administration into the BLA also not downregulating miR-134 levels in the dDG 30 min later when rats were pre-exposed to the shock box 1 min before inhibitory avoidance training in that same box (mean $\pm$ s.e.m., $n=9$). ** $P<0.01$ versus saline; # $P<0.05$, ## $P<0.01$ versus shock box. The bars show mean $\pm$ s.e.m. Individual data points are represented as small circles.

CONCLUSIONS

Our findings indicate that noradrenergic activation of the BLA not only enhances the strength of memory (McGaugh, 2000; McIntyre et al., 2002; Roozendaal & McGaugh, 2011), but also, via the involvement of a second mechanism, induces memory specificity after training on a task with two similar events. These findings are consistent with reported evidence that emotional memories are often remembered vividly and distinctly (Krenz et al., 2021; Sharot et al., 2004). Human studies have shown that changes in context during emotional memory encoding may elicit a transient burst of noradrenergic arousal, which may support the segmentation of continuous experience into discrete and memorable episodes (Clewett & Murty, 2019; Dunsmoor et al., 2018), but that selective episodic memory for emotionally arousing experiences may also depend on a subsequent period of consolidation (Dunsmoor et al., 2018). We have identified that downregulation of miR-134 within the dDG critically regulates a consolidation process that mediates noradrenergic arousal effects on memory specificity. However, the potential contribution of other miRNAs cannot be discounted from our results.

How this miR-134-mediated mechanism accomplishes this function is unclear. The dDG receives nonspatial contextual information from the lateral entorhinal cortex via

the lateral perforant path (LPP) (Hargreaves et al., 2005). In response to LPP inputs, it has been reported that adult-born, immature granule cells exert monosynaptic inhibition of dDG granule cells (Luna et al., 2019). This inhibitory control may promote sparse neural activity (Myers & Scharfman, 2009), which can enhance pattern separation (GoodSmith et al., 2017; Leutgeb et al., 2007; Senzai & Buzsáki, 2017; Yassa & Stark, 2011). Previous findings have implicated miR-134 in regulating long-term associative plasticity (Baby et al., 2020; Gao et al., 2010) and dendritic spine development within the dHPC (Bicker et al., 2014; Schratt et al., 2006). We propose that BLA activity-induced downregulation of miR-134, via its stimulatory effect on CREB synthesis and gene expression, could facilitate long-term plasticity in LPP–dDG cell synapses during the consolidation period (Ikegaya et al., 1997; Joilin et al., 2014; Vouimba & Richter-Levin, 2013), which could then potentiate the inhibitory control of dDG granule cells to facilitate pattern separation and improve discrimination behavior during the later retention test (McHugh et al., 2022). Moreover, *de novo* synthesis of BDNF, acting on immature neurons, was critically involved in the consolidation of overlapping memories (Bekinschtein et al., 2013, 2014). Therefore, the miR-134 effect on enhancing BDNF, eventually via the CREB pathway (Baby et al., 2020; Gao et al., 2010; Li et al., 2020), could further contribute to a suppression of the response of dDG granule cells to LPP inputs (Luna et al., 2019). This effect of noradrenergic activation of the BLA on enhancing memory specificity could act in parallel with its well-established influence on brain regions such as the dHPC, striatum and cortical areas to enhance memory strength of different components of the acquired information (McGaugh, 2000; McIntyre et al., 2002; Roozendaal & McGaugh, 2011).

We examined the effect of noradrenergic activity on memory specificity only in male rats, but investigating this mechanism also in females would be of great interest. Previous work has indicated sex-specific differences in the noradrenergic system and its regulation by stress and emotional arousal (Bangasser et al., 2016; Borodovitsyna et al., 2022). Higher levels of training-induced NE in the BLA and dHPC in females (Joshi & Chandler, 2020) may underlie, in part, sex-specific differences in fear learning, contextual pattern separation and the ability to discriminate a shock context from a safe context (Yagi et al., 2016, 2022), as well as a sex-specific modulation of this performance by stress and emotional arousal (Edwards et al., 2021).

These results provide solid evidence to strongly indicate that noradrenergic activation of the BLA supports a consolidation process that facilitates the separation of memories. Such a modulatory mechanism may protect emotional memories from competing experiences encoded close in time and enable the selective strengthening of correct associations into long-term memory.

METHODS

Subjects

Male adult Sprague–Dawley rats (280–320 g at time of surgery) from Charles River Breeding Laboratories were kept individually in a temperature-controlled (22°C) vivarium room and maintained on a 12-h:12-h light:dark regimen (07:00–19:00 lights on) with free access to food and water. We restricted our study to males because it was built on previous experiments and settings optimized for male rats only (Atucha et al., 2017; Atucha & Roozendaal, 2015; LaLumiere et al., 2003; Roozendaal et al., 2008; Roozendaal & Mirone, 2020). Fear learning, including contextual pattern separation and the ability to discriminate a shock context from a safe context, differs between sexes (Edwards et al., 2021; Yagi et al., 2016, 2022) and varies with the estrous cycle phase in females (do Nascimento et al., 2019; Graham & Scott, 2018; Kirry et al., 2019). Moreover, sex-specific differences in the noradrenergic system and its regulation by stress and emotional arousal have been reported (Bangasser et al., 2016; Borodovitsyna et al., 2022), which may result in higher levels of training-induced NE in females (Joshi & Chandler, 2020). Therefore, performing these studies in females may require different experimental settings (for example, drug doses, shock intensity, interval) as well as testing animals at different stages of the estrous cycle. Training and testing were performed during the light phase of the cycle between 10:00 and 15:00. All experimental procedures were in compliance with European Union Directive no. 2010/63/EU and approved by the Central Authority for Scientific Procedures on Animals (CCD), the Hague, the Netherlands and/or the Institutional Animal Care and Use Committee of Radboud University, Nijmegen, the Netherlands.

Cannula implantation

Rats were anesthetized with a subcutaneous injection of ketamine (37.5 mg per kg of body weight; Alfasan) and dexmedetomidine (0.25 mg kg⁻¹; Orion), and received the nonsteroidal analgesic carprofen (4 mg kg⁻¹; Pfizer). The rat was positioned in a stereotaxic frame (Kopf Instruments) and two stainless steel guide cannulae (23G; Component Supply Co/SKU Solutions) were implanted bilaterally with the cannula tips 2.0 mm above the BLA (15 mm; coordinates: anteroposterior (AP): 2.8 mm posterior to bregma; mediolateral (ML): ± 5.0 mm lateral to midline; dorsoventral (DV): 6.5 mm below skull surface), 2.0 mm above the central amygdala (15 mm; AP: -2.2 mm; ML: ± 4.1 mm; DV: -5.8 mm) or 1.5 mm above the dHPC (11 mm; AP: -3.6 mm; ML: ± 1.9 mm; DV: -2.6 mm) (Paxinos & Watson, 2006). The cannulae were affixed to the skull with surgical screws and dental cement. Stylets (15- or 11-mm-long 00-insect dissection pins) were inserted into each cannula to maintain patency and were removed only for the infusion of drugs. After surgery, rats were administered atipamezole hydrochloride (0.25 mg kg⁻¹, subcutaneously; Orion) to reverse anesthesia and 3 ml of sterile saline to facilitate clearance of drugs. The rats were allowed to recover for a minimum of 7 days before commencement of training and were handled 3 $\times$ for 1 min each during this recovery period.

Dual-event inhibitory avoidance task

Rats were consecutively trained in two contextually distinct, inhibitory avoidance apparatuses within a single training session, but footshock was delivered only in the latter context. On the retention test, rats were tested in the two training contexts as well as in a new context. Each apparatus had the same geometry and consisted of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top and 6.4 cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor. The starting compartment (31 cm) was made of opaque white plastic and was well lit; the shock compartment (60 cm) was made of two dark, electrifiable metal plates and was not illuminated. The training context in which footshock was given (shock box) did not have any contextual modifications. The other two apparatuses had some contextual modifications that made them a distinctly different training and/or test context. The safe training context (non-shock box) had four vertical white stripes (2 cm wide) taped in the dark compartment together with tape placed on the floor, closing the gap between the two plates. The new box (used on the retention test only) had two white circles (9 cm diameter) taped on each wall of the dark compartment and the gap between both plates was closed with tape. The three inhibitory avoidance apparatuses were located next to one other in a sound- and light-attenuated room. For a detailed description of this task, also termed inhibitory avoidance discrimination task, see ref. Atucha & Roozendaal (2015).

For training, rats were initially placed into the starting compartment of the non-shock box, facing away from the door, and could freely explore the apparatus, including the dark compartment, for 20 s, without a footshock being delivered. Afterwards, the rats were removed from the apparatus and, after a brief delay of 1 or 2 min, placed into the starting compartment of the second inhibitory avoidance apparatus (shock box). When the rat had stepped into the dark compartment, the sliding door was closed and a single inescapable footshock (0.60 mA for rats with cannulae into the BLA or central amygdala and 0.38 or 0.60 mA for rats with cannulae into the dHPC) was delivered for 1 s. The rats were removed from the apparatus 20 s after termination of the footshock and, after drug treatment, returned to their home cages. In a control experiment, the order of training in the non-shock box and shock box was reversed, so rats were trained first in the shock box (with footshock) and subsequently in the non-shock box (without footshock). Previous findings indicated that the duration of the interval between the two training events determines the difficulty of the task. Rats trained with a short 1-min interval, causing a temporal overlap of the two memory traces, are unable to associate the shock experience with the correct training context, whereas rats trained with an increased interval of 2 min show accurate memory of the shock–context association (Atucha & Roozendaal, 2015; Roozendaal & Mirone, 2020). Some rats were sacrificed at 15, 30 or 60 min after training and drug treatment for assessment of miRNA and mRNA changes or at 3 h after training for immunohistochemistry. Other rats were left undisturbed until the retention test 48 h later. For retention testing, retention latencies were assessed, in a randomized order and without delay, in the two training contexts (that is, shock box and non-shock

box) and in the new box that they had not visited before. Previously, we have shown that the order of retention testing in the different test environments does not affect retention latencies (Atucha & Roozendaal, 2015). For all three boxes, the rats were placed into the starting compartment, facing away from the door, and their latency to enter the dark compartment with all four paws (maximum latency of 600 s) was recorded. Stronger memory was defined as increased retention latencies in the shock box. Memory specificity for the two training events was defined as longer latencies in the shock box relative to the non-shock box, whereas latencies in the non-shock box and new box do not differ from each other. Linking of memory (or lack of memory specificity) for the two training events was defined as similar latencies in the shock box and non-shock box, which are longer than those in the new box, whereas memory generalization was defined as similar latencies in all three test contexts.

Single-event inhibitory avoidance task

Rats were placed into the starting compartment of the shock box, facing away from the door, and allowed to explore the apparatus freely. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.60 mA for BLA or 0.38 mA for dHPC) was delivered for 1 s. Rats were removed from the apparatus 20 s later and, after drug treatment, returned to their home cages. Some rats were pre-exposed to the shock box 1 min before inhibitory avoidance training. During the pre-exposure, rats were placed into the starting compartment and allowed to freely explore the apparatus, including the dark compartment, for 20 s, without a footshock being delivered. Some rats were sacrificed at 30 min after training and drug treatment for molecular analysis. On the retention test, 48 h later, rats were placed, in a randomized order, into the starting compartment of the shock box or the new box and the latency to enter the dark compartment with all four paws (maximum latency of 600 s) was measured. Immediately after testing in the first context, rats were placed in the other apparatus and retention latencies were measured.

Drug and infusion procedures

NE (Sigma-Aldrich) was dissolved in saline and infused into the BLA (1.0 µg in 0.2 µl), central amygdala (1.0 µg in 0.2 µl) or dHPC (2.5 µg in 0.5 µl) immediately after the training session. Some rats received intra-BLA infusions of NE or saline control 3 h after the training. The β-adrenoceptor antagonist propranolol (0.3 µg in 0.2 µl; Sigma-Aldrich) was also dissolved in saline and infused into the BLA immediately after the training session. Doses of NE and propranolol were selected based on their well-established memory-enhancing or -impairing effect, respectively (Atucha et al., 2017; LaLumiere et al., 2003; Roozendaal et al., 2008). NE and propranolol were freshly prepared before each experiment. Cholesterol-tagged LNA-antagomir targeting miR-134 (Ant-134; TGGTCAACCAGTCACA-cholesterol-TEG; 10 pmol in 0.5 µl; Exiqon A/S) (Jimenez-Mateos et al., 2012) or a nontargeting scrambled version of the antagomir

(Scr; 10 pmol in 0.5 μ l; Exiqon A/S) (Jimenez-Mateos et al., 2012) was stored as 20- μ M aliquots at -20°C and diluted in saline on the day of the experiment to reach the final concentration. Ant-134 or Scr was administered into the dHPC either immediately or 3 h after the training session. Overexpression of miR-134 in the dHPC was performed by Syn-rno-miR-134-5p (Mimic-134; 5'-UGUGACUGGUUGACCAGAGGGG-3'; 0.25 pmol in 0.5 μ l; QIAGEN) or a nontarget control (NT; 0.25 pmol in 0.5 μ l; QIAGEN). Mimic-134 or NT (5 nmol in 100 μ l) was diluted in 150 μ l of reagent mixture, consisting of 40 μ l of Screenfect reagent (Screenfect A) mixed with 110 μ l of Screenfect buffer (Screenfect A). This RNA mixture was incubated for 20 min at room temperature and aliquots of 0.5 nmol in 25 μ l were stored at -20°C . On the day of the experiment, an aliquot was thawed and saline was added to achieve the final concentration, which contained 14% of reagent mixture.

For behavioral assessment, bilateral infusions of drug or an equivalent volume of control solution was made using 30G injection needles connected to 10- μ l Hamilton microsyringes by poly(ethylene) (PE-20) tubing. For molecular assessment, a unilateral infusion of drug was administered into one hemisphere and an equivalent volume of control solution into the other hemisphere in a randomized manner across rats. For BLA and central amygdala infusions, the injection needles protruded 2.0 mm beyond the cannula tips and a 0.2- μ l injection volume per hemisphere was infused during 30 s by an automated syringe pump (Stoelting Co.). For dHPC infusions, the injection needles protruded 1.5 mm beyond the cannula tips and a 0.5- μ l injection volume per hemisphere was infused over a period of 75 s. The injection needles were retained within the cannulae for 20 s after drug infusion to maximize diffusion.

Cannula placement verification

Rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.9% saline, followed by 4% formaldehyde. The brains were removed and immersed in 4% formaldehyde. At least 24 h before sectioning, the brains were placed in a 25% sucrose solution for cryoprotection. Coronal sections of 50 μ m were cut on a cryostat, mounted on gelatin-coated slides, stained with Cresyl Violet and examined by light microscopy by an observer blind to drug treatment. Rats with needle tips located outside the boundaries of the BLA, central amygdala or dHPC, or with extensive tissue damage at the target areas, were excluded from the final analyses.

RNA isolation, miRNA microarray and qPCR

Rats were administered an overdose of sodium pentobarbital at 15, 30 or 60 min after the training session. Within 90 s after the pentobarbital injection, rats were decapitated, the brains rapidly removed and flash frozen by submersion for 2 min in a beaker filled with pre-cooled isopentane on dry ice. Flash-frozen brains were stored at -80°C until tissue processing. For verification of cannula placement of flash-frozen brains, 50- μ m-thick coronal sections were cut, collected on gelatin-coated slides and left to dry. Sections were then fixed in 100% acetone for 30 min, stained with Cresyl

Violet and processed as mentioned above.

Brain tissue containing the dHPC was cut into 350- μ m-thick coronal slices and further dissected using a 0.75-mm brain puncher (Stoelting Co.). Bilateral punches from the different subfields of the dHPC (dDG, vDG, CA1 and CA3) (AP: -2.64 to -3.86 mm) were collected from three consecutive slices to a total of six punches. Punches were preserved at -20°C for at least 16 h and later at -80°C in RNALater-ICE (Ambion, Life Technologies). After tissue dissection, RNALater-ICE was removed and total RNA was isolated using a miRNAeasy kit (QIAGEN). The concentration and purity of total mRNA were assessed using Nanodrop and measurements exceeding mean $\pm$ 2s.d were considered outliers and, thus, excluded. For the miRNA microarray, half of the sample from each animal ($n=9-10$) was pooled per treatment condition. The microarray was performed at the Dutch Genomics Service & Support Provider, University of Amsterdam, the Netherlands (www.dutchgenomics.nl).

The remainder of the sample from each animal was used for qPCR analysis of miRNAs and mRNAs. For optimized detection, first DNase treatment (Thermo Fisher Scientific) was carried out. Small RNA-seq was performed with Ion Proton System (Thermo Fisher Scientific). Second, complementary DNA was synthesized from total RNA using miScript II kit (QIAGEN) according to the manufacturer's protocol. Using first-strand cDNA as a template, qPCR reactions were performed for each miRNA, using miRNA-specific forward primers of the entire mature miRNA sequence and the universal qPCR reverse primer. Hairpin miR-134-specific primers were designed using criteria described previously (Schmittgen, 2004). For mRNA qPCR reactions, mRNA-specific forward and reverse primers were used (Table 1). qPCR analysis was performed with miScript SYBR Green PCR kit (QIAGEN) in a Rotorgene qPCR thermocycler. All take-off C_t values used for analysis were averaged except for those considered outliers, being >2 s.d. higher or lower than the mean. Relative expression was calculated using the comparative C_t method and normalized to the expression of U6 single-nucleus RNA and with the $\Delta\Delta C_t$ method (Schmittgen et al., 2008).

Table 1 – Primer sequences used for mRNA and miR qRT-PCR

Primer Sequences Used for mRNA qRT-PCR		
Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
CREB	TCAGCCGGGTACTACCATTC	TTCAGCAGGCTGTGTAGGAA
BDNF	GGTCACAGCGGCAGATAAAAGAC	TTCGGCATTGCGAGTTCCAG
Arc	AAGAAAGCAGACTTCGGCTCC	TGAGACCTGGTGTCTCTCTCT
Npas4	CTGCATCTACACTCGCAAGG	GCCACAATGTCTTCAAGCTCT
c-Fos	ACGGAGAATCCGAAGGGAAAGGAA	TCTGCAACGCAGACTTCTCGTCTT
18S	ACGGACCAGAGCGAAAGCAT	TGTAATCTGTCTCCGTGTCC
Coxiv	ACTACCCCTTGCTGATG	ACTCATTGGTGCCCTTGTC

Primer Sequences Used for mirR qRT-PCR		
Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
miR-124	TAAGGCACGCGGTGAATGCC	Universal Primer
miR-134-5p	TGTGACTGGTTGACCAGAGGGG	Universal Primer
hairpin miR-134	TGTGACTGGTTGACCAGAGGGG	TGGTGACTAGGTGGCCAC
miR-132	TAACAGTCTACAGCCATGGTCG	Universal Primer
miR-137	TTATTGCTTAAGAATACGCGTAG	Universal Primer
miR-138	AGCTGGTGTGTGAATCAGGCCG	Universal Primer
U6	CTCGCTTCGGCAGCACA	Universal Small RNA Control Primer

Immunohistochemistry

For immunohistochemical experiments, rats received NE (1.0 μ g in 0.2 μ l) into the BLA or Ant-134 (10 pmol in 0.5 μ l) into the dHPC of one hemisphere and saline or scrambled control into the other hemisphere, randomized across rats, immediately after the training session. After 3 h, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with ice-cold 0.9% saline solution followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The brains were removed, postfixed overnight at 4 °C and then transferred to a 25% sucrose solution in 0.1 M PBS for at least 24 h at 4 °C. Frozen coronal sections at the level of the dHPC (AP: -3.1 to -3.3 mm) were cut at a thickness of 20 μ m on a cryostat and collected in Tris-buffered saline (TBS) with 0.1% sodium azide and phosphatase inhibitors (20 mM sodium fluoride and 2 mM sodium orthovanadate). Free-floating sections were rinsed in 0.3% Triton X-100 in TBS with phosphatase inhibitors. To block nonspecific binding, sections were incubated with TBS containing phosphatase inhibitors, 0.3% Triton X-100 and 5% normal donkey serum (NDS, Jackson ImmunoResearch) and 1% bovine serum albumin (BSA, Jackson ImmunoResearch) for 1 h.

Free-floating sections were incubated with primary antibodies for CREB (rabbit anti-CREB, 1:1,000; Cell Signaling, cat. no. 9197S) or pCREB (rabbit anti-(Ser133)-pCREB, 1:100; Cell Signaling, cat. no. 9198L) in TBS containing phosphatase inhibitors, 0.3% Triton X-100, 5% NDS and 1% BSA for 24 h at room temperature. Sections were then rinsed 3 $\times$ with TBS and incubated with a fluorochrome-conjugated secondary antibody (Alexa Fluor-488 donkey, anti-rabbit, 1:200) and DAPI (dihydrochloride) fluorescent dye at 455 nm when bound to double-stranded DNA (Thermo Fisher Scientific) for 2 h in the dark. Sections were rinsed again in TBS, mounted on gelatin-coated slides, using Fluor-save reagent to preserve fluorescence (Merck-Millipore, cat. no. 345789) and nonfading mounting medium for coverslipping, and stored in the dark for further analysis.

Images were acquired at $\times$ 20 magnification with TissueFaxes, ZEN black 3.7 v.3.7 (Zeiss), Axio Observer Z1 Microscope System TCS SP2 AOBS system (Leica). The number of pCREB-positive nuclei was quantified with Image J 1.48 software (National Institutes of Health (NIH)). In total, four random, square-shaped measurements per dDG were

taken. The borders of the layer were determined by visual judgment (Paxinos & Watson, 2006). For CREB, an average fluorescence intensity for the dDG was calculated from the four obtained values. CREB fluorescence intensities were normalized to DAPI fluorescence intensity. We defined the intensity of the control-treated hemisphere as 100% and calculated the percentage of the drug-treated hemisphere relative to the control condition. These percentages of the different slices were averaged across each individual rat to get a single percentage relative to the control condition.

Dual-luciferase reporter assay

Luciferase reporter assays were performed and analyzed by Creative Biogene (<https://www.creative-biogene.com>). To evaluate the binding of miR-134 to CREB and BDNF mRNA, pmirGLO luciferase reporter constructs were created (Table 2) by cloning the specific miR-binding sequence (wild-type or mutants) of CREB 3'-UTR and BDNF 3'-UTR into the XhoI/XbaI site located at 3'-UTR of pmirGLO-control vector (Promega). Co-transfection of luciferase reporter constructs with miR-134 mimic (rno-miR-134-5p, UGUGACUGGUUGACCAGAGGGG) and NT control mimic (UUCUCCGAACGUGUCACGUUU) was performed in HEK293T cell culture. Luciferase activity was measured using the Dual-Glo Luciferase assay system, as recommended by the manufacturer (Promega). All experiments were performed in triplicate.

Table 2 – Wild-type and mutant CREB 3'-UTR and BDNF 3'-UTR sequences

WT CREB-3'UTR sequence (miR binding sites)

GTCATTTTTAAAACTGGTTTAACAGAAATCAAGCAAAGTCACAAATATGTTACAAG
TTGGAATTATTTATTGAGTCAAAATGTCGAATACAAAGCACAACTAGAACTTTTTGTTG
GGAGGCTTACATACATCTTGAATATTCTTAATGTAATAATGTTGACTATTAAGTTGGCT
ACACAGTCACTGTATGTACTAGGAAGTGGTTTCCTTGACATTCTGAATCAATGGCTAGG
AGAGGCATTAGTGAAATATTGTCATAAAGTGCTTTTTCTTACTGTAATCTTTGTGGTATC
AACTGTCATAATGCTCTTTTTACACAAACATTTATGTGCAGTCACATAAACATGCTTTT
AAAAACTC

Mutant CREB 3'UTR sequence (mutant sites)

GTCATTTTTAAAACTGGTTTAACAGAAATCAAGCAATTTTTTAAATATGTTCAAAG
TTGGAATTATTTATTGAGTCAAAATGTCGAATACAAAGCACAACTAGAACTTTTTGTTG
GGAGGCTTACATACATCTTGAATATTCTTAATGTAATAATGTTGACTATTAAGTTGGCT
ACATTTTTTTTGATGTACTAGGAAGTGGTTTCCTTGACATTCTAGAATCAATGGCTAG
GAGAGGCATTAGTGAAATATTGTCATAAAGTGCTTTTTCTTACTGTAATCTTTGTGGTAT
CAACTGTCATAATGCTCTTTTTACACAAACATTTATGTGTTTTTTTTAAACATGCTTTT
AAAAACTC

WT BDNF 3'UTR sequence (miR binding sites)

GAACTGCATGTATAAATGAAGTTTATACAGTACAGTGTTCTACAATCTATTTATTGGA
 CATATCCATGACCAGAAAGAAACAGTCATTTGCGCACAACCTTTAAAAGTCTGCATTAC
 ATTCCTCGATAATGTTGTGGTTTGTGGCGTTGCCAAGAATCCAATCAGATGACTGGAA
 AACGAATAAAAATTAAGGCAACTGAATAAAATGCTCACACAACACTGCCCATGATGTA
 TCTCCCTGGTCCCCCAGGTCCTCTTCTGGCATGGGTCAGGGAAAGCTGCTTTTATTGG
 AAAGACCAGCATTTGTTTAAAGCACATTCTTCCCTCCCTCCTCCCA

Mutant BDNF 3'UTR sequence (mutant sites)

GAACTGCATGTATAAATGAAGTTTATACAGTACAGTGTTCTACAATCTATTTATTGGA
 CATATCCATGACCAGAAAGAAATTTTTTTTGGCGCACAACCTTTAAAAGTCTGCATTAC
 ATTCCTCGATAATGTTGTGGTTTGTGGCGTTGCCAAGAATCCAATCAGATGACTGGAA
 AACGAATAAAAATTAAGGCAACTGAATAAAATGCTCACACAACACTGCCCATGATGTA
 TCTCCTTTTTTCCCCAGTTTTTCTTCTGGCATGGGTCAGGGAAAGCTGCTTTTATTGG
 AAAGACCAGCATTTGTTTAAAGCACATTCTTCCCTCCCTCCTCCCA

Small RNA sequencing and data processing

Twelve samples, across four conditions (1-min versus 2-min interval, saline versus propranolol, $n = 3$ per condition), were processed for small RNA-seq. Small RNA-seq was performed at Lexogen (www.lexogen.com/services/small-rna-seq).

RNA extraction and quality control: Total RNA was extracted from the sample material using a column-based extraction kit (mirVana miRNA Isolation Kit). The RNA extraction process included an on-column DNase treatment to remove contaminating genomic DNA. The quality and concentration of the extracted RNA were assessed using a Bioanalyzer (Agilent Technologies) and RNA integrity number values were recorded.

Small RNA enrichment: To enrich for small RNAs, the total RNA was subjected to size selection. This was achieved by using a gel-based extraction method or the TraPR (trans-activator RNA precipitation) method (Lexogen). The latter involves a convenient column-based centrifugation step to enrich small RNAs in their functional protein-bound form, removing any free RNA and DNA from the sample, as described by Lexogen-RNA Lexicon-eBook (Lexogen). The resultant enriched small RNA fraction was used for library preparation.

Library preparation: The small RNA library preparation was performed using the Small RNA-Seq Library Prep Kit (Lexogen). The procedure involved the following steps: (1) adapter ligation: the 3'- and 5'-adapters were sequentially ligated to the enriched small RNAs. The 3'-adapter was first ligated to the small RNAs using T4 RNA ligase. After the 3'-ligation, the 5'-adapter was ligated using T4 RNA ligase again. Adapter-ligated small RNAs were then purified using a gel extraction method. (2) Reverse transcription: reverse transcription was carried out using the adapter-ligated small RNAs as templates. The first-strand cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and a primer complementary to the 3'-adapter. (3) PCR amplification: the cDNA was amplified by PCR using primers complementary to the 5'- and 3'-adapters. This step also incorporated the sequencing

indices required for multiplexing. PCR conditions were optimized to minimize amplification bias and ensure adequate library complexity. The PCR products were purified using a spin column-based method (AMPure XP beads, Beckman Coulter). (4) Library quality control: the final libraries were assessed for quality and concentration using a Bioanalyzer (Agilent UK). Libraries with adapter dimers or other byproducts were further purified. Only libraries with a satisfactory concentration and size distribution were used for sequencing.

Sequencing: Sequencing was performed on an Illumina platform using single-end 50-bp reads. The libraries were denatured and diluted to the appropriate concentration for loading on to the flow cell. Cluster generation, followed by high-throughput sequencing, was carried out according to the manufacturer's protocols (Illumina platform and Lexogen-RNA Lexicon-eBook, Lexogen).

Small RNA data processing: Small RNA data processing was performed at the Bioinformatics Technology Center of Radboud University Medical Center. The nf-core/smrnaseq v.2.2.4 pipeline was utilized for data processing. The following steps were taken by the pipeline.

Quality control and trimming: Initial raw read quality was assessed using FastQC v.0.11.9. Adapter trimming and quality filtering were performed using Trim Galore! v.0.6.6, which removes low-quality sequences and adapter contaminants. Trim Galore! was configured with the following parameters:

- Adapter sequence: automatically detected and removed
- Quality cutoff: reads were trimmed at a Phred score threshold of 20
- Minimum read length: reads shorter than 17 nt after trimming were discarded.

Read alignment: Filtered reads were aligned to the reference genome using Bowtie1 v.1.3.0 and Bowtie2 v.2.3.5 for contamination filtering. Alignments were performed against miRBase mature and hairpin miRNAs sequences from the public database (mirbase.org/download/CURRENT, retrieved March 2024), with unaligned reads being removed. Postalignment steps included basic statistics generation, analysis of miRBase and MirGeneDB hairpin counts using edgeR v.3.32.1 for normalization and clustering and miRNA/iso-miRNA annotation using mirtop v.0.4.20. Further alignment against the host reference genome and new miRNA discovery was performed using MiRDeep2 v.0.1.3.

Differential expression analysis: Differential expression analysis was conducted using edgeR. This included normalization, calculation of $\log_2(\text{FC})$ and adjusted $P(P_{\text{adj}})$ values. The miRNA expression levels refer to edgeR normalized read counts as performed by default in the nf-core/smrnaseq v.2.2.4 pipeline. MA plots and principal component analysis plots were generated for visualization.

Small RNA quality control and reporting: Comprehensive small RNA quality control was conducted using mirtrace v.1.0.1 and MultiQC v.1.9 to present the results of raw reads, alignment and expression analysis. Key quality control steps are listed in a quality control report, including:

- Assessing read quality and adapter content
- Ensuring that the distribution of read lengths fell within the expected range (18–

30 nt)

- Filtering low-complexity reads.

miRNA length analysis: Data analysis was conducted using the UNIX tools GREP, CUT and AWK to extract the Bowtie2 mapped read lengths. Data formatting and processing were performed by Libreoffice Calc 3.6. Further calculations and plotting within this analysis were performed by using the R project v.4.1.1 and default R functions like `t.test()` and `boxplot()`.

Statistics

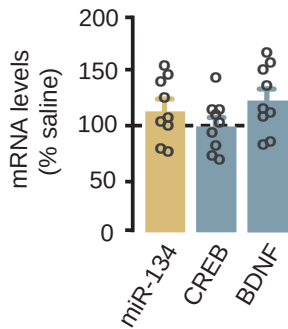
Data are presented as mean $\pm$ s.e.m. Data analyses were performed with SPSS v.27 for windows. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in previous publications (Atucha et al., 2017; Atucha & Roozendaal, 2015; Bahtiyar et al., 2020; Roozendaal & Mirone, 2020). The data distribution was assumed to be normal but this was not formally tested. Data collection and the assignment of groups for individual animals were done in a random or blocked manner to guarantee that groups are equally distributed in both behavioral and molecular experiments. Furthermore, data collection and analysis were performed blind to the conditions of the experiments.

Inhibitory avoidance retention latencies were analyzed with a mixed-model ANOVA with drug treatment (in either one or two brain regions) as between-subject variables and latencies in the different test contexts (shock, non-shock and new boxes) as within-subject measurements. Post hoc analysis used unpaired Student's *t*-tests (two tailed) to compare drug conditions and paired Student's *t*-tests (two tailed) to compare test contexts or retention versus training latencies, when appropriate. For analyses of miRNA, mRNA and immunofluorescence data, paired Student's *t*-tests were used to compare normalized levels in the drug-treated hemisphere versus control hemisphere or Student's unpaired *t*-tests to compare miR-134 levels of rats trained with a 2- or 1-min interval. Unpaired Student's *t*-tests were also used to analyze luciferase reporter assay data. For all comparisons, $P < 0.05$ was accepted as statistical significance. The number of rats per group is indicated in the figure legends.

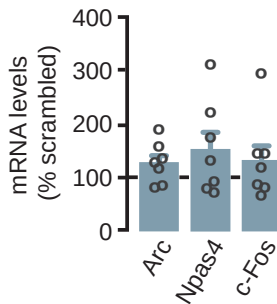
ACKNOWLEDGMENTS

Funding was provided by Radboud University Topfund (to B.R.); Dutch Research Council Open Research Area grant (no. 464.18.110 to B.R.), and European Community's Seventh Framework Programme (no. FP7/2007–2013) and Horizon 2020 Programme under grant agreement nos. 603016 and 667302 (to J.C.G.).

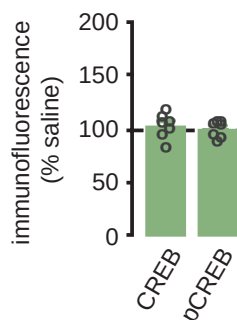
SUPPLEMENTARY INFORMATION



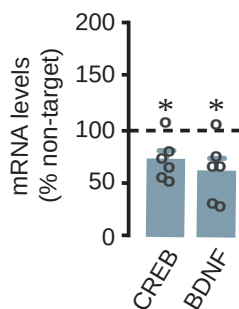
Extended Data Fig. 1. NE administration into the BLA of home-cage control rats does not affect miR-134, CREB or BDNF levels within the dDG. NE (1.0 μg in 0.2 μl) was administered unilaterally into one BLA and saline into the other BLA, counterbalanced across animals, of home-cage control rats. NE administration did not affect miR-134, CREB or BDNF levels within the dDG 30 min later. mRNA levels are normalized to the saline-treated hemisphere (mean + s.e.m.) ($n = 9$, paired t -test: miR-134: $t_8 = 0.52$, $P = 0.62$; CREB: $t_8 = 0.46$, $P = 0.66$; BDNF: $t_8 = 0.98$, $P = 0.36$).



Extended Data Fig. 2. Ant-134 administration into the dHPC does not alter Arc, Npas4 or c-Fos levels within the dDG. Ant-134 (10 pmol in 0.5 μl) was administered unilaterally into one dHPC and scrambled control (Scr, 10 pmol in 0.5 μl) into the other dHPC, counterbalanced across animals, immediately after training on the dual-event inhibitory avoidance task. Ant-134 administration did not affect mRNA levels of several plasticity-related genes that are considered non-targets of miR-134 within the dDG 30 min later. mRNA levels are normalized to the scrambled-treated hemisphere (mean + s.e.m.) ($n = 7$, paired t -test: activity-regulated cytoskeleton-associated protein (Arc): $t_6 = 1.73$, $P = 0.13$; neuronal PAS domain protein 4 (Npas4): $t_6 = 1.86$, $P = 0.11$; c-Fos: $t_6 = 1.09$, $P = 0.32$).



Extended Data Fig. 3. Noradrenergic activation of the BLA does not increase CREB or pCREB protein levels within the ventral blade of the DG. NE (1.0 μ g in 0.2 μ l) was administered unilaterally into one BLA and saline into the other BLA, counterbalanced across animals, immediately after training on the dual-event inhibitory avoidance task. NE administration did not affect CREB or pCREB protein levels within the ventral blade of the DG granule cell layer 3 h later. Protein levels are normalized to the saline-treated hemisphere (mean + s.e.m.) ($n = 7$, paired t -test: CREB: $t_6 = 1.43$, $P = 0.20$; pCREB: $t_6 = 1.01$, $P = 0.35$).



Extended Data Fig. 4. Overexpression of miR-134 in the dHPC induces an efficient down-regulation of CREB and BDNF mRNA levels within the dDG. Mimic-134 (0.25 pmol in 0.5 μ l) was administered unilaterally into one dHPC and non-target control (NT, 0.25 pmol in 0.5 μ l) into the other dHPC, counterbalanced across animals, immediately after training on the dual-event inhibitory avoidance task. Mimic-134 administration resulted in a 6.60 ± 0.91 log2FC (mean $\pm$ s.e.m) increase in total-tissue miR-134 levels within the dDG 30 min later ($n = 6$, paired t -test: $t_5 = 7.26$, $P < 0.01$). mRNA levels are normalized to the non-target-treated hemisphere (mean + s.e.m.) ($n = 6$, paired t -test: CREB: $t_5 = 3.22$, $P < 0.05$; BDNF: $t_5 = 3.28$, $P < 0.05$). * $P < 0.05$ vs non-target.

Extended Data Tables 1-3 can be accessed via: <https://doi.org/10.1038/s41593-025-02014-0>.



CHAPTER 3

Strong hypothalamic–pituitary–adrenal-axis negative feedback regulation determines resistance to glucocorticoid enhancement of extinction memory in rats

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Manuscript in preparation

ABSTRACT

Exposure-based therapies for post-traumatic stress disorder (PTSD) rely on the formation of extinction memories, yet treatment outcomes vary widely and relapse often occurs when traumatic cues are encountered outside the therapeutic context. Exogenous glucocorticoids have been shown to enhance extinction memory consolidation and generalization, however, the impact of interindividual differences in endogenous glucocorticoid and their regulation on extinction memory remains unclear. The hypothalamic–pituitary–adrenal (HPA) axis regulates endogenous glucocorticoid levels and may therefore contribute to treatment variability by influencing extinction memory and responsiveness to glucocorticoid-based interventions. Here, we investigated whether stronger HPA-axis negative feedback regulation and blunted post-stressor glucocorticoid responses influences extinction memory strength, generalization, and response to post-extinction glucocorticoid administration. Using two rat lines selectively bred for different constitutive post-stressor corticosterone levels, endocrine phenotypes were assessed by stress-induced corticosterone responses and a dexamethasone suppression test (DST), establishing the Low line as presenting stronger HPA-axis negative feedback regulation. Rats then underwent extinction training in a context distinct from the conditioning, enabling assessment of extinction strength and generalization. Glucocorticoids were administered immediately after extinction training, and neural and molecular correlates were examined. Stronger HPA-axis regulation did not impair fear learning, extinction learning, or extinction recall in the Low line under the present training conditions. However, post-extinction glucocorticoid administration transiently enhanced extinction memory strength and generalization in Control rats, reducing fear renewal at a 1-day recall, but not at a 14-day recall test. In contrast, Low line rats were resistant to glucocorticoid enhancement of extinction memory. At the neural level, glucocorticoid treatment in Control rats was associated with reduced dentate gyrus (DG) activity, whereas Low line rats showed increased DG–prelimbic cortex connectivity. These findings demonstrate that interindividual differences in HPA-axis regulation critically affect the behavioral and neural effects of glucocorticoids on extinction memory, with implications for personalized treatments for PTSD.

Keywords: extinction memory, generalization, fear renewal, glucocorticoids, HPA-axis regulation, interindividual differences

INTRODUCTION

Exposure therapy, a first-line treatment for post-traumatic stress disorder (PTSD), relies on successful safety learning through repeated exposure to trauma-related cues in the absence of threat. However, many patients fail to acquire or adequately express safety learning (Schottenbauer et al., 2008; Wessa & Flor, 2007; Wicking et al., 2016). Even among initial responders, pathological fear often returns when individuals encounter trauma-related stimuli outside the therapeutic context (Bouton et al., 2012), highlighting a vulnerability to relapse. These observations point to substantial interindividual variability in treatment outcome, yet the biological mechanisms driving such variability in safety learning and relapse remain poorly understood.

Variability in safety learning and relapse following exposure-based interventions can be conceptualized as reflecting differences in extinction memory expression, consistent with the growing recognition that stress-related psychopathology depends on memory processes (Brewin, 2011; Rigoli et al., 2016; Rubin et al., 2008). Extinction training is thought to form a new memory that coexists and competes with the original fear memory (Lacagnina et al., 2019), which varies qualitatively in its consolidation strength and its ability to generalize across contexts. These aspects critically determine long-term maintenance of extinction, as weakly consolidated extinction memories are prone to rapid fear return, whereas poor extinction generalization promotes fear renewal (i.e., relapse) when cues are encountered outside the extinction context. At the neural level, extinction memory expression relies on coordinated interactions between the hippocampus, ventromedial prefrontal cortex (vmPFC) and basolateral amygdala (BLA) (Bouton et al., 2021; Corcoran & Quirk, 2007). Understanding what mechanisms determine strength and generalization of extinction memory is therefore critical for supporting successful safety learning and preventing relapse.

Extensive evidence from both animal and human studies demonstrates that glucocorticoids enhance memory consolidation in a dose-dependent manner, including extinction memory (Bohus & Lissák, 1968; De Quervain et al., 2019; McGaugh & Roozendaal, 2002; Meir Drexler et al., 2019; Roozendaal & McGaugh, 2011). Building on these findings, glucocorticoids have been explored as potential adjuvants to extinction-based interventions, with many experimental studies supporting that glucocorticoid administration may facilitate extinction consolidation and reduce fear responses (Brueckner et al., 2019; De Quervain et al., 2017, 2019; Meir Drexler et al., 2019; Merz et al., 2018). Emerging evidence further indicates that glucocorticoids promote not only memory strength but also memory generalization (Bahtiyar et al., 2020; Roozendaal & Mirone, 2020; van Ast et al., 2014). However, randomized controlled trials have not yet consistently demonstrated an augmentation effect of glucocorticoid administration, pointing instead to heterogeneous, subgroup-dependent outcomes (Inslicht et al., 2022; Lehrner et al., 2021; Yehuda, Bierer, et al., 2015). Notably, endogenous glucocorticoid levels have been suggested to determine treatment success (Olf & van Zuiden, 2017; van Gelderen et al., 2020; Yehuda, Bierer, et al., 2015). While the numerous administration studies have been critical in

establishing glucocorticoids as memory modulators, comparatively few studies have systematically examined how endogenous differences in glucocorticoids and their regulation influence memory processes. As such, it remains unclear to what extent endogenous glucocorticoid signaling contributes to variability in extinction memory strength and generalization, and to differential responsiveness to glucocorticoid-based treatments.

The hypothalamic–pituitary–adrenal (HPA) axis determines the magnitude, duration, and termination of glucocorticoid responses to stress via negative feedback processes (Herman et al., 2016). Importantly, glucocorticoid fluctuations regulated by the HPA axis occur within the time window critical for memory consolidation, positioning endocrine dynamics as a likely source of interindividual variability in memory processes. Further, stronger HPA-axis negative feedback regulation—commonly assessed using the dexamethasone suppression test (DST)—and associated blunted post-stressor glucocorticoid responses have been described in a subset of PTSD patients, as well as following chronic and early life stress (Yehuda, 2009; Zoladz & Diamond, 2016). These endocrine patterns have been linked to genetic and epigenetic variation in glucocorticoid signaling pathways, including the co-chaperone FKBP5, a key regulator of glucocorticoid receptor (GR) sensitivity (Klengel et al., 2013; Mehta, 2011). Together, these observations suggest that HPA-axis regulation of glucocorticoid signaling, rather than glucocorticoid output alone, may be a key mechanism through which endogenous glucocorticoids modulate both memory consolidation and generalization.

The present study aimed to determine whether stronger HPA-axis negative feedback regulation and low post-stressor circulating glucocorticoid levels influence extinction memory strength and generalization, and how these effects interact with exogenous glucocorticoid administration following extinction training. To this end, we used two rat lines selectively bred on the basis of their constitutive post-stressor glucocorticoid levels (Walker et al., 2017): a Low line with blunted post-stressor corticosterone levels compared to a Control line. This model enables investigation of constitutive differences in glucocorticoid signaling in experimental animals without the confounding influence of prior stress exposure or trauma. In line with a previous study showing that blunted glucocorticoid responsiveness impairs extinction memory (Monari et al., 2024), here we hypothesized that Low line rats might show impairments in the strength and generalization of extinction memory compared to the Control line, which may be subsequently corrected by post-extinction glucocorticoid administration.

First, we examined HPA-axis negative feedback regulation in both rat lines by assessing stress-induced corticosterone response profiles, conducting a low-dose DST, and measuring the expression of glucocorticoid signaling-related genes in blood. Next, we assessed the strength and generalization of extinction memory using a fear conditioning and extinction task, in which extinction was performed in a context that was distinct from the conditioning context, and the recall test consisted of conditioned stimulus (CS) presentations in both contexts. To examine how exogenous glucocorticoids affect the strength and generalization of extinction memory in

both lines, corticosterone was administered immediately after extinction training. Finally, we evaluated the effect of post-extinction corticosterone administration on hippocampal neural activity and GR activation (i.e., phosphorylation).

MATERIALS & METHODS

1. Subjects

Adult male rats (270-330 g at the start of behavioral testing) from two selectively bred rat lines with Wistar Han background were used: a line with low post-stressor corticosterone levels (i.e., Low line) and a line with normal post-stressor corticosterone levels (i.e., Control line). These rat lines were originally developed in the laboratory of Dr. Carmen Sandi (École Polytechnique Fédérale de Lausanne (EPFL), Switzerland) (Walker et al., 2017), and then bred in our in-house animal facility. Rats were maintained on a standard 12-h/12-h light/dark cycle (lights on: 6:30 - 18:30 h) in a temperature (21 ± 1 °C) and humidity-controlled (55 ± 5 %) housing room, with *ad libitum* access to food and water. Rats were individually housed starting one week prior to the experiment to minimize potential testing order and social hierarchy effects (Bahtiyar et al., 2025). Testing order was block randomized and maintained for every procedure. Researchers were (semi-)blind to the phenotype and drug conditions until data analysis. All procedures were performed during the light phase between 8:00 and 16:00 h, at the nadir of the circadian cycle of corticosterone. Rats were habituated to handling for 2 min on 3 consecutive days prior to the start of experimental procedures. All procedures were in compliance with European Union Directive 2010/63/EU and approved by the Central Authority for Scientific Procedures on Animals (CCD), The Hague, The Netherlands.

2. Selective breeding

To maintain HPA-axis characteristics and corticosterone levels of the two rat lines across generations, both male and female candidate breeder rats were subjected to a standardized stress test for breeding selection: the Stress Adaptation Test (SAT) (Walker et al., 2017). The SAT consisted of three consecutive days of stress exposure during the peripubertal period, i.e., postnatal day (PND) 28-30, and subsequent measurement of plasma corticosterone levels. Rats were selected for breeding based on their plasma corticosterone levels at 50 min after stress onset on PND30: <100 ng/ml for the Low line, and 100-200 ng/ml for the Control line. Breeding was performed strictly within each line. To minimize effects of genetic drift, the breeding scheme strictly excluded sibling mating (for more details see Supplementary Methods and Figure S1A).

3. Fear conditioning and extinction task

To assess both strength and generalization of extinction memory, rats underwent a fear conditioning and extinction protocol conducted in two identical rodent conditioning chambers (30 cm width $\times$ 24 cm depth $\times$ 21 cm height, MED Associates, Vermont, USA). Each chamber was made of aluminum and Plexiglas walls and ceiling, with a

4-mm thick stainless-steel grid floor, which delivered the footshock (unconditioned stimulus, US). A speaker for delivery of the auditory cue (CS) was mounted on the wall of the chamber. A house light was mounted on the ceiling and another one on the wall. Each chamber was contained inside a sound-attenuating cabinet equipped with a fan that provided background noise (65 dB). The cabinets were located in a room that was adjacent to the housing room. Two different contexts were created by altering several visual and tactile features. Context A had a rectangular shape and maintained the original chamber appearance (i.e., no wall alterations and grid floor). Context B had a semi-rounded shape and contained black and white stripes mounted on the walls and flat floors. Other features such as lighting and transport to the room also differed between contexts. For a detailed overview and illustration of context characteristics, see Table S1 and Figure S1.

The behavioral task consisted of three sessions, i.e., fear conditioning, extinction training and a recall test, that took place on different days (Table S1). The first 2 min of each session were always reserved for exploration and habituation to the context (i.e., no stimuli were presented). Sessions ended 1 min after the last CS presentation. Both fear conditioning and extinction training were performed in the morning between 8:00 and 13:00 h, whereas the recall test was performed in the afternoon between 13:00 and 16:00 h. During fear conditioning (context A), rats received five paired presentations of a tone CS (9 kHz, 80 dB, 20 s) co-terminating with a footshock US (0.7 mA, 1 s) via the grid floor, with a constant inter-tone interval (ITI) of 60 s. During extinction training (context B), 1 day later, the 20-s CS was presented 20 times without footshock, with an ITI ranging from 40 to 120 s (Plendl & Wotjak, 2010). Either 1 or 14 days after extinction training, extinction recall was tested by placing the rats back first in context B for 6 min and subsequently in context A for 6 min. In both contexts, the 20-s CS was presented 3 times with an ITI of 60 s. Other groups of rats underwent fear conditioning in context A but not extinction training (i.e., remained in home cage), and were tested 48 h later in context B followed by context A. In both contexts, the 20-s CS was presented 3 times with an ITI of 60 s. After every single session, rats were returned to their home cage and the chambers were cleaned with a 5% ethanol solution.

Videos were recorded using near infrared light. Freezing behavior was defined as the absence of movement, and was scored manually using BORIS (Friard & Gamba, 2016). Each session was segmented in 20-s sections (i.e., the duration of CS presentation) and freezing behavior was calculated into a percentage for each segment. In the extinction session, every two consecutive CS presentations were averaged, resulting in a total of ten CS blocks (CSB). In the recall test, freezing scores prior to CS presentation represent the 20-s segment immediately before the first CS delivery in each context (i.e., representing freezing to the context only). Freezing scores during CS presentation represent an average across the three CS presentations in each context. During this phase, freezing scores in context B were indicative of the strength of extinction memory, whereas freezing scores in context A were indicative of the generalization of extinction memory (i.e., attenuation of fear renewal).

4. Assessment of HPA-axis function

4.1. Corticosterone response after fear conditioning

To determine the magnitude of the corticosterone response after fear conditioning, the rats underwent fear conditioning as previously described, after which they were returned to their home cage until blood collection. Blood was collected via tail-nick at 15, 30, 60 and 90 min after the start of the fear conditioning session. See section: 6. Corticosterone Assay for details on sample processing. Negative feedback regulation was assessed by comparing plasma corticosterone levels acquired at 90 min with those of undisturbed animals collected at the same time of day (between 9 h and 13 h). Measuring basal corticosterone levels in a different group of animals allowed for the assessment of the conditioning-induced corticosterone response without the confounding influence of prior blood collection procedure.

4.2. Low-dose DST

To determine the magnitude of negative feedback regulation of the HPA axis, a low-dose DST, adapted for rats, was performed (Zoladz et al., 2021; Zoladz & Diamond, 2016). Low doses of the synthetic glucocorticoid dexamethasone (25 or 50 µg/kg) or vehicle control (2% ethanol in saline) were administered subcutaneously between 10:00 and 11:00 h, after which the rats were returned to their home cage. Dexamethasone (D1756, Sigma-Aldrich) dosages were freshly diluted from a previously prepared stock solution of 1 mg/ml dexamethasone in 2% ethanol and saline stored at -20 °C. Six hours after the dexamethasone administration, the rats were exposed to a stress induction procedure of synthetic fox odor (trimethylthiazoline, TMT; W332518, Sigma-Aldrich) for 20 min in a different room. Blood was collected via tail-nick immediately prior to stress induction (0 min) as well as at 30, 60 and 90 min after stressor onset. See section: 6. Corticosterone Assay for details on sample processing. Dexamethasone-induced suppression of HPA-axis activity was assessed by normalizing corticosterone levels at 30 min (reflecting HPA-axis activation) of both dexamethasone doses to the mean corticosterone level of the vehicle group for each line.

5. Systemic administration

Corticosterone (0.3, 1 and 3 mg/kg; C2505, Sigma-Aldrich) was first dissolved in heated absolute ethanol and then diluted in 0.9% saline to reach a final ethanol concentration of 5% (Cai et al., 2006; De Quervain et al., 1998; Roozendaal et al., 2006). The vehicle control consisted of 5% ethanol in saline only. Drugs were administered subcutaneously, in a volume of 2 ml/kg, immediately after the fear extinction session. Drug solutions were freshly prepared on the day of administration. Rats from each line were randomly assigned to each drug condition.

6. Corticosterone assay

Blood samples were collected in ice-cold lithium heparin-coated tubes (Sarstedt or Greiner Bio One), and kept on ice until centrifugation (10,000 rpm for 4 min at 4 °C). Then, plasma was isolated into separate tubes and stored at -20 °C until analysis.

Corticosterone ELISA immunoassay (sensitivity: 27.0 pg/ml, ADI-900-097, Enzo Life Sciences) was performed according to the manual. Plasma samples were diluted 1:40 in Assay Buffer without the addition of steroid displacement reagent solution. All samples were measured in duplicate, except those used for breeding selection. The average coefficient of variance among the duplicates equaled 2.9%. Each plate contained a balanced number of samples from each group. Corticosterone levels were calculated using linear fitting and logarithmic plotting software (Enzo Life Sciences) for standard curve and data extrapolation.

7. Collection of fresh tissue

Fresh brain tissue for molecular analyses of glucocorticoid signaling was collected after rapid decapitation at defined experimental timepoints. For basal measurements, tissue was collected one day after the last handling day between 9 and 13 h. For post-extinction treatment measurements, tissue was collected 30 min after extinction training and systemic corticosterone administration. To minimize stress, all rats were first administered an overdose of sodium pentobarbital. After 90 s, rapid decapitation was performed, the brain and adrenal glands were rapidly removed and flash frozen by submersion for 2 min in a beaker filled with pre-cooled isopentane on dry-ice, and then stored at -80 °C until tissue processing. Before storage, the adrenal glands were cleaned and weighed in a microscale. Trunk blood was also collected for corticosterone measurements and/or blood mRNA analysis.

8. Blood mRNA analysis

To assess basal expression of glucocorticoid signaling-related genes (i.e., *Nr3c1*, *Nr3c2*, *Fkbp5*), trunk blood was collected under undisturbed conditions one day after the last handling session between 9 and 13 h (as described in section: 7. Collection of fresh tissue). Samples were collected in 100- μ l RNeasy Protect Animal Blood tubes (76544, QIAGEN) at room temperature (RT). RNA extraction was performed according to RNeasy Protect Animal Blood kit manual instructions (73224, QIAGEN), using the QIAcube extraction robot (QIAGEN). After RNA quantification (Tecan), RNA concentrations were normalized and cDNA was synthesized from total RNA using SensiFAST cDNA Synthesis kit (BIO-65054, Bioline). Primer efficiency (90–120%) and specificity were verified by standard curve analysis and melt-curve peak inspection, respectively (see Table S2 for target/reference genes and primer sequences). Real-time PCR analysis was performed with an annealing temperature of 60 °C, according to the SensiFast SYBR No-ROX kit instructions (BIO-98020, Bioline) using the QIAgility pipetting robot (QIAGEN) and Rotor-Gene Q quantitative real-time PCR thermo-cycler (QIAGEN). All samples were run in triplicate. When the coefficient of variance across technical replicates exceeded 0.5, individual replicate values were examined for potential outliers. Outlier replicates, when identified, were excluded from further analysis (6.5 % of replicates). Reference gene stability was assessed using the geNorm algorithm (Vandesompele et al., 2002) (average M = 0.53; average coefficient of variance = 0.20). Relative expression was calculated using the comparative Ct method and normalized

to the arithmetic mean Ct values of the reference genes with the $\Delta\Delta\text{Ct}$ method (Livak & Schmittgen, 2001).

9. Western blot

To examine hippocampal GR expression and activation, brain tissue was collected 30 min after post-extinction corticosterone administration (as described in section 7. Collection of fresh tissue). Two-hundred-micrometer thick coronal sections were cut on a cryostat (Leica Biosystems). Bilateral punches of the dorsal hippocampus were made with a 1.25-mm punching needle (57401, Stoelting Co) in six consecutive slices between -2.52 and -4.08 mm anteroposterior (AP) coordinates from Bregma (Paxinos & Watson, 2006). Punches were temporarily stored on dry ice, weighed and then stored at -80 °C until further procedures. Protein was extracted by adding neuronal protein extraction reagent (1 g sample: 10 ml reagent ratio, 87792, Thermo Scientific) and halt protease inhibitor cocktail (1% of total volume, 78438, Thermo Scientific) to the sample, according to the manufacturer's instructions. After protein quantification (micro bicinchoninic acid (BCA) protein assay, 23235, Thermo Scientific), sample concentration was normalized. Tissue samples and biotinylated ladder (81851S, Cell Signaling Technology) were mixed with 2x Tris-Glycine SDS Sample Buffer (1:1 ratio, LC2676, Novex) and heated to 85 °C for 3 min for protein denaturation. Of each sample, 20 µg of protein was loaded on a 4% stacking and 7.5% resolving Tris-Glycine gel. Each gel included a similar number of samples from each experimental group. Electrophoresis was first run at 60 V for 30 min, then at 125 V for 70 min (Biorad), and finally protein was transferred to a methanol-activated-polyvinylidene fluoride (PVDF) membrane using the Power Blotter Station (Invitrogen). As a loading control, total protein was labeled with no-stain protein labeling reagent (A44449, Invitrogen). Immunoblotting was performed against Serine-232 (S232) and Serine-246 (S246) phospho-GR protein on separate membranes and, after antibody stripping, against total GR protein for normalization. For the first immunoblotting, the membranes were blocked using 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) solution (Blocker BSA TBS, 37520, Thermo Scientific) for 1 h, then incubated with a cocktail of rabbit anti-Ser211-phospho-GR (1:1,000, #4161, Cell Signaling Technology) and rabbit anti-Ser226-phospho-GR (D9D3V) (1:1000, #97285, Cell Signaling Technology) primary antibodies overnight at 4 °C, which target the orthologous rodent GR phosphorylation sites S232 and S246, respectively (Gallagher-Beckley & Cidlowski, 2009). The next day, the membranes were washed 3x for 10 min with 0.1% Tween-20 in TBS, and then incubated for 1 h with a cocktail of peroxidase-conjugated antibodies: donkey anti-rabbit peroxidase (1:20,000, 715-035-150, Jackson ImmunoResearch) and goat anti-biotin peroxidase (1:2,000, #7075, Cell Signaling Technology) in 5% BSA in TBS. Next, the membranes were washed 4x for 10 min in TBS-Tween and then once for 10 min in TBS. After imaging (see below for details), the membranes were stored overnight in TBS at 4 °C.

To allow for the second immunoblotting, the membranes were stripped in undiluted restore Western blot stripping buffer (21059, Thermo Scientific) at 37 °C for 20 min, and then washed 3x for 5 min in TBS. Verification of complete removal of primary and

secondary antibodies was done on a random membrane in each batch. The second immunoblotting was performed as previously described using 5% non-fat dry milk in TBS blocking solution, and incubating with primary antibody rabbit anti-GR (D8H2) (1:1,000, #3660, Cell Signaling Technology).

Imaging was performed using iBright Western Blot Imager (Invitrogen) and SuperSignal West Atto Ultimate Substrate (A38554, Thermofischer), according to the manufacturer's instructions. The membranes were first imaged for chemiluminescence and then for total protein stain. Exposure time was automatically defined for each membrane by the imager's Smart Capture algorithm.

All images were processed and analyzed using FIJI (NIH, version 1.52p) (Schindelin et al., 2012). In inverted color images, regions-of-interest (ROIs) of the same dimensions were drawn around the target bands and total protein lanes, and the integrated density was extracted. All samples were run in duplicate. Only samples with a coefficient of variance below 15% were considered for further analysis. The average coefficient of variance among the duplicates equaled 5.9%. Total GR was calculated by dividing the signal intensity of the GR band by the signal intensity of the same total protein lane. Phosphorylated GR (%) was calculated by dividing the signal intensity of the target phosphorylated GR band by the total GR band of the same lane.

10. Immunohistochemistry

Some experimental groups of rats were deeply anesthetized with sodium pentobarbital one hour after post-extinction corticosterone administration and transcardially perfused with phosphate-buffered saline (PBS) with heparin (400 U/l) followed by 4% paraformaldehyde (PFA) in PBS. Trunk blood was collected for corticosterone measurement. The brain was extracted, post-fixed in 4% PFA overnight at 4 °C, and then transferred to 30% sucrose in PBS for cryoprotection. Finally, brains were flash-frozen in dry ice and placed at -80 °C for long-term storage.

Fifty micrometer-thick coronal slices were cut on a cryostat (Leica Biosystems) and stored in 0.01% sodium azide in PBS at 4 °C. To avoid possible differences in activity along the AP axis, analyses were restricted to coordinates (from Bregma) between +3.2 mm and +2.8 mm for the vmPFC, -3.1 mm and -3.6 mm for the dorsal hippocampus, and between -2.8 mm and -3.3 mm for the BLA (Paxinos & Watson, 2006). This criteria minimized variability within each region, allowing data averaging from the selected coronal sections (i.e., minimum 3 sections per ROI). Sections were washed 3x for 10 min with 0.3% Triton X-100 in PBS, and then incubated in 1% BSA and 0.3% Triton X-100 in PBS for 1 h, followed by an overnight incubation with primary antibody guinea pig anti-c-Fos (1:750, 226-308, Synaptic Systems) in the same solution. The next day, sections were washed 3x for 10 min in PBS, and then incubated with fluorochrome-conjugated secondary antibody donkey anti-guinea pig Alexa Fluor 647 (1:200, 706-605-148, Jackson ImmunoResearch) in 1% BSA in 0.3% Triton X-100 in PBS. All procedures starting from the secondary antibody incubation onwards were performed in the dark. Subsequently, sections were incubated with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI, 1:6,000, 62248 Thermo Scientific) in PBS for 10

min, then washed 3x for 10 min in PBS, mounted on gelatin-coated slides, left to dry, and finally coverslipped with Fluorsave (345789, Millipore). All immunohistochemistry steps were performed at RT. The slides were stored at 4 °C in the dark. Each staining batch included a similar number of samples from each experimental group.

11. Imaging and quantification

Fluorescent images were acquired at 20x magnification (0.8 NA objective) using the Axio Observer with AI Sample Finder (Zeiss). Image processing was performed both in ZEN (Zeiss) and FIJI (NIH, version 1.52p) (Schindelin et al., 2012). First, image tiles were corrected for background signal differences due to bleaching and subsequently stitched into a single image using the ZEN software, followed by a 22% resolution downsampling in FIJI. ROIs were delineated based on DAPI staining and the Paxinos & Watson Rat Brain Atlas (Paxinos & Watson, 2006), using a combination of manual tracing and the BrainSeg2D algorithm (Amil, 2025). All algorithm-generated ROIs were subsequently inspected and manually corrected to ensure accurate labeling. The dorsal hippocampus was divided into three subregions: granule cell layer of the dentate gyrus (DG), pyramidal cell layer of cornu ammonis 1 (CA1) and cornu ammonis 3 (CA3) (Figure 4B). The vmPFC was divided into the prelimbic (PrL) and infralimbic (IL) cortex, and the BLA was also delineated (Figure S4B and S4D). The area of each subregion was extracted, and c-Fos⁺ cells were automatically identified using the StarDist algorithm (Schmidt et al., 2018). Cell counts were normalized to subregion area and averaged per ROI to obtain a mean value per animal.

12. Statistical analysis

Corticosterone and gene expression levels were logarithmically transformed to approximate normal distributions. Normality was verified by visually inspecting the residual Q-Q plots, and/or by testing the normality of data or residuals using the D'Agostino-Pearson and Shapiro-Wilk tests.

For comparisons of two groups (e.g., corticosterone measurements), two-tailed independent sample *t*-tests were used. For analyses containing within-subject (e.g., time point) and between-subject variables (e.g. rat line), mixed-model ANOVAs were conducted with Greenhouse-Geisser correction applied by default to account for any violations of the sphericity assumption. Compliance with the homogeneity of covariance matrices assumption was verified by Box's test. Datasets violating this assumption were analyzed using a Linear Mixed Model (unstructured covariance matrix). *Post hoc* comparisons were adjusted for multiple testing using the Holm-Šidák method.

Pearson correlations between regional c-Fos⁺ cell numbers were computed separately for each experimental group. Linearity was assessed by Run's test, and bivariate normality was verified by visual inspection of scatterplots and the Shapiro-Wilk test. Individual correlation coefficients were tested for significance using Pearson's correlation test. Differences between correlation coefficients were evaluated by a z-test after a Fisher's *r*-to-*z* transformation. *P*-values were corrected for multiple

testing using the Benjamini–Hochberg False Discovery Rate (FDR) method.

Analyses with more than two variables were performed in SPSS (IBM, version 27), and correlation analyses were run in Python (3.12.0). All other analyses were conducted in GraphPad Prism (Dotmatics, version 10.1.2.). Whenever appropriate, effect sizes are reported as Cohen's d for t -tests and η^2 -based measures for ANOVAs and linear mixed models. The significance threshold was set at $P < 0.05$.

RESULTS

Phenotypic characterization of glucocorticoid signaling

Post-stressor corticosterone levels in breeding rats across generations

Candidate breeding rats of both the Low and Control lines were subjected to the standardized 3-day SAT at PND 28–30 (Figure S1A). Plasma corticosterone levels measured at 50 min after stressor onset on PND30 were used for breeding selection. Post-stressor plasma corticosterone levels of both male and female rats of the Low line were consistently lower than those of the Control line across generations (male: $F_{1,200} = 215.6$, $P < 0.0001$; female: $F_{1,235} = 150$, $P < 0.0001$; Figure S1B). From this pool of candidate breeders, in every generation 10–15 couples from each line were selected for breeding according to the following criteria: <100 ng/ml for the Low line, and between 100 and 200 ng/ml for the Control line (Walker et al., 2017).

Basal circulating corticosterone levels and adrenal weight

In adult experimental animals, morning basal plasma corticosterone levels, collected between 9:00 and 13:00 h, did not differ between the two lines (independent sample t -test: $t_{28} = 0.90$, $P = 0.37$, Figure 1A). Also, average adrenal gland weight (adjusted to body weight) did not differ between the two lines (independent sample t -test: $t_{28} = 0.70$, $P = 0.49$, Figure 1B).

Stress-induced corticosterone response profiles

To assess the magnitude and temporal dynamics of stress-induced HPA-axis activation in both rat lines, blood samples were collected at 15, 30, 60 and 90 min after the start of the fear conditioning session (Figure 1C). A mixed-model ANOVA for plasma corticosterone levels revealed significant main effects of rat line ($F_{1,28} = 46.90$, $P < 0.0001$) and time point ($F_{1,65,46,07} = 66.30$, $P < 0.0001$), as well as a significant rat line $\times$ time point interaction effect ($F_{3,84} = 11.41$, $P < 0.0001$). *Post hoc* analyses showed that corticosterone levels of the Low Line were consistently lower than those of the Control line at all time points (independent sample t -tests: all P s ≤ 0.002). Moreover, the two rat lines displayed distinct temporal response profiles (Figure 1D). Corticosterone levels of the Control line peaked at 30 min post-stressor onset (15 min vs 30 min: $t_{14} = 5.36$, $P < 0.001$), followed by a gradual decline (30 min vs 60 min: $t_{14} = 3.50$, $P < 0.02$; 60 min vs 90 min: $t_{14} = 6.70$, $P < 0.0001$). In contrast, corticosterone levels of the Low line

peaked already between 15 and 30 min after stress onset (15 min vs 30 min: $t_{14} = 1.90$, $P = 0.15$), and then rapidly declined (30 min vs 60 min: $t_{14} = 9.70$, $P < 0.0001$), reaching potential basal levels at 60 min (60 min vs 90 min: $t_{14} = 0.72$, $P = 0.48$).

To determine whether corticosterone levels had returned to baseline at 90 min after stress onset, a follow-up two-way ANOVA compared corticosterone levels measured 90 min post-stressor onset with basal levels obtained from a separate group of rats at the same time of day, ensuring that blood collection prior to fear conditioning did not itself act as a confounding stressor. This analysis showed a significant rat line $\times$ time point interaction effect ($F_{1,56} = 6.88$, $P < 0.05$). *Post hoc* analyses indicated that corticosterone levels at 90 min in the Low line did not differ from basal levels ($t_{56} = 1.79$, $P = 0.08$), whereas levels in the Control line remained elevated ($t_{56} = 5.49$, $P < 0.0001$). The faster return to basal corticosterone levels in the Low line suggests that these rats present stronger HPA-axis negative feedback regulation.

HPA-axis regulation in response to dexamethasone administration

To formally test whether Low line rats present stronger HPA-axis negative feedback regulation, rats from both lines underwent a low-dose DST using two doses of dexamethasone (25 and 50 $\mu\text{g/kg}$) and a stress challenge of exposure to synthetic fox odor 6 h later (Figure 1E)(Zoladz & Diamond, 2016). First, we assessed dose- and time-dependent effects of dexamethasone administration on plasma corticosterone levels in the two lines separately, followed by a direct comparison of the suppressive effects of dexamethasone administration on HPA-axis activation between the two lines.

In the Control line, a significant dexamethasone dose $\times$ time point interaction effect was found ($F_{6,63} = 5.13$, $P < 0.0002$), as well as significant main effects for dexamethasone dose ($F_{2,21} = 4.52$, $P < 0.05$) and time point ($F_{1,16,24,34} = 67.83$, $P < 0.0001$; Figure 1F). *Post hoc* tests further showed that, before the stress challenge (0 min), both dexamethasone doses significantly reduced corticosterone levels compared to vehicle (vehicle vs 25 $\mu\text{g/kg}$: $t_{13,98} = 2.59$, $P = 0.05$; vehicle vs 50 $\mu\text{g/kg}$: $t_{13,71} = 2.59$, $P < 0.03$). At 30 min post-stressor onset, only the 50 $\mu\text{g/kg}$ dose continued to suppress corticosterone (vehicle vs 50 $\mu\text{g/kg}$: $t_{8,50} = 3.07$, $P < 0.05$), while the 25 $\mu\text{g/kg}$ did not (vehicle vs 25 $\mu\text{g/kg}$: $t_{11,66} = 0.63$, $P = 0.54$), supporting a dose-dependent and activity-sensitive partial suppression. No differences were found in either dose compared to vehicle at the 60 and 90 min time points ($t_{12,12} < 1.80$, $P_s > 0.05$).

In the Low line, a significant dose $\times$ time point interaction effect was found ($F_{6,63} = 7.83$, $P < 0.0002$), as well as significant main effects for dexamethasone dose ($F_{2,21} = 16.66$, $P < 0.0001$) and time point ($F_{2,14,45} = 36.67$, $P < 0.0001$; Figure 1G). *Post hoc* tests showed that at baseline (0 min) both dexamethasone doses significantly reduced corticosterone levels compared to vehicle (vehicle vs 25 $\mu\text{g/kg}$: $t_{9,75} = 5.23$, $P < 0.001$; vehicle vs 50 $\mu\text{g/kg}$: $t_{10,64} = 5.68$, $P < 0.0005$). At 30 min post-stressor onset, both dexamethasone doses also suppressed corticosterone levels (vehicle vs 25 $\mu\text{g/kg}$: $t_{10,29} = 3.32$, $P < 0.02$; vehicle vs 50 $\mu\text{g/kg}$: $t_{7,37} = 4.81$, $P < 0.02$). At 60 min, a significant reduction was still observed for the 50 $\mu\text{g/kg}$ dose compared to vehicle (vehicle vs 50

µg/kg: $t_{7,42} = 3.11$, $P < 0.05$), but not in the 25 µg/kg (vehicle vs 25 µg/kg: $t_{9,80} = 2.50$, $P = 0.06$). No differences were found in either dose compared to vehicle at the 90 min time point ($t_{8,78} < 2.47$, $P_s > 0.1$).

To compare the dose-dependent dexamethasone-induced suppression of HPA-axis activity between lines, corticosterone levels at 30 min post-stressor onset (reflecting HPA-axis activation) of both dexamethasone doses were normalized to the mean corticosterone level of the vehicle group within each line (Figure 1H). A two-way ANOVA revealed significant main effects of rat line ($F_{1,28} = 12.27$, $P < 0.002$) and dexamethasone dose ($F_{1,28} = 13.25$, $P < 0.002$), together with a non-significant rat line $\times$ dexamethasone dose interaction effect ($F_{1,28} = 0.36$, $P = 0.55$). *Post hoc* analyses showed that the higher dose of dexamethasone (50 µg/kg) induced greater suppression of HPA-axis activity than the lower (25 µg/kg) dose in both lines (Control line: $t_{28} = 3.00$, $P < 0.05$; Low line: $t_{28} = 2.15$, $P < 0.05$). Critically, across doses, the Low line exhibited significantly stronger suppression of HPA-axis activity than the Control line (25 µg/kg: $t_{28} = 2.90$, $P < 0.05$; 50 µg/kg: $t_{28} = 2.05$, $P < 0.05$). One-sample *t*-tests confirmed that corticosterone levels were significantly suppressed relative to vehicle in all groups ($t_7 \geq 4.28$, $P_s \leq 0.005$) except in Control rats treated with the lower dexamethasone dose (25 µg/kg: $t_7 = 0.35$, $P = 0.74$), indicating a lack of suppression at this dose in the Control line. Together, these findings indicate that the Low line is more sensitive to glucocorticoid negative feedback, consistent with stronger HPA-axis regulation.

Nr3c1, Nr3c2 and Fkbp5 gene expression in blood

To investigate whether the expression of glucocorticoid signaling-related genes in blood differed between the two lines, blood was collected from undisturbed rats from each line (between 9 and 13 h) and processed for the assessment of blood mRNA levels of *Nr3c1* (encoding GR), *Nr3c2* (encoding the mineralocorticoid receptor (MR)) and *Fkbp5* (encoding a corticosteroid receptor co-chaperone) (Le-Niculescu et al., 2020; Zannas et al., 2016).

Nr3c1 mRNA levels did not differ between the two rat lines (independent samples *t*-test: $t_{27} = 0.23$, $P = 0.81$) (Figure 1I). In contrast, both *Nr3c2* and *Fkbp5* mRNA levels were significantly elevated in the Low line compared to the Control line (*Nr3c2*: independent samples *t*-test: $t_{27} = 2.13$, $P < 0.05$; *Fkbp5*: independent samples *t*-test: $t_{27} = 2.56$, $P < 0.05$).

Next, associations between glucocorticoid signaling-related markers in blood were assessed using Pearson correlations calculated separately for each line. In line with MR-driven *Fkbp5* modulation in basal (i.e., non-stressed) conditions (Hartmann et al., 2021), *Nr3c2* and *Fkbp5* mRNA blood levels were significantly positively correlated in the Control line ($r_{15} = 0.66$, $P < 0.01$). In contrast, no significant correlation was observed in the Low line ($r_{14} = 0.32$, $P = 0.27$; Figure 1J). Other correlations between glucocorticoid signaling-related markers levels were not significant in either line (Table S3).

Moreover, the relationship between circulating corticosterone and *Fkbp5* mRNA

blood levels was examined. In the Control line, corticosterone levels were positively correlated with *Fkbp5* expression ($r_{15} = 0.61$, $P < 0.05$), while no association was detected in the Low line ($r_{14} = 0.00$, $P = 0.99$; Figure 1K). Other correlations between corticosterone and glucocorticoid signaling-related markers levels were not significant in either line (Table S3).

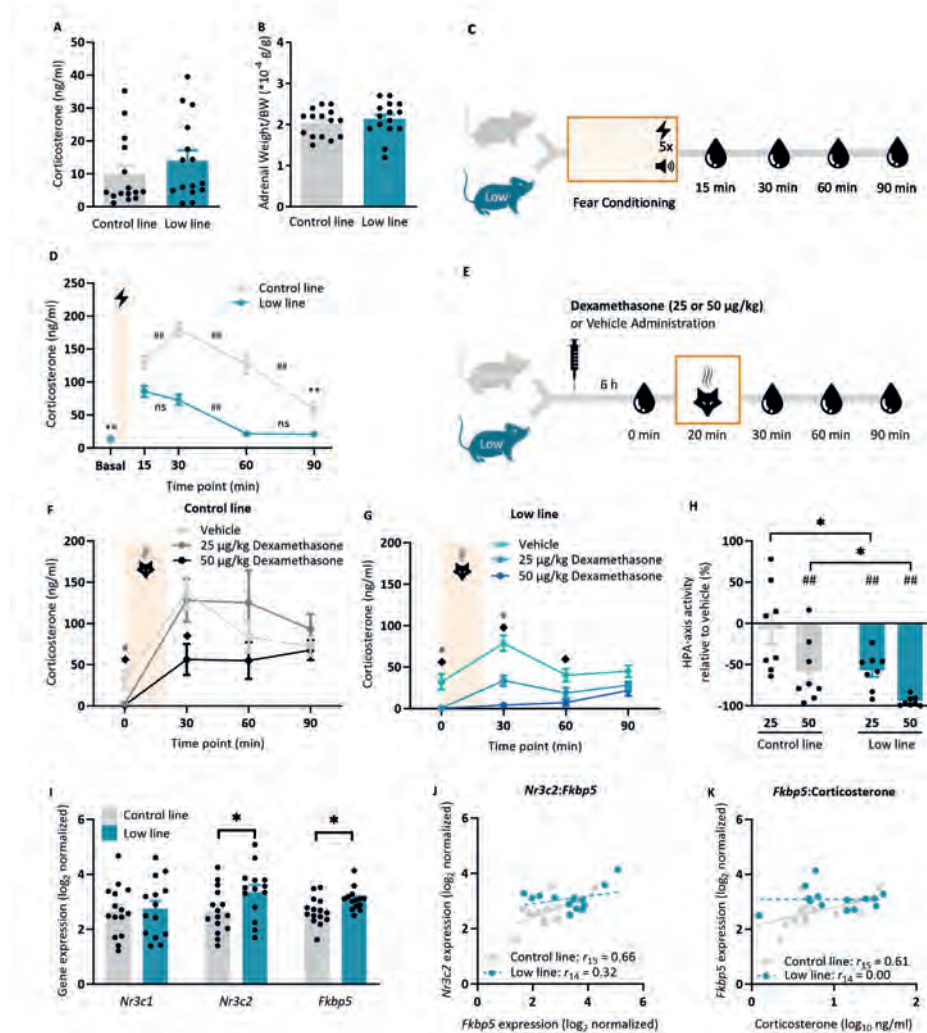


Figure 1 – Phenotypic characterization of glucocorticoid signaling in the Low and Control lines

A. Morning basal plasma corticosterone levels (mean $\pm$ s.e.m.) did not differ between the Low and Control lines ($n = 15$ rats per line). **B.** Adrenal gland weight adjusted to body weight (mean $\pm$ s.e.m.) did not differ between the Low and Control lines ($n = 15$ rats per line). **C.** Experimental protocol for the assessment of the magnitude

and temporal dynamics of the post-stressor corticosterone response. After rats underwent fear conditioning, blood was collected for corticosterone measurement 15, 30, 60 and 90 min post-stressor onset. **D.** Low line showed a peak in post-stressor corticosterone levels (mean $\pm$ s.e.m.) that was earlier (at 15 min) and lower compared to the Control line (at 30 min), as well as a faster return to baseline ($n = 15$ rats per line, $** P < 0.01$, $## P < 0.01$). **E.** Experimental protocol for the low-dose DST. In the morning, different groups of rats from both lines were subcutaneously administered two dexamethasone dosages (25 and 50 $\mu\text{g/kg}$) or vehicle. Six hours later, blood was collected for basal plasma corticosterone measurement, followed by an immediate 20-min exposure to synthetic fox odor. Blood was collected again 30 min, 60 min and 90 min after stressor onset. **F.** Corticosterone (mean $\pm$ s.e.m.) response blunted by 50 $\mu\text{g/kg}$ dexamethasone dose, but not 25 $\mu\text{g/kg}$, in the Control line ($n = 8$ rats per treatment, $^{\#} P < 0.05$ vehicle vs 50 $\mu\text{g/kg}$ dexamethasone). **G.** Corticosterone (mean $\pm$ s.e.m.) response blunted by both the 25 and 50 $\mu\text{g/kg}$ doses of dexamethasone in the Low line ($n = 8$ rats per treatment, $^{\#} P < 0.05$ vehicle vs 25 $\mu\text{g/kg}$ dexamethasone, $^{\diamond} P < 0.05$ vehicle vs 50 $\mu\text{g/kg}$ dexamethasone). **H.** Low line presented a stronger suppression of HPA-axis activity compared to the Control line ($n = 8$ rats per line and treatment, $^* P < 0.05$, $## P < 0.01$ vs chance level). **I.** Low line presented higher *Nr3c2* (MR) and *Fkbp5* expression (mean $\pm$ s.e.m.), but not *Nr3c1* (GR), in blood compared to the Control line ($n = 14$ -15 rats per line, $^* P < 0.05$). **J.** Control line exhibited a significant positive correlation between *Nr2c3* and *Fkbp5* mRNA levels in blood ($P < 0.01$), but not the Low line ($P = 0.27$; $n = 14$ -15 rats per line). **K.** Control line exhibited a significant positive correlation between plasma corticosterone and *Fkbp5* mRNA levels in blood ($P < 0.05$), but not in the Low line ($P = 0.99$; $n = 14$ -15 rats per line). Dashed regression line represents a non-significant correlation, and a solid line represents a significant correlation. Dots represent individual values.

Strength and generalization of extinction memory under non-treated conditions

To determine whether fear conditioning and extinction memory differed between the Low and Control lines, rats from both lines were trained on a fear conditioning and extinction paradigm (Figure 2A). Rats underwent fear conditioning in context A, and then extinction training in context B one day later. Extinction memory was assessed in a recall test consisting of sequential exposure to context B followed by context A. During this test, freezing during CS presentation in context B was taken as measure of strength of extinction memory, whereas freezing during CS presentation in context A was taken as a measure of generalization of extinction memory (i.e., prevention of fear renewal). An additional group of fear conditioned rats from each line did not undergo extinction training and served as no-extinction controls. In these animals, freezing during CS presentation in context A was taken as a measure of strength of fear memory strength, whereas freezing in context B was taken as a measure of generalization of fear memory.

Fear conditioning

Rats from both lines underwent fear conditioning in context A, where 5 CS-US pairings were delivered. A two-way mixed-model ANOVA for freezing levels during CS presentation revealed a significant main effect of time point ($F_{3.59, 201.30} = 98.55$, $P < 0.0001$), but no effect of rat line ($F_{1, 56} = 1.16$, $P = 0.28$) or rat line $\times$ time point interaction effect ($F_{5, 280} = 1.43$, $P = 0.22$, Figure 2B). *Post hoc* comparisons showed that freezing increased significantly from the first to the last CS presentation (CS1 vs CS5: $t_{57} = 14.96$, $P < 0.0001$), reflecting an average increase of $60.4 \% \pm 4.0 \%$ ($d^z = 1.96$). These results indicate that fear conditioning was effective and that within-session fear learning was similar between lines.

Extinction training

On the following day, half of the rats from each line underwent extinction training in context B, where the CS was presented 20 times. Changes in freezing levels across CS presentations were analyzed as a measure of within-session extinction learning. A two-way mixed-model ANOVA for freezing levels during extinction training showed no significant effects of rat line ($F_{1, 28} = 0.03$, $P = 0.87$), time point ($F_{5.76, 161.20} = 1.75$, $P = 0.12$), or rat line $\times$ time point interaction ($F_{10, 280} = 0.83$, $P = 0.60$, Figure 2C). These results indicate that freezing levels did not change throughout the extinction session in either line.

One-day extinction recall

One day after extinction training, all rats underwent a recall test to assess extinction memory, where they were first exposed to 3 CS presentations in context B followed by 3 CS presentations in context A.

First, to evaluate between-session extinction memory between the two lines, freezing levels during CS presentations at the start of extinction training in context B were compared to freezing levels during CS presentations in the same context during the recall test. These two measurements constituted the within-subject factor "session". The mixed-model ANOVA for freezing levels revealed a significant main effect of session ($F_{1, 28} = 14.11$, $P < 0.001$), but no significant effects of rat line ($F_{1, 28} = 0.05$, $P = 0.83$) or rat line $\times$ session interaction ($F_{1, 28} = 0.00$, $P = 0.96$). Subsequent analysis showed that individual freezing levels in context B were reduced by an average of $24.1 \pm 6.4 \%$ ($d^z = 0.69$) from extinction training to recall test. Together, these results indicate effective between-session extinction that was comparable between the two rat lines.

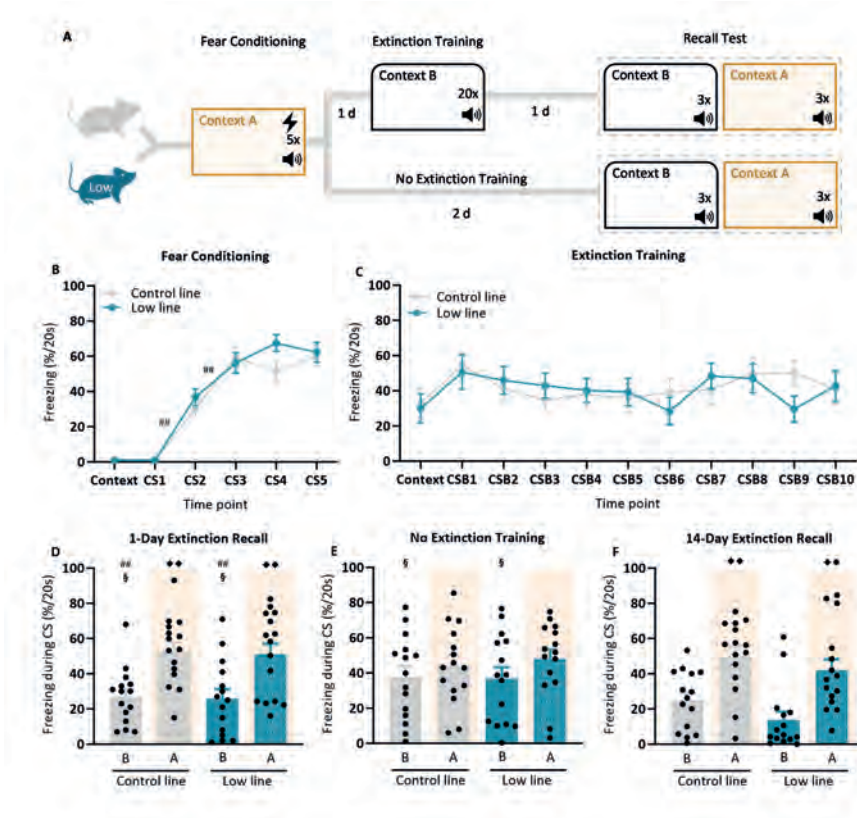


Figure 2 – Strength and generalization of extinction memory at a 1-day and 14-day recall test in the Low and Control lines.

A. Experimental protocol of the fear conditioning and extinction task. Rats underwent fear conditioning in context A, where a 20-s auditory conditioned stimulus (CS) was presented 5 times, co-terminating with a 1-s footshock (unconditioned stimulus; US). The next day, fear extinction training was conducted in context B, which differed in both visual and tactile features. In this context, the CS was presented 20 times without the US with an unpredictable ITI. Finally, one day later, the strength and generalization of the extinction memory was assessed by sequentially re-exposing the rats to context B followed by context A, with 3 CS presentations in each context. Additional groups of rats from each line did not undergo extinction training after fear conditioning and were subsequently tested using the same recall protocol. **B.** Rat lines presented similar within-session fear learning, as freezing levels (mean $\pm$ s.e.m.) significantly increased after the first CS presentation ($n = 29$ rats per line, ## $P < 0.01$). **C.** The rat lines presented similar freezing levels (mean $\pm$ s.e.m.) prior to and during CS presentation, with no evidence of within-session extinction ($n = 15$ rats per line). **D.** In a 1-day recall test, the rat lines presented similarly reduced freezing levels during CS block (CSB) presentation (mean $\pm$ s.e.m.) in context B compared to context A, displaying robust between-session extinction ($n = 15$ rats per line, ## $P < 0.01$ vs extinction training, ♦♦

$P < 0.01$ context B vs A, [§] $P < 0.05$ extinction training vs no training). **E.** No-extinction controls showed similar freezing levels during CS presentation between lines (mean $\pm$ s.e.m.) in context B compared to context A ($n = 15$ rats per line). **F.** In the 14-day recall test, the rat lines presented a similarly reduced freezing levels during CS presentation (mean $\pm$ s.e.m.) in context B compared to context A, displaying comparable context-dependent extinction memory ($n = 15$ rats per line, $\blacklozenge$ $P < 0.01$ context B vs A). Black dots represent individual values.

Second, to assess whether the strength and generalization of extinction memory differed between the two rat lines, a three-way mixed-model ANOVA compared average freezing levels during CS presentation in rats that underwent extinction training (Figure 2D) versus rats that did not receive training (i.e., no-extinction controls), but were tested in the same two contexts (Figure 2E). This analysis revealed a significant interaction effect of extinction training $\times$ test context ($F_{1,56} = 7.02$, $P < 0.02$) and a significant main effect of test context ($F_{1,56} = 29.11$, $P < 0.0001$). No other effects were significant, including the main effect of extinction training ($F_{1,56} = 0.29$, $P = 0.59$). Crucially, no effects involving rat line were observed, namely of rat line ($F_{1,56} = 0.00$, $P = 0.97$), rat line $\times$ test context ($F_{1,56} = 0.08$, $P = 0.77$), rat line $\times$ extinction training ($F_{1,56} = 0.12$, $P = 0.73$) or rat line $\times$ extinction training $\times$ test context ($F_{1,56} = 0.19$, $P = 0.67$), indicating similar fear and extinction recall between lines.

To assess the context-dependency of the extinction memory, the simple effects of the significant extinction training $\times$ test context interaction were examined. Consistent with extinction-specific learning, *post hoc* comparisons showed that extinction-trained rats from both lines froze significantly less during CS presentation in context B compared to context A (context B vs A: $t_{58} = 5.78$, $P < 0.0001$, $d^2 = 1.05$), whereas in no-extinction control rats from both lines the context difference was less pronounced and only approached statistical significance (context B vs A: $t_{58} = 1.97$, $P = 0.05$, $d^2 = 0.36$). To further characterize the strength and generalization of the extinction memory, between-group comparisons were performed within each context. In context B, freezing levels during CS presentation were significantly lower in extinction-trained rats compared to no-extinction controls (no training vs training: $t_{116} = 1.99$, $P < 0.05$), indicating robust extinction memory expression. In contrast, in context A, extinction trained-rats presented similar freezing levels to no-extinction controls in both lines (no training vs training: $t_{116} = 1.08$, $P = 0.28$), indicating that the extinction memory did not generalize.

Moreover, freezing levels measured prior to CS presentation (i.e., context only) supported a main context effect with freezing higher in context A compared to context B, without evidence for an effect of extinction training or rat line (Figure S3A and B).

Together, these analyses demonstrate that the two rat lines did not differ in between-session extinction learning, or in the strength and generalization of the extinction memory. Across lines, despite the absence of detectable within-session extinction during training, the extinction protocol successfully produced between-session

extinction in context B, but did not enable generalization to context A, the original conditioning context.

Fourteen-day extinction recall

Given that both strength and generalization of extinction memory can change with the passage of time (Bouton et al., 2021; Krenz et al., 2023; Wiltgen & Tanaka, 2013), we next assessed whether the two rat lines differed in the long-term maintenance of the extinction memory. Therefore, separate groups of rats from both lines underwent fear conditioning and extinction training, but recall of extinction was tested 14 days later (Figure 2F). Fear conditioning and extinction training results replicated those observed in the 1-day recall cohort. A two-way mixed-model ANOVA for average freezing levels during CS presentation at the 14-day extinction recall test revealed a significant main effect of test context ($F_{1,28} = 31.07, P < 0.0001$), but not of rat line ($F_{1,28} = 0.17, P = 0.68$) or rat line $\times$ test context interaction ($F_{1,28} = 2.07, P = 0.16$). Moreover, freezing levels measured prior to CS presentation (i.e., context only) mirrored this difference in freezing between test contexts together with the absence of a rat line effect (Figure S3C). These results support that the rat lines displayed similar context-dependent extinction at this timepoint.

Overall, across experiments, no evidence was found for differences between the Low and Control lines in within-session fear learning, within- or between-session extinction learning, or in the strength and generalization of extinction memory at either a 1-day or 14-day extinction recall test.

Strength and generalization of extinction memory following glucocorticoid administration

We next examined whether glucocorticoid administration would differentially affect the strength and generalization of extinction memory in the Low line compared to the Control line. To this end, rats from both lines were subcutaneously administered vehicle or one of three doses of corticosterone (0.3, 1 or 3 mg/kg) immediately after extinction training, in order to selectively modulate the consolidation phase (Figure 3A). Strength and generalization of extinction memory were then tested 1 or 14 days later in separate groups of rats.

One-day extinction recall

To assess the effects of corticosterone administration immediately after extinction training on subsequent extinction memory, a linear mixed model was used to compare average freezing levels during CS presentation in a 1-day recall test (Figure 3B), with corticosterone treatment included as an additional factor alongside rat line and test context.

This analysis revealed significant interactions between rat line $\times$ test context ($F_{3,105} = 23.29, P < 0.001$) and rat line $\times$ corticosterone treatment ($F_{3,105} = 3.30, P < 0.05$), as

well as significant main effects of rat line ($F_{1,105} = 16.36, P < 0.001$) and test context ($F_{1,105} = 314.65, P < 0.001$). No other main or interaction effects reached significance, including corticosterone treatment ($F_{3,105} = 1.58, P = 0.19$), corticosterone treatment $\times$ test context ($F_{3,105} = 1.28, P = 0.29$), rat line $\times$ corticosterone treatment $\times$ test context ($F_{3,105} = 0.45, P = 0.72$).

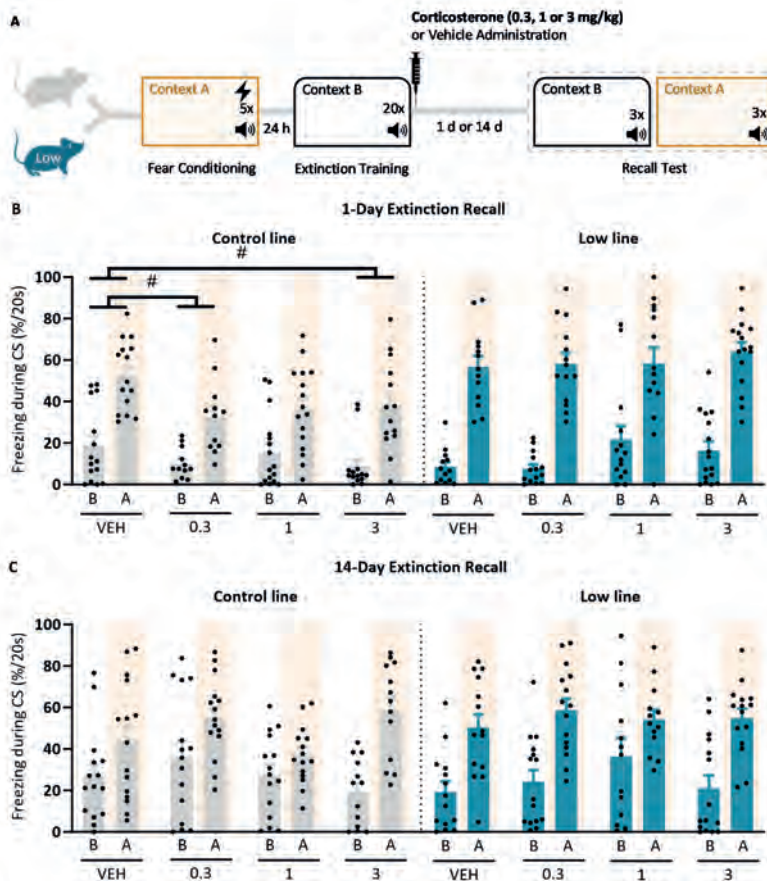


Figure 3 – Effect of post-extinction corticosterone administration on the strength and generalization of extinction memory at 1-day and 14-day recall test in the Low and Control lines.

A. Experimental protocol of the fear conditioning and extinction task with post-extinction administration of corticosterone. Fear conditioning and extinction training were conducted as previously described. Immediately after extinction training, rats were subcutaneously administered one of three doses of corticosterone (0.3, 1 or 3 mg/kg) or vehicle. Extinction recall was then tested either 1 day or 14 days later. **B.** At the 1-day recall test, corticosterone administration dose-dependently reduced CS-induced freezing levels (mean $\pm$ s.e.m.) across contexts in the Control line, whereas

corticosterone treatment was ineffective in the Low line ($n = 12$ -16 rats per line and treatment, $^{\#} P < 0.05$). **C.** At the 14-day recall test, corticosterone administration did not affect freezing levels at extinction recall in the two rat lines ($n = 13$ -15 rats per line and treatment). Black dots represent individual values.

Follow-up univariate tests examined the simple effects of the significant interaction terms. For the rat line $\times$ corticosterone treatment interaction, univariate analyses identified a significant corticosterone treatment effect in the Control line ($F_{3, 105} = 2.95, P < 0.05$). Pairwise comparisons showed that both the lowest (0.3 mg/kg; $t_{105} = 2.63, P < 0.05, d = 1.02$) and highest (3 mg/kg; $t_{105} = 2.41, P < 0.05, d = 0.90$) dose of corticosterone significantly reduced freezing relative to vehicle. The intermediate dose (1 mg/kg) did not produce a reliable effect compared to vehicle ($t_{105} = 1.90, P = 0.06, d = 0.68$). In contrast, no significant corticosterone treatment effect was found in the Low line ($F_{3, 105} = 2.95, P = 0.13$). Given the absence of corticosterone treatment $\times$ test context or three-way interaction effects, these results suggest that corticosterone treatment reduced freezing in the Control line independently of context, whereas this modulatory effect was absent in the Low line.

Consistent with previous experiments (Figure 1D), additional univariate tests confirmed that freezing levels of vehicle-treated groups did not differ between lines across contexts ($F_{1, 105} = 0.34, P = 0.56$). In contrast, following corticosterone administration, Control line rats exhibited significantly lower freezing than Low line rats across contexts at every dose tested (0.3 mg/kg: $F_{1, 105} = 4.41, P < 0.05$; 1 mg/kg: $F_{1, 105} = 10.63, P < 0.005$; 3 mg/kg: $F_{1, 105} = 11.38, P < 0.005$). These comparisons confirm that corticosterone treatment only reduced freezing levels during the extinction recall test in the Control line, but not the Low line.

For the rat line $\times$ test context interaction, freezing levels during CS presentation were significantly lower in context B compared to context A in both lines, indicating robust context-dependency of extinction memory. Notably, this test context effect was less pronounced in the Control line (Low line: $F_{1, 105} = 252.87, P < 0.001$, partial η^2 : 0.71; Control line: $F_{1, 105} = 83.93, P < 0.001$, partial η^2 : 0.44), suggesting a reduction in context-dependence of extinction memory in this line. Consistent with this, freezing levels in context B did not differ between lines ($F_{1, 105} = 0.07, P = 0.80$), whereas Control line rats exhibited significantly lower freezing than Low line rats in context A ($F_{1, 105} = 30.92, P < 0.001$). This pattern is in line with a greater attenuation of fear renewal in the Control line, but not in the Low line.

Notably, freezing levels measured prior to CS presentation (i.e., context only) mirrored the pattern of fear renewal attenuation observed during CS presentation, with lower freezing specifically in context A, but not context B, in the Control line compared to the Low line ($F_{3, 105.48} = 7.18, P < 0.01$, Figure S4A). However, in contrast to freezing during CS presentation, no corticosterone-related main or interaction effects were detected prior to CS presentation, suggesting that corticosterone modulation of extinction memory in the Control line was more robust after cue presentation than during context-only presentation.

In sum, while differences between lines were not previously observed in non-treated animals, differences in extinction expression emerged after post-extinction glucocorticoid challenge. In the Control line, corticosterone reduced freezing and facilitated expression of extinction memory in both test contexts, indicating a non-specific enhancement of strength and generalization of extinction memory. In contrast, corticosterone treatment did not affect freezing in the Low line in either test context, revealing a reduced behavioral sensitivity to glucocorticoid modulation.

Fourteen-day extinction recall

To assess whether the differential effect of corticosterone administration on extinction memory between lines persisted over time, in other groups of rats extinction recall was tested 14 days after extinction training and immediate post-extinction corticosterone administration. A linear mixed model comparing average freezing during CS presentation (Figure 3C) revealed a significant corticosterone treatment $\times$ test context interaction effect ($F_{3,107.28} = 3.20, P < 0.05$) alongside a significant main effect of test context ($F_{1,107.30} = 104.74, P < 0.001$). In contrast to the 1-day recall test, rat line $\times$ test context ($F_{3,107.30} = 2.79, P = 0.10$) and rat line $\times$ corticosterone treatment ($F_{3,107.93} = 1.12, P = 0.34$) were not significant. Other main and interaction effects were also not significant, including corticosterone treatment ($F_{3,107.93} = 1.15, P = 0.33$), rat line ($F_{1,107.94} = 0.25, P = 0.62$) and rat line $\times$ corticosterone treatment $\times$ test context ($F_{3,107.28} = 0.92, P = 0.43$).

Follow-up univariate tests examined the simple effects of the significant corticosterone treatment $\times$ test context interaction. Freezing levels were significantly lower in context B compared to context A across all treatment groups, indicating preserved context-dependence of extinction memory. However, the magnitude of this context effect varied as a function of corticosterone dose. The difference in freezing levels between contexts was comparable under vehicle (vehicle: $F_{1,106.41} = 23.48, P < 0.001$, partial $\eta^2: 0.18$) and the lowest corticosterone dose (0.3 mg/kg: $F_{1,106.41} = 30.48, P < 0.001$, partial $\eta^2: 0.22$). In contrast, this difference was attenuated at the intermediate dose ($F_{1,109.88} = 8.29, P < 0.01$, partial $\eta^2: 0.07$) and markedly enhanced at the highest dose ($F_{1,106.41} = 52.46, P < 0.001$, partial $\eta^2: 0.33$). Importantly, corticosterone dose did not reliably affect freezing levels within either context when simple effects were analyzed (context B: $F_{3,107.24} = 1.40, P = 0.25$; context A: $F_{3,107.13} = 2.49, P = 0.07$), indicating that corticosterone effects were mainly manifested as changes in the magnitude of contextual discrimination rather than context-specific changes in freezing levels.

In contrast to results during CS presentation, freezing levels measured prior to CS presentation (i.e., context only) revealed a main effect of test context ($F_{1,107.11} = 96.33, P < 0.001$), but no related interaction effects (Figure S4B). Along with the absence of effects involving corticosterone or rat line, this supports that corticosterone treatment modulated context-cue associations, rather than response to context only.

Together, these findings indicate that the facilitating effects of post-extinction corticosterone administration on extinction memory observed in the Control line, but

not Low line, at 1-day recall did not persist over time.

Activity and connectivity of hippocampal circuits following glucocorticoid administration

Given the divergent behavioral effects of post-extinction corticosterone administration between lines, we next examine whether these effects were accompanied by differences in neural activity. Because contextual modulation of extinction memory involves the hippocampus (Bouton et al., 2006; Ji & Maren, 2007), we assessed hippocampal activity following post-extinction corticosterone treatment. To this end, rats from both lines were perfused one hour after post-extinction corticosterone administration (Figure 4A) and brain sections were processed for c-Fos immunostaining, as a marker of activity across dorsal hippocampal subregions: DG, CA3 and CA1 (Figure 4B). Based on the behavioral results, the lowest effective dose of corticosterone (0.3 mg/kg) in the Control line was selected.

Subregion activity

In the DG granule cell layer (Figure 4C), two-way ANOVA for the number of c-Fos⁺ cells revealed a significant rat line × corticosterone treatment interaction effect ($F_{1,52} = 5.19, P < 0.05$), with no main effects of either rat line ($F_{1,52} = 3.25, P = 0.08$) or corticosterone treatment ($F_{1,52} = 1.84, P = 0.18$). *Post hoc* tests showed that vehicle-treated rats of both rat lines had a similar number of c-Fos⁺ cells ($t_{52} = 0.34, P = 0.74$). In the Control line, corticosterone significantly reduced the number of c-Fos⁺ cells compared to vehicle (corticosterone vs vehicle: $t_{52} = 2.57, P < 0.05$). In contrast, in the Low line, corticosterone administration had no effect on the number of c-Fos⁺ cells (corticosterone vs vehicle: $t_{52} = 0.65, P = 0.51$). Further, rats of the Control line treated with corticosterone displayed a significantly lower number of c-Fos⁺ cells in the DG than corticosterone-treated rats of the Low line (Low vs Control: $t_{52} = 2.89, P < 0.05$).

In the CA1 pyramidal cell layer (Figure 4E), there were no significant effects of corticosterone treatment ($F_{1,52} = 0.04, P = 0.83$) or rat line × corticosterone treatment interaction ($F_{1,52} = 0.02, P = 0.88$). The main effect of rat line approached significance ($F_{1,52} = 3.58, P = 0.06$), suggesting a trend toward higher number of c-Fos⁺ cells in the CA1 in the Low line compared to the Control line.

Together, these results show that, during the consolidation phase after extinction training, corticosterone reduced DG activity in the Control line, but failed to suppress DG activity in the Low line. In addition, the Low line presented significantly higher CA3 and (non-significantly) higher CA1 activity, independently of treatment condition.

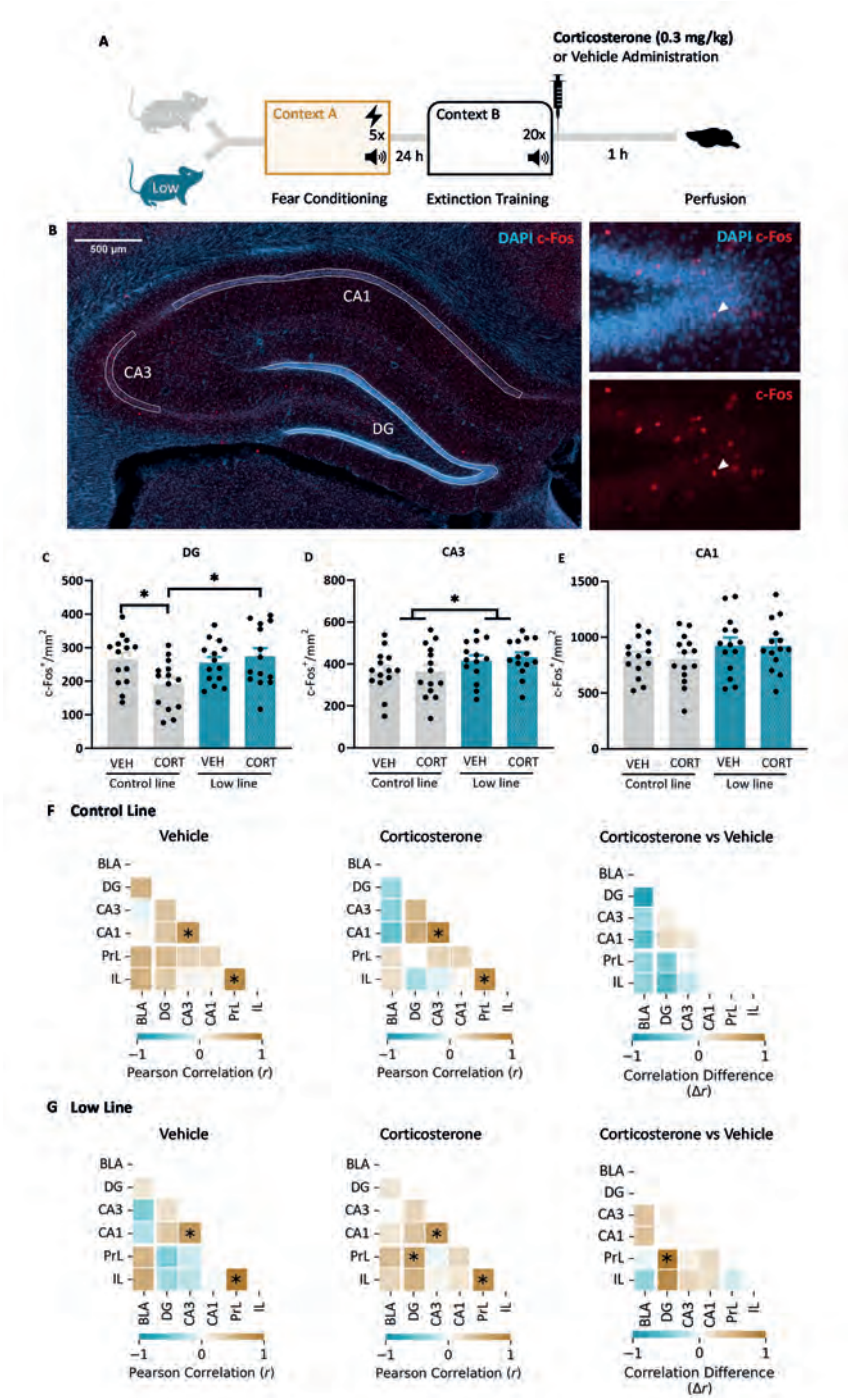


Figure 4 – Activity and connectivity of hippocampal circuits following glucocorticoid administration in the Low and Control lines.

A. Experimental protocol of brain sample collection 60 min after post-extinction corticosterone administration (0.3 mg/kg). **B.** Representative images of c-Fos- and DAPI-stained cells in hippocampal subregions DG, CA3 and CA1. **C.** Post-extinction corticosterone administration significantly decreased the number of c-Fos⁺ cells (mean $\pm$ s.e.m.) in the DG of the Control line, but not in the Low line ($n = 14$ rats per line and treatment, $* P < 0.05$). **D.** Low line displayed significantly higher overall number of c-Fos⁺ cells in the CA3 (mean $\pm$ s.e.m.) compared to the Control line ($n = 14$ rats per line and treatment, $* P < 0.05$). **E.** Low line displayed a trend towards higher overall number of c-Fos⁺ cells in the CA1 (mean $\pm$ s.e.m.) compared to the Control line ($n = 14$ rats per line and treatment). **F.** In the Control line, there was no difference in correlations (Δr) between vehicle and corticosterone treatments ($n = 14$ per treatment). **G.** Corticosterone-treated Low line displayed a significant positive correlation (r) between DG and PrL, which was significantly greater than in vehicle-treated rats (Δr ; $n = 14$ rats per treatment, $* P < 0.05$). Black dots represent individual values.

Functional connectivity

We also examined whether corticosterone treatment might induce differential effects on activity in other brain regions involved in contextual modulation of extinction memory (Bouton et al., 2006). However, no significant differences in the number of c-Fos⁺ cells between rat line or treatment condition were found in the BLA (Figure S5B), PrL (Figure S5D), or IL (Figure S5E).

We next investigated whether corticosterone treatment and rat line might influence correlations in activity across brain regions involved in extinction as a proxy for their functional connectivity. Therefore, Pearson correlations were calculated for c-Fos expression data between each hippocampal subregion, vmPFC subregion and the BLA within each line and treatment group. In the Control line (Figure 4F), no significant correlations between different brain regions were detected in either treatment group. In the Low line (Figure 4G), corticosterone-treated rats displayed a significant positive correlation between the number of c-Fos⁺ cells in the DG and PrL ($r_{14} = 0.69$, $P < 0.05$), which was absent in vehicle-treated rats ($r_{14} = -0.42$, $P = 0.38$). Interestingly, coordinated activity between the dorsal hippocampus and PrL has been implicated in the contextual regulation of extinction memory (Mourão et al., 2026). A direct comparison of these correlations by z-test after Fisher's r -to- z transformation indicated that the DG-PrL correlation was significantly stronger in corticosterone-treated rats compared to vehicle-treated rats of the Low line ($\Delta r = 1.11$, $z = 3.18$, $P < 0.05$). These findings suggest that corticosterone selectively enhanced DG-PrL connectivity in the Low line, whereas this connectivity was not affected by corticosterone treatment in the Control line.

Glucocorticoid receptor protein and phosphorylation in the dorsal hippocampus

Given that post-extinction corticosterone produced differential effects on hippocampal activity in the two rat lines, we next examined whether these effects were associated with altered GR signaling in the dorsal hippocampus. Rats from both lines were trained on the fear conditioning and extinction task with corticosterone (0.3 mg/kg) or vehicle treatment immediately after extinction training (Figure 5A). Tissue was collected 30 min later via rapid decapitation, and dorsal hippocampus samples were processed for detection of GR protein and its phosphorylation levels using Western Blot (Figure 5B).

Plasma corticosterone levels

To verify circulating corticosterone levels at the time of tissue collection, plasma corticosterone was measured in trunk blood samples obtained at the same time as the brain samples. A two-way ANOVA on plasma corticosterone levels revealed a significant main effect of rat line ($F_{1,56} = 16.89, P < 0.0005$) and corticosterone treatment ($F_{1,56} = 242.80, P < 0.0001$), as well as an almost significant rat line $\times$ corticosterone treatment interaction effect ($F_{1,56} = 3.86, P = 0.05$) (Figure 5C). *Post hoc* tests indicate that plasma corticosterone levels after vehicle administration in the Low line were significantly lower than those in the Control line ($t_{56} = 4.30, P < 0.0001$). Corticosterone administration significantly increased plasma corticosterone levels in both rat lines compared to vehicle controls (Control line: $t_{56} = 9.63, P < 0.0001$; Low line: $t_{56} = 12.41, P < 0.0001$). Further, plasma corticosterone levels after corticosterone administration did not differ significantly between the two rat lines ($t_{56} = 1.52, P = 0.14$).

Total glucocorticoid receptor protein

To determine whether the two lines differed in GR abundance in the dorsal hippocampus, a two-way ANOVA on total GR levels (Figure 5D) showed no significant effects of rat line ($F_{1,56} = 0.02, P = 0.88$), corticosterone treatment ($F_{1,56} = 0.13, P = 0.72$) or rat line $\times$ corticosterone treatment interaction ($F_{1,56} = 0.08, P = 0.77$). This indicates that total GR expression levels were similar in both rat lines, and not affected by the corticosterone administration.

Phosphorylated sites of the glucocorticoid receptor

GR phosphorylation at distinct serine residues is known to regulate receptor transcriptional activity, nuclear translocation, and glucocorticoid sensitivity (Adzic et al., 2009; Rogatsky et al., 1998). As such, we next examined GR phosphorylation at Serine 232 (pSer232) and Serine 246 (pSer246) sites, as they have been implicated in promoting or repressing GR-dependent transcriptional activity, respectively (Wang et al., 2002). pSer232 and pSer246 levels were normalized to total GR protein levels to determine the proportion of phosphorylated GR.

A two-way ANOVA on the percentage of pSer232 (Figure 5E) showed no significant

effects of rat line ($F_{1,42} = 0.04$, $P = 0.83$), corticosterone treatment ($F_{1,42} = 1.78$, $P = 0.19$) or rat line $\times$ corticosterone treatment interaction ($F_{1,42} = 2.61$, $P = 0.11$). Similarly, analysis of pSer246 (Figure 5F) showed no significant effects of corticosterone treatment ($F_{1,47} = 0.57$, $P = 0.45$) or rat line $\times$ corticosterone treatment interaction ($F_{1,47} = 0.15$, $P = 0.70$). A trend toward a main effect of rat line was observed ($F_{1,47} = 3.11$, $P = 0.08$), suggesting reduced S246 phosphorylation in the Low line.

Additionally, the ratio of pSer232 to pSer246 was calculated, as it is commonly interpreted as a proxy for GR activation. A two-way ANOVA of the phosphorylation ratio (Figure 5G) showed no significant effects of rat line ($F_{1,41} = 0.17$, $P = 0.68$), corticosterone treatment ($F_{1,41} = 0.14$, $P = 0.71$) or rat line $\times$ corticosterone treatment interaction ($F_{1,41} = 0.80$, $P = 0.37$).

Together, these results indicate that post-extinction corticosterone administration did not produce detectable differences between lines in total GR expression or phosphorylation in the dorsal hippocampus.

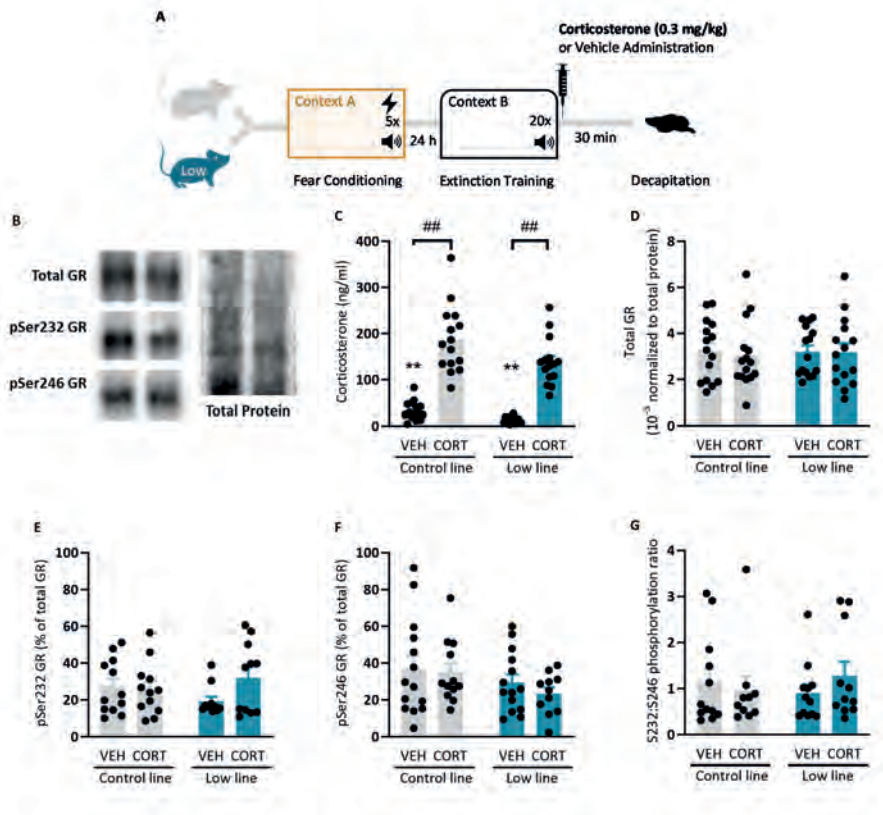


Figure 5 – Glucocorticoid receptor expression and phosphorylation in the dorsal hippocampus in the Low and Control lines.

A. Experimental protocol of brain sample collection 30 min after post-extinction corticosterone administration (0.3 mg/kg). **B.** Representative blots for total GR, and

phosphorylated GR at the Serine 232 (pSer232 GR) or 246 (pSer246 GR) sites. **C.** Plasma corticosterone levels (mean $\pm$ s.e.m.) were significantly higher after corticosterone administration ($n = 15$ rats per line and treatment, $^{##}P < 0.01$, $^{**}P < 0.01$ Low vs Control line). **D.** No difference in hippocampal GR levels (mean $\pm$ s.e.m.) between lines ($n = 15$ rats per line and treatment). **E.** No difference in phosphorylation levels at the S232 site (mean $\pm$ s.e.m.) between lines ($n = 11-12$ rats per line and treatment). **F.** No difference in phosphorylation levels of S246 site (mean $\pm$ s.e.m.) between lines ($n = 11-14$ rats per line and treatment). **G.** No difference in S232:S246 phosphorylation ratio (mean $\pm$ s.e.m.) between lines ($n = 10-12$ rats per line and treatment). Black dots represent individual values.

DISCUSSION

Individuals differ in their capacity to acquire and retain extinction memories. This study examined whether variation in glucocorticoid signaling – specifically stronger HPA-axis feedback regulation and low post-stressor corticosterone levels – contributes to differences in strength, generalization and maintenance of extinction memory, as well as to variability in responsiveness to exogenous glucocorticoid administration. We first showed that the Low line presented stronger HPA-axis negative feedback regulation and lower post-stressor corticosterone levels compared to the Control line. Contrary to expectations, stronger HPA-axis regulation did not alter extinction memory *per se*, but instead determined responsiveness to glucocorticoid augmentation. Post-extinction glucocorticoid administration selectively enhanced both strength and generalization of extinction memory in Control line rats, whereas it was ineffective in Low line rats. At the neural level, the Control line displayed reduced DG activity after glucocorticoid treatment, whereas in the Low line this reduction was absent and was accompanied by greater DG-PrL connectivity. Finally, no significant differences were observed in hippocampal GR phosphorylation patterns between lines and treatment conditions.

We first characterized HPA-axis feedback regulation in the rat lines. Consistent with previous descriptions (Walker et al., 2017), stress-induced corticosterone levels were lower in the Low line than the Control line, whereas basal (i.e., non-treated, non-stressed) plasma corticosterone levels and adrenal weights did not differ. In the Low line, the corticosterone response showed an early, blunted peak, accompanied by a faster return to basal levels, suggesting enhanced feedback sensitivity. This interpretation was supported by robust corticosterone suppression in the Low line, but not in the Control line, following administration of the lowest dose of dexamethasone (25 μ g/kg) in the DST, consistent with increased GR sensitivity. Gene expression analyses on basal blood samples revealed higher mRNA levels of both *Nr3c2* (MR) and *Fkbp5*, but not *Nr3c1* (GR), in the Low line. These findings align with prior evidence of an increased MR:GR ratio in the CA1 and DG in the Low line (Monari et al., 2024). Elevated basal *Fkbp5* expression is consistent with reduced FKBP5 methylation observed in PTSD (Klengel et al., 2013). In the Control line, *Nr3c2* and *Fkbp5* expression were positively correlated, as were *Fkbp5* levels and circulating corticosterone, consistent with MR-driven regulation of *Fkbp5* under basal conditions and its role in modulating

GR sensitivity (Hartmann et al., 2021). Notably, these associations were absent in the Low line, suggesting altered MR signaling. Together, these findings indicate that the Low line exhibits stronger HPA-axis negative feedback, potentially driven by altered MR-dependent regulation of GR sensitivity via FKBP5.

Given the role of glucocorticoids in memory consolidation (De Quervain et al., 2019; Roozendaal & McGaugh, 2011), we assessed whether this differential endogenous regulation impairs extinction memory strength and generalization in the Low line. To this end, we used a fear conditioning and extinction task, where extinction training occurred in a distinct context to the conditioning, and extinction recall was tested in both contexts. The extinction protocol employed variable intertone intervals (ITIs), which can produce effective between-session extinction even without within-session extinction (Plendl & Wotjak, 2010). Contrary to the findings of a study using a constant ITI during extinction (Monari et al., 2024), we did not observe differences in extinction memory between the two rat lines. One possibility is that Low line rats may engage compensatory mechanisms under our training conditions, as unpredictable CS presentations may increase arousal and noradrenergic activation. Low line rats exhibit heightened arousal relative to Control rats, as demonstrated by elevated norepinephrine levels in the DG during REM sleep (Monari et al., 2024), reduced vagal tone and increased heart rate in response to stress (Huzard et al., 2019). Given that noradrenergic activity promotes memory consolidation and specificity (Atucha et al., 2025; Cain et al., 2004; Mueller et al., 2008; Roozendaal & Mirone, 2020), such an enhanced noradrenergic activation in the Low line may normalize behavioral output in this task (i.e., context-dependent extinction memory), despite underlying differences in glucocorticoid signaling. This interpretation is supported by increased CA3 activity and a trend toward increased CA1 activity in saline-treated Low line rats relative to Control rats.

We initially hypothesized that Low line rats may particularly benefit from post-extinction glucocorticoid treatment, by presumably normalizing low corticosterone levels. At a 1-day recall test, post-extinction glucocorticoid treatment reduced freezing levels across both test contexts in the Control line, indicating enhancement of both the strength and generalization of extinction, thereby attenuating fear renewal. These findings support increasing evidence that glucocorticoids may be important modulators memory generalization and context dependency (Guo, 2024; Roozendaal & Mirone, 2020; van Ast et al., 2014). However, contrary to expectations and previous results (Monari et al., 2024), glucocorticoid treatment did not affect freezing in the Low line across multiple hormone doses, supporting a behavioral resistance to glucocorticoid treatment rather than an effect of suboptimal dosing. At a 14-day recall time point, these differential effects of post-extinction glucocorticoid administration between lines were not observed, indicating that extinction generalization did not persist in the Control line.

At the neural level, glucocorticoid enhancement of extinction memory strength and generalization in the Control line was accompanied by reduced DG activity during consolidation. The hippocampus plays a central role in contextual modulation of

memory, including context-dependent extinction (Bouton et al., 2006; Ji & Maren, 2007). This function relies in part on pattern separation in the DG and pattern completion in CA3, which together support discrimination between similar experiences (Yassa & Stark, 2011). These processes are critical for balancing specificity and generalization of fear and extinction memories (Asok et al., 2019; Laing & Dunsmoor, 2023; Lange et al., 2017; Neudert et al., 2023), and are implicated in related deficits observed in PTSD (Kheirbek et al., 2012). Accordingly, glucocorticoids may facilitate extinction generalization by reducing DG-dependent pattern separation, increasing overlap between fear and extinction representations and promoting transfer across contexts. In the Low line, however, resistance to glucocorticoid effects was associated with a lack of DG suppression during consolidation and increased DG–PrL connectivity. Dorsal hippocampus–PrL interactions support context-dependent extinction expression (Mourão et al., 2026), and activation of direct projections can strengthen fear while suppressing extinction memories (Ye et al., 2017). As such, this neural shift suggests that glucocorticoid administration in the Low line reinforces circuits that maintain established fear representations rather than promoting extinction.

Despite differences in hippocampal activity between lines, Western Blot analyses showed no evidence of altered hippocampal GR abundance or activation between lines, as total GR protein and phosphorylation at Ser232 and Ser246 sites were comparable across lines and treatment conditions. As these analyses were conducted on whole dorsal hippocampus homogenates, it cannot be excluded that region-specific alterations in GR signaling were masked by bulk tissue sampling (Ponce-Lina et al., 2020). However, the absence of differences in hippocampal GR expression between lines is consistent with the comparable blood *Nr3c1* mRNA expression in our study, as well as with previous reports of comparable GR levels across hippocampal subregions (Monari et al., 2024; Walker et al., 2017). As such, these results raise the possibility that glucocorticoid effects on hippocampal activity and connectivity are mediated indirectly via actions on other regions, such as the BLA, as observed in other studies (McGaugh, 2004; Roozendaal et al., 2009).

From a translational perspective, these findings suggest that interindividual differences in HPA-axis negative feedback regulation influence extinction memory augmentation by glucocorticoids, with implications for patient stratification in PTSD. Our findings suggest that glucocorticoid-based adjuncts may be beneficial primarily for individuals with intact HPA-axis negative feedback regulation. Of note, glucocorticoid enhancement of strength and generalization of extinction memory did not persist over time in the Control line, suggesting that repeated glucocorticoid treatment may be necessary for a long-lasting effect. In contrast, individuals with stronger HPA-axis negative feedback regulation profiles may require alternative interventions targeting downstream signaling pathways or interacting systems (e.g., endocannabinoid agonists) (Campolongo et al., 2009; Hill & McEwen, 2009).

Complementary human studies assessing the relationship between HPA-axis regulation and exposure therapy outcomes, as well as responses to glucocorticoid augmentation, will be crucial for translational validation. In parallel to these rodent

studies, we are conducting an observational study investigating associations between stronger HPA-axis negative feedback regulation, early life stress, and PTSD treatment outcome, followed by a pharmacological intervention study examining the effects of exogenous glucocorticoids on extinction memory strength and generalization in a stratified PTSD population (de Nooij et al., 2024). Notably, ongoing efforts to delineate PTSD subtypes based on glucocorticoid signaling have identified a “GR super sensitivity” phenotype mediated by a FKBP5 polymorphism, characterized by unchanged basal glucocorticoid levels during the early sleep phase, stronger HPA-axis suppression following dexamethasone administration, and altered transcriptional response to glucocorticoids (Mehta, 2011). This phenotype closely parallels the HPA-axis profile of the Low line observed in the present study.

In summary, this study demonstrates that glucocorticoid treatment enhances strength and generalization of extinction memory in Control line rats but fails to do so in Low line rats. These findings highlight the importance of considering individual endocrine phenotypes when developing pharmacological adjuvants to extinction-based interventions in stress-related disorders, such as PTSD.

ACKNOWLEDGMENTS

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SUPPLEMENTARY INFORMATION

Selective breeding

All male and female candidate breeder rats were subjected to the Stress Adaptation Test (SAT) performed on PND28 to PND30 (Figure S1A) (Walker et al., 2017). After weaning on PND21, rats were housed in groups of three or four, separated by sex. All rats were habituated to handling and gentle restraining for 2 min on 3 consecutive days (PND25-27). On PND28, they were initially placed on an open field (50 x 50 cm) for 5 min and subsequently on an elevated platform (10 cm width x 10 cm depth x 100 cm height) under bright light (400 lux) for 25 min. On PND29, the rats were exposed to synthetic fox odor TMT (W332518, Sigma-Aldrich) for 25 min, and then were placed again on the elevated platform for 25 min. On PND30, they were first placed on the elevated platform for 25 min and then exposed to synthetic fox odor for 25 min. On PND28 and 29, stress induction procedures were conducted between 13 and 16 h, and on PND30 they were conducted between 9 and 13h. Blood samples were collected via tail-nick on both PND28 and PND30 at 50 and 80 min after the start of stress induction. The rats were returned to their home cage after every blood collection. Only the blood samples collected on PND30 at 50 min after the start of the stress induction were analyzed for selection of breeding rats. For every breeding selection round, 10-15 couples per line were selected based on their plasma corticosterone levels: >100 ng/ml for the Low line, and 100-200 ng/ml for the Control line. Weight was monitored until adulthood to verify similar development of rat lines. Once the animals reached sexual maturity (PND90), males and females within each line were paired for breeding. Breeding was performed strictly within each line. To minimize effects of genetic drift, the breeding scheme strictly excluded sibling mating. The first generation (F1) of breeders was obtained from F16 breeders imported from EPFL, Switzerland (F0). Breeder rats were kept for a maximum of one year and females underwent a maximum of four breeding rounds. Note that only breeder candidates underwent the SAT during puberty; their offspring used for experiments did not undergo any stress procedure before behavioral testing began in adulthood.

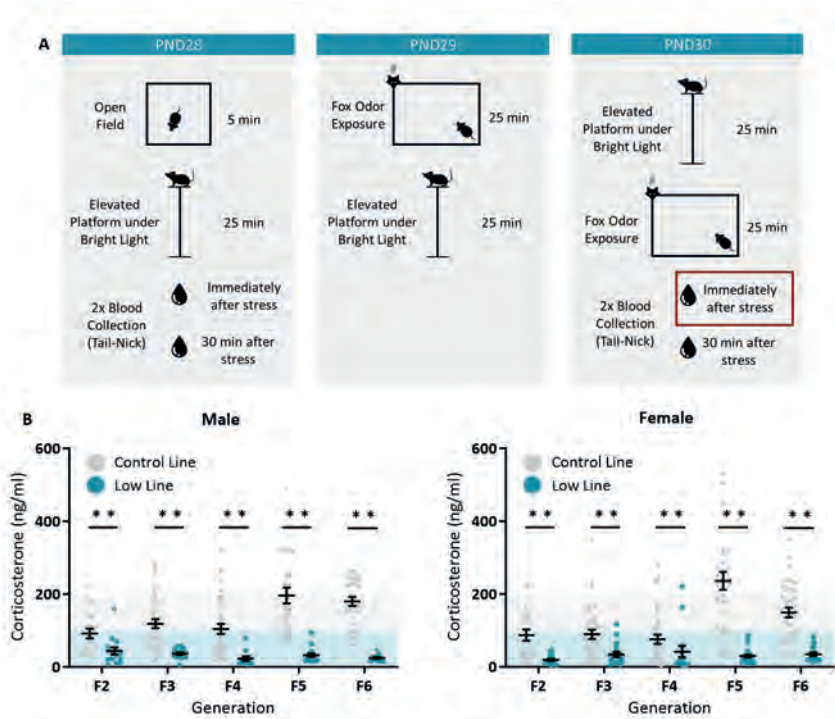


Figure S1 – Post-stressor corticosterone levels in male and female candidate breeding rats across generations.

A. Experimental protocol of the Stress Adaptation Test (SAT), consisting in the unpredictable presentation of different stressors for 3 consecutive days during postnatal day (PND) 28-30. Only corticosterone levels measured 50 min after stress onset on PND30 were used for selection of breeding rats. **B.** Plasma corticosterone levels (mean $\pm$ s.e.m.) obtained 50 min after stress onset on PND30 in male and female candidate breeding rats of each line ($n = 11-36$ rats per sex per generation, $** P < 0.01$). Blue and grey areas denote the corticosterone range for selection for breeding in the Low and Control lines, respectively. Dots represent individual values.

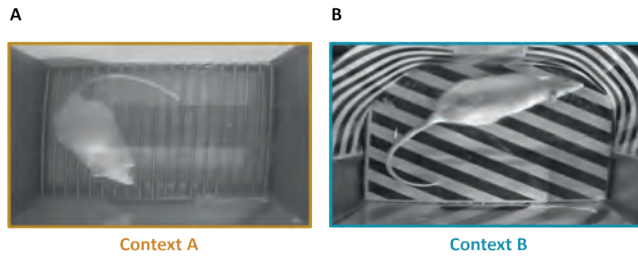


Figure S2 – Contextual alterations of the fear conditioning and extinction task.

For context A, the chamber maintained its original appearance: aluminum and Plexiglas walls and ceiling, with stainless-steel grid floor, and a rectangular context shape. **B.** For context B, the chamber was altered to present black and white stripes on plastic walls and flat floors, and a semi-rounded context shape.

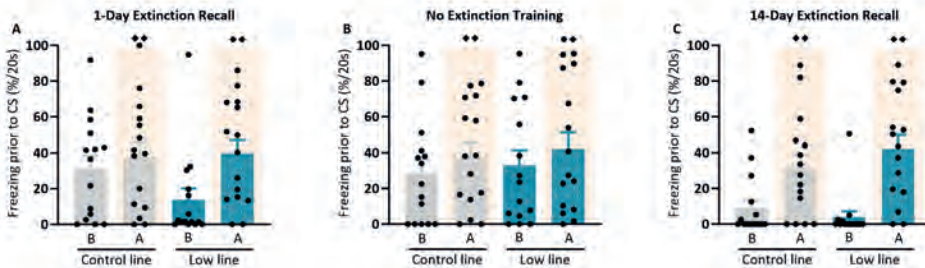


Figure S3 – Strength and generalization of extinction memory at a 1-day and 1-day recall test under non-treated conditions in the Low and Control lines.

Context only freezing scores represent the 20-s segment immediately prior to the first CS presentation. **A.** In line with freezing during CS presentation results, rats of both the Low and Control line showed lower freezing levels (mean $\pm$ s.e.m.) prior to CS presentation in context B compared to context A during the 1-day recall test ($n = 15$ rats per line, $F_{1,54} = 7.81$, $\diamond\diamond P < 0.01$ context B vs A). **B.** No-extinction training controls from both lines presented a reduction in freezing levels (mean $\pm$ s.e.m.) prior to CS presentation in context B compared to context A ($n = 15$ rats per line, $F_{1,54} = 7.81$, $\diamond\diamond P < 0.01$ context B vs A). **C.** In line with freezing during CS presentation results, both rat lines displayed lower freezing levels (mean $\pm$ s.e.m.) prior to CS presentation in context B compared to context A during the 14-day recall test ($n = 15$ rats per line, $F_{1,28} = 31.07$, $\diamond\diamond P < 0.01$ context B vs A). Black dots represent individual values.

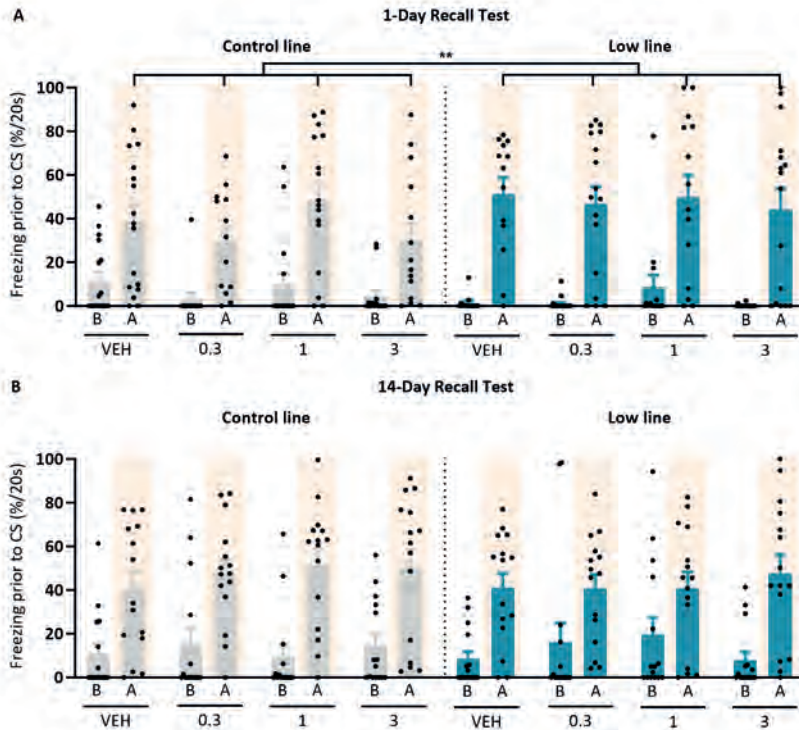


Figure S4 – Strength and generalization of extinction memory following glucocorticoid administration at a 1-day and 14-day recall test.

A. At the 1-day recall test, freezing levels (mean $\pm$ s.e.m.) prior to CS presentation showed no main or interaction effects involving corticosterone treatment (all P s $>$ 0.19). However, a significant rat line $\times$ test context interaction was observed, where the Control line rats presented significantly lower freezing levels compared to Low line rats prior to CS presentation in context A, but not to context B ($n = 12$ -16 rats per line and treatment, $F_{3,105.48} = 7.18$, $** P < 0.01$). **B.** At the 14-day recall test, freezing levels (mean $\pm$ s.e.m.) prior to CS presentation revealed only main but no interaction effects of test context ($F_{1,107.11} = 96.33$, $P < 0.001$). No significant main or interaction effects involving corticosterone treatment (all P s $>$ 0.66) or rat line (all P s $>$ 0.44) were found, indicating comparable freezing levels across lines following corticosterone administration ($n = 13$ -15 rats per line and treatment). Black dots represent individual values.

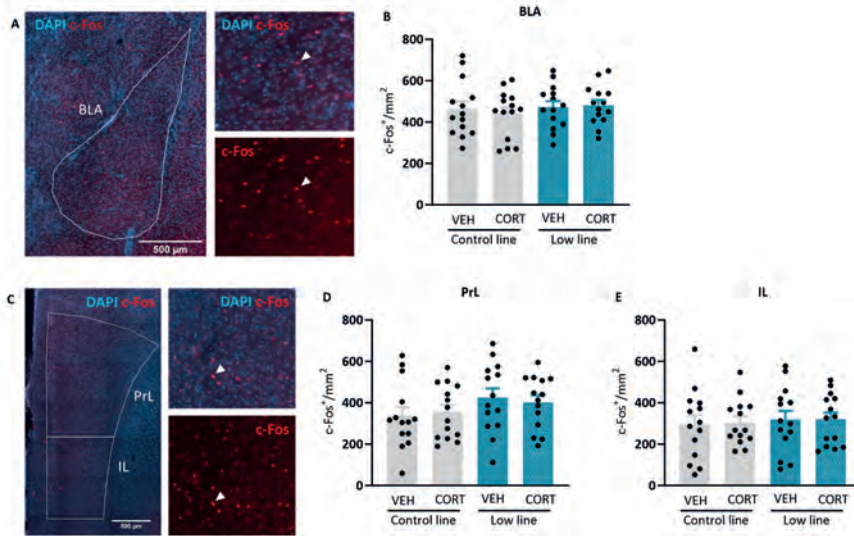


Figure S5 – Effect of post-extinction corticosterone administration on BLA and medial prefrontal cortex activity in Low and Control lines.

A. Representative images of c-Fos- and DAPI-stained cells in the BLA. **B.** The number of c-Fos⁺ cells (mean $\pm$ s.e.m.) in the BLA did not differ between lines or in response to treatment ($n = 14$ rats per line and treatment, all P s ≥ 0.39). **C.** Representative images of c-Fos- and DAPI-stained cells in prefrontal cortex subregions. **D.** Low line presented a non-significant trend towards higher number of c-Fos⁺ cells (mean $\pm$ s.e.m.) in the PrL compared to the Control line ($F_{1,52} = 3.02$, $P = 0.08$), independent of treatment ($n = 14$ rats per line and treatment). **E.** The number of c-Fos⁺ cells (mean $\pm$ s.e.m.) in the IL did not differ between lines or in response to treatment ($n = 14$ rats per line and treatment, all P s ≥ 0.62). Black dots represent individual values.

Table S1 – Overview on the contextual details and parameters of different sessions of the fear conditioning and extinction task

	Fear Conditioning				Extinction Training		Recall Test	
	Day 1		Day 2		Day 3 or 16			
	A	B	A	B	A	B	A	B
Context								
Floor Characteristics	Grid	Stripes, flat	Stripes, flat	Stripes, flat	Grid	Grid	Grid	Grid
Wall Characteristics	Aluminum, Plexiglas	Stripes	Semi-rounded	Semi-rounded	Aluminum, Plexiglas	Aluminum, Plexiglas	Aluminum, Plexiglas	Aluminum, Plexiglas
Context Shape	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular
Chamber Lighting	House light on	Wall light on	House light on	Wall light on	House light on	House light on	House light on	House light on
Room Lighting	Light on (50 lux)	Light off	Light on (50 lux)	Light off	Light on (50 lux)	Light off	Light on (50 lux)	Light off
Transport to Room	Wrapped in a towel	In plastic container	In plastic container	In plastic container	Wrapped in a towel	Wrapped in a towel	Wrapped in a towel	Wrapped in a towel
CS Characteristics		Pure tone, 9 kHz, 80 dB, 20 s		Pure tone, 9 kHz, 80 dB, 20 s		Pure tone, 9 kHz, 80 dB, 20 s		Pure tone, 9 kHz, 80 dB, 20 s
CS Presentations	5x	20x	3x	3x	3x	3x	3x	3x
US Characteristics	5x, 0.7 mA, 1 s	-	-	-	-	-	-	-
ITI	60 s	Variable: 40-120 s	60 s	60 s	60 s	60 s	60 s	60 s
Session Duration	8 min 20 s	32 min	6 min	6 min	6 min	6 min	6 min	6 min
Time of Day	8-13 h	8-13 h	13-16 h	13-16 h	13-16 h	13-16 h	13-16 h	13-16 h

Table S2 – Primer sequences used for qPCR.

Target	Forward Primer	Reverse Primer
<i>Nr3c1</i> (GR)	5'–AAA GCT TCT GGA CTC CAT GC–3'	5'–TCA ATA CTC ATG GTC TTA TCC AAA AA–3'
<i>Nr3c2</i> (MR)	5'–CAA GCT GGC ATG AAC TTA GGA–3'	5'–TCC TCG TGG AGG CCT TTT–3'
<i>Fkbp5</i>	5'–GGG AAC GGT GTA CTT CAA GG–3	5'–TTC TGA CAA GCC GTA CTC CA–3'
<i>Actb</i> (Actin B) Reference Gene 1	5'–TCA CCA ACT GGG ACG ATA TG–3'	5'–GGT ACG ACC AGA GGC ATA CAG–3'
<i>B2-m</i> (β2-Microglobulin) Reference Gene 2	5'–CTC GGT GAC CGT GAT CTT TC–3'	5'–ATT TGA GGT GGG TGG AAC TG–3'
<i>Rpl13a</i> (Ribosomal Protein L13a) Reference Gene 3	5'–GGA TCC CTC CAC CCT ATG ACA–3'	5'–CTG GTA CTT CCA CCC GAC CTC–3'

Table S3 – Pearson correlations between blood glucocorticoid signaling-related markers (log₂ normalized) and circulating corticosterone (log₁₀ ng/ml).

	Control line	Low line
<i>Nr3c1:Nr3c2</i>	$r_{15} = 0.25, P = 0.36$	$r_{14} = -0.14, P = 0.64$
<i>Nr3c1:Fkbp5</i>	$r_{15} = 0.44, P = 0.09$	$r_{14} = 0.48, P = 0.08$
<i>Nr3c2:Fkbp5</i>	$r_{15} = 0.66, P < 0.01$	$r_{14} = 0.32, P = 0.27$
<i>Nr3c1:Corticosterone</i>	$r_{15} = 0.17, P = 0.54$	$r_{14} = 0.14, P = 0.62$
<i>Nr3c2:Corticosterone</i>	$r_{15} = 0.17, P = 0.55$	$r_{14} = -0.41, P = 0.14$
<i>Fkbp5:Corticosterone</i>	$r_{15} = 0.61, P < 0.05$	$r_{14} = 0.00, P = 0.99$



CHAPTER 4

Resting-state functional and structural brain imaging in a rat line with strong hypothalamic-pituitary-adrenal-axis negative feedback regulation

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Manuscript in preparation

ABSTRACT

Post-traumatic stress disorder (PTSD) develops in only a subset of trauma-exposed individuals, suggesting the presence of pre-existing vulnerability factors. Alterations in glucocorticoid signaling as well as default mode network (DMN)-related changes in brain structure and functional connectivity have been suggested as candidate correlates of vulnerability. Here, we tested whether a predisposition toward altered glucocorticoid signaling is associated with enduring structural and functional differences in the DMN and other brain networks in the absence of trauma. Using a combination of resting-state functional MRI with *ex vivo* structural and diffusion tensor imaging MRI, we assessed functional and structural differences in selectively bred rat line (i.e., Low line) characterized by stronger hypothalamic-pituitary-adrenal (HPA)-axis negative feedback regulation and lower post-stressor corticosterone levels compared to a Control line.

Both lines displayed intact DMN-like activity, with no differences in connectivity within the DMN, or DMN segregation from the rest of the brain, but the Low line displayed a smaller volume of the temporal association cortex (TeA), a subregion of the DMN. Consistent with glucocorticoid modulation of the DMN, Control line rats displayed a positive correlation between DMN segregation and circulating corticosterone levels. In contrast, this relationship was absent in Low line, suggesting a dissociation between DMN engagement and glucocorticoid signaling. Whole-brain analyses revealed enhanced functional connectivity between cortical and hippocampal regions, and retrosplenial and temporal regions in the Low line relative to the Control line, alongside reduced connectivity between insular and midbrain regions. Structurally, the Low line showed smaller volume in subregions of the hippocampus and temporal cortex, but larger volumes in the bed nucleus of the stria terminalis (BNST) and fimbria of the hippocampus. No evidence of differences in white matter microstructural integrity between lines was found.

These findings indicate that a predisposition toward stronger HPA-axis negative feedback and lower post-stressor glucocorticoid levels is associated with alterations in functional connectivity and regional brain volumes even in the absence of stress exposure, suggesting that altered glucocorticoid signaling may represent a pre-existing vulnerability factor for PTSD.

Keywords: glucocorticoids, HPA-axis regulation, default-mode network, MRI, DTI

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating condition that develops in a subset of individuals following trauma exposure, leading to persistent impairments in emotional regulation and mnemonic processing. Critically, it remains unclear why only 25–35% of trauma-exposed individuals develop PTSD (Ressler et al., 2022; Shalev et al., 2017). Addressing this issue requires identifying pre-existing vulnerability factors and clarifying the mechanisms through which they may increase risk.

PTSD patients have consistently been shown to exhibit systematic neuroendocrine and neural alterations (Pitman et al., 2012). First, significant variations in glucocorticoid levels and hypothalamic–pituitary–adrenal (HPA) axis negative feedback regulation have been widely reported (Morris et al., 2012; Schumacher et al., 2019; Yehuda, 2009; Yehuda, Hoge, et al., 2015), although findings show substantial heterogeneity across studies (Zoladz & Diamond, 2013). Most prominently, a subset of PTSD patients exhibits blunted glucocorticoid responses and stronger HPA-axis negative feedback regulation (Meewisse et al., 2007; Yehuda, 2009; Yehuda, Hoge, et al., 2015). Interindividual differences in glucocorticoid signaling are increasingly considered a candidate vulnerability factor for PTSD (Galatzer-Levy & Bryant, 2013; Szeszko et al., 2018). For example, the BclI polymorphism of the glucocorticoid receptor gene, which is linked to lower glucocorticoid levels, is associated with greater PTSD symptom severity (Hauer et al., 2011).

Second, PTSD has been associated with widespread alterations in brain structure and functional connectivity, both locally and across large-scale networks (Akiki et al., 2017; Sripada et al., 2012; van Oort et al., 2020; Xiao et al., 2022). Neuroimaging studies have identified distinctive patterns of functional connectivity within the default mode network (DMN) in PTSD, which have been associated with re-experiencing and negative self-processing symptoms (Dégeilh et al., 2017; Lanius et al., 2020). These alterations include reduced connectivity between DMN nodes, reflecting lower network coherence (Akiki et al., 2017; Doucet et al., 2020; Miller, Hayes, et al., 2017; Y. Zhang et al., 2015), and, to a lesser extent, greater network segregation, indicating increased functional isolation of the DMN from the rest of the brain (Akiki et al., 2018; Lei et al., 2015; Sartin-Tarm et al., 2018). Importantly, DMN connectivity correlates with and predicts overall PTSD symptom severity (Harnett et al., 2021; Lanius et al., 2010).

Beyond the DMN, functional and structural alterations in regions central to emotion regulation and mnemonic processing have been reported in PTSD. For example, reduced hippocampal volume has been the most consistent finding, but widespread changes in regions such as the retrosplenial and temporal cortices have also been reported (Karl et al., 2006; Kribakaran et al., 2020; Lambert & McLaughlin, 2019; O'Doherty et al., 2015; Sherin & Nemeroff, 2011; Wang et al., 2021). In addition, white matter tracts such as the cingulum bundle and corpus callosum have been shown to present lower fractional anisotropy (FA) in PTSD patients, indicative of microstructural changes (Daniels et al., 2013; Dennis et al., 2021; Ju et al., 2020; Siehl et al., 2018; Weis et al., 2018). Although both DMN-specific and widespread changes have been

associated with the trauma experience itself (DiGangi et al., 2016), it remains unclear whether they may represent pre-existing differences related to interindividual differences in glucocorticoid signaling.

Glucocorticoids exert acute, chronic, and programming effects on brain structure and function (Lupien et al., 2009; McEwen, 2007; Sapolsky, 2003). Under basal conditions, glucocorticoid actions are primarily mediated by mineralocorticoid receptors (MRs), which are highly expressed in limbic regions, whereas under stress they are predominantly mediated by glucocorticoid receptors (GRs), which are more widely distributed and particularly dense in the hippocampus and neocortex (Koning et al., 2019; Matsusue et al., 2014; Reul & de Kloet, 1985, 1986). Through these receptors, glucocorticoids modulate local dendritic branching, spine density, neurogenesis, and myelination (Fuchs & Gould, 2000; Liston & Gan, 2011; Miguel-Hidalgo, 2023). Further, it has been shown that stress and glucocorticoids modulate the engagement of large-scale brain networks, such as suppression of the DMN (Hermans et al., 2014; W. Zhang et al., 2019; Y. Zhang et al., 2020, 2021). Therefore, enduring differences in glucocorticoid signaling—particularly during neural development—may scale from local cellular effects to long-lasting brain-wide patterns in functional connectivity, volumetry, and white matter microstructure (Hall et al., 2015; Lupien et al., 2009; Wang et al., 2019; Zeev-Wolf et al., 2019). This interpretation is supported by evidence from patients which present persistent pathological alterations in HPA-axis function (Long et al., 2024; Papakokkinou & Ragnarsson, 2023).

Here, we investigate whether a predisposition for stronger HPA-axis negative feedback regulation contributes to differences in the DMN as well as widespread differences in brain structure and functional connectivity, under basal (i.e., non-stressed, non-treated) conditions. To this end, we used two rat lines selectively bred based on their constitutive glucocorticoid levels (Walker et al., 2017): a Low line characterized by blunted post-stressor corticosterone levels and stronger HPA-axis negative feedback regulation compared to a Control line (Chapter 3). These rat lines enable the study of the effects of HPA-axis regulation on neurocircuitry without the confounding effects of trauma, environmental stress and high genetic heterogeneity present in human cohorts. Initial evidence from a previous study indicated that the Low line exhibits reduced hippocampal volume (Monari et al., 2024). In the present study, we provide a more comprehensive characterization of functional connectivity and structural differences between the two lines, with an *a priori* confirmatory analysis focused on the DMN as well as an exploratory whole-brain analysis. To this end, we employed a multimodal magnetic resonance imaging (MRI) approach, combining resting-state functional MRI under light sedation to assess functional connectivity with *ex vivo* structural and diffusion tensor imaging (DTI) MRI to evaluate regional brain volumes and white matter microstructure.

MATERIALS & METHODS

1. Subjects

Twenty-four adult male rats (280–420 g) were used, comprising 12 rats from the Low line and 12 rats from the Control line. These two selectively bred lines are defined by their constitutive post-stressor corticosterone levels during the recovery phase and were originally developed in the laboratory of Dr. Carmen Sandi (École Polytechnique Fédérale de Lausanne (EPFL), Switzerland) from commercial Wistar Han rats (Walker et al., 2017). Experimental rats were bred in-house from F16 breeder rats imported from EPFL. Breeding candidates were selected based on the following criteria: <100 ng/ml post-stressor corticosterone levels in the Low line, and between 100 and 200 ng/ml post-stressor corticosterone levels in the Control line. In Chapter 3, we further found that the Low line exhibits stronger HPA-axis negative feedback regulation compared to the Control line. See Chapter 3 for a detailed description of the breeding selection procedure. Rats were maintained on a standard 12-h/12-h light/dark cycle (lights on: 6:30 - 18:30 h) in a temperature ($21 \pm 1^\circ\text{C}$) and humidity-controlled ($55 \pm 5\%$) colony room, with *ad libitum* access to food and water. Rats were group housed until one week prior to the fMRI scans, where they changed to individual housing in order to minimize potential testing order and social hierarchy effects (Bahtiyar et al., 2025). All procedures were performed during the light phase between 9:00 and 15:00 h, at the nadir of the diurnal cycle of corticosterone. Testing order was block-randomized and maintained during the whole experimental protocol. Researchers were blinded to the rat line during all experimental procedures and subsequent image analysis. All procedures were in compliance with European Union Directive 2010/63/EU and were approved by the Central Authority for Scientific Procedures on Animals (CCD), The Hague, The Netherlands.

2. *In vivo* Resting-State Functional Magnetic Resonance Imaging (rs-fMRI)

Rats were habituated to handling for 2 min each on 3 consecutive days. fMRI was conducted under a standardized light sedation protocol to maintain a resting state and ensure immobility while scanning (Grandjean et al., 2023). Anesthesia was induced with 4% isoflurane (1:2 oxygen:air ratio) and, after gradual decrease, maintained with 0.4% isoflurane together with a subcutaneous bolus of dexmedetomidine (0.025 mg/kg, Zoetis), an α_2 -adrenergic receptor agonist. Fifteen minutes after the bolus, a slow and steady injection of dexmedetomidine was started (0.05 mg/kg/h) via an injection line placed in the flank. Body temperature was kept at $37 \pm 1^\circ\text{C}$ using a heating pad, and breathing was monitored continuously.

Functional scans were made in a 7-T ClinScan MRI system (Bruker Biospin MRI GmbH) with a 83 mm transmit volume coil and 2x2 phased array rat head receiver coil, following a standardized protocol (Vrooman et al., 2025). First, an anatomical scan was ran using a turbo spin-echo sequence with parameters: Repetition Time (TR)/Echo Time (TE) = 2650/25 ms, field of view (FOV) = 26x26 mm, matrix size = 128x128, flip angles = 90, 180 and 18 slices. After the anatomical scan, shimming was performed using MAPSHIM following the B0 maps acquisition. Next, task-free functional scans

were acquired with a single-shot gradient echo sequence using echo planar imaging readouts (GE-EPI). To ensure the rat was in a steady sedative state, the scanning sequence used for data analysis started 40 min after the bolus injection, with the following parameters: TR/TE = 1000/17 ms, FOV = 26x26 mm, matrix size = 64x64, 18 slices and 400 repetitions plus 10 dummy scans (Vrooman et al., 2025).

3. *Ex vivo* Structural Magnetic Resonance Imaging

For volumetry and DTI analysis, scans were performed *ex vivo* with fixated brains perfused with the contrast agent gadolinium (Guerbet), to improve image contrast. As soon as the fMRI scans were finished, the rat was quickly moved to an inhalation mask flowing with 5% isoflurane in air, to increase the depth of anesthesia. Then, the rats were transcardially perfused with phosphate-buffered saline (PBS) with heparin (400 U/l, Sigma-Aldrich) and gadolinium (2 mM), followed by 4% formaldehyde with 2 mM gadolinium in PBS (pH = 7.4). The brain/head was extracted and kept in 4% formaldehyde with 2 mM gadolinium in PBS overnight at 4 °C, then in PBS with 2 mM gadolinium at 4 °C for a week until scanning.

The structural scans were performed in a 11.7-T MRI system (Bruker Biospin MRI GmbH) with a 23 mm inner diameter transmit/receiver volume coil, running Paravision 6.0.1. The brains were wrapped in gauze to prevent movement, and submersed in Galden Perfluorinated Fluid (1937SN, Solvay) in a closed tube, carefully avoiding air bubbles to minimize air and water distortion. First, a high-resolution anatomical scan was acquired with the following parameters: TR/TE = 2500/16 ms, matrix size = 240x240x240 and a resolution of 0.1x0.1x0.1 mm³. Then, scans for DTI were acquired by a single-shot spin-echo sequence with the following parameters: TR/TE = 2000/17.5 ms, flip angle = 90, matrix size = 200x160, resolution = 0.12x0.15 mm², number of slices = 28, slice thickness = 0.75 mm, b-values = 500, 900 mm²/ms, number of direction encoding per b-value = 36, and number of b0 volumes = 5.

4. *In vivo* Image Processing and Parameter Estimation

Raw anatomical and functional scans were converted to the Brain Imaging Data Structure, using BIDScoin 3.7.3 (Zwiers et al., 2022). The scans were processed with RABIES 0.4.7 (Desrosiers-Grégoire et al., 2024) using default parameters. Briefly, RABIES applies inhomogeneity correction, brain masking, motion correction, functional to anatomical registration, and registration to the SIGMA rat brain template (version 1.1) (Barrière et al., 2019). Following pre-processing quality control, functional scans were corrected for confounds in RABIES, including temporal filtering with a 0.01 - 0.1 Hz bandwidth, spatial filtering with a 0.5 mm² smoothing kernel, and denoising with white-matter and cerebrospinal fluid signal regression. Cleaned time series were extracted using a resampled SIGMA atlas and were used to estimate functional connectivity. Using RABIES, seed time courses were extracted for each parcel, and functional connectivity was computed as the Pearson correlation coefficient (*r*) between all region pairs.

5. *Ex vivo* Image Processing and Parameter Estimation

Raw structural and diffusion-weighted scans were converted to the Brain Imaging Data Structure using BrkRaw 0.3.5 (Lee et al., 2022). Due to poor image quality, two scans from each line were excluded from the volumetric analysis. For the DTI analysis, three Control line scans and one Low line scan were excluded. Structural scans were processed using ANTs 2.4.0, including correction for B1 field inhomogeneity, and registration to the SIGMA template (version 1.1). The jacobians were estimated from the transformation maps, extracted using a resampled SIGMA atlas and used to compute volumes (mm³), without total brain volume correction. Diffusion-weighted scans were processed using FSL 6.0.0, including correction for eddy current-induced distortions followed by tensor estimation. Fractional anisotropy (FA) values were extracted from selected regions manually drawn in FSL software following the Paxinos & Watson Rat Brain Atlas (Paxinos & Watson, 2006) (Figure 5A).

6. Region-of-interest (ROI) analysis

6.1. Resting-state functional connectivity analysis

To assess whether the two rat lines exhibited differences in functional connectivity, we performed both a targeted analysis of the DMN and an exploratory whole-brain analysis to detect effects beyond this *a priori* defined network.

DMN analysis

For the ROI-based analysis of the DMN, 12 regions (Table S1 and Figure 1C) were selected based on the anatomical structures previously identified as components of the rat DMN, which exhibits homology with the human DMN (Hsu et al., 2016; Lu et al., 2012; Tsai et al., 2020). ROIs were selected to cover five major nodes of the rodent DMN, namely the prelimbic system (including orbital cortex; PrL), cingulate cortex (Cg), retrosplenial cortex (RSC), posterior parietal cortex (PPC) and auditory/temporal association cortices (Au/TeA) by comparing identified components mapped in the Paxinos & Watson Rat Brain Atlas (Lu et al., 2012) to ROI coverage in the SIGMA atlas (Barrière et al., 2019). ROIs were analyzed separately by hemisphere, resulting in a total of 24 ROIs.

For every subject, connectivity within the DMN (i.e., DMN–DMN connectivity) was computed as the average Fisher's z-transformed correlation coefficient between all ROIs defined as part of the DMN, whereas connectivity of DMN nodes with the rest of the brain (i.e., DMN–Other connectivity) was computed as the average Fisher's z-transformed correlation coefficient between DMN ROIs and all non-DMN ROIs (Chan et al., 2014). Computations included intra- and cross-hemisphere connections. These metrics enabled verification that DMN-like activity could be detected (i.e., stronger DMN–DMN than DMN–Other connectivity) in both rat lines, and allowed assessment of differences in DMN coherence and connectivity with regions outside the network. A third metric, the DMN segregation index, was calculated for each subject to integrate these measures into a single index reflecting the relative balance between within-

network connectivity and connectivity with the rest of the brain, thereby quantifying the extent to which the DMN operates as a functionally distinct network. The network segregation index was mathematically defined as: $(\text{DMN-DMN connectivity} - \text{DMN-Other connectivity}) / \text{DMN-DMN connectivity}$; where values close to 1 indicate high network segregation (i.e., greater functional isolation from the rest of the brain), and values close to 0 indicate low network segregation (i.e., greater functional integration with the rest of the brain) (Chan et al., 2014; Pierce et al., 2024; Schulz et al., 2024; Tays et al., 2025).

Whole-brain analysis

We performed a whole-brain analysis on functional connectivity matrices containing all 79 grey matter regions from the SIGMA atlas (i.e., excluding regions classified as white matter tracts and cerebrospinal fluid) (Barrière et al., 2019). This resulted in a total of 12,403 unique ROI pairs, as left and right hemispheres were considered separately.

Given the large number of comparisons (12,403 unique ROI pairs) and limited sample size ($n = 12$ per line), a multiplicity-adjusted null hypothesis significance testing framework for whole-brain analysis would be underpowered, reflecting the strong dependence of P -values on both sample size and the number of tests. This limitation is well recognized in neuroimaging, where stringent correction procedures can obscure meaningful variation in effect magnitude and inflate *post hoc* effect estimates (Poldrack et al., 2008; Reddan et al., 2017). In line with the exploratory nature of the analysis, recommendations to emphasize estimation over dichotomous inference (Lakens, 2013) and the recognition that effect sizes enable assessment of clinical and practical relevance (Hojat & Xu, 2004; Schober et al., 2018), we therefore report effect size estimates and their associated uncertainty.

For every ROI pair, Hedges' g and corresponding 95% confidence intervals (CIs) were computed for the mean difference between rat lines to allow assessment of the magnitude, direction and precision of the observed effects (see section 8. Statistical analysis for more details). All functional connectivity effect sizes and 95% CIs are reported in Supplementary Table S2.

6.2. Volumetry analysis

Paralleling the functional connectivity analysis, we performed both a targeted analysis of DMN regions and an exploratory whole-brain analysis of grey and white matter structures to assess volume differences between the two rat lines.

DMN analysis

For the DMN volumetry analysis, the same 12 grey matter regions were selected (Table S1). In addition, the SIGMA ROI named "corpus callosum and associated subcortical white matter" (SIGMA #: 891, 892) was selected for analysis of DMN-associated white matter, based on previous reports identifying the cingulum and corpus callosum as

major fiber tracts connecting DMN nodes (Hsu et al., 2016). Left and right hemisphere volume measurements of each ROI were summed per animal for total subregion volume.

Whole-brain analysis

We performed a whole-brain analysis on volumes of all 79 grey and 37 white matter regions from the SIGMA atlas template (i.e., excluding regions classified as cerebrospinal fluid) (Barrière et al., 2019). This resulted in a total of 158 grey and 74 white matter ROIs, as left and right hemispheres were considered separately. Similar to the functional connectivity analysis, Hedges' g and corresponding 95% CIs were computed for the mean difference between rat lines for every ROI (see section 8. Statistical analysis for more details). All functional connectivity effect sizes and 95% CIs are reported in Supplementary Table S3. For visualization of consistent effects, left and right hemisphere volume measurements of each ROI were summed per animal for total subregion volume.

6.3.DTI analysis

For the DTI analysis, 9 ROIs corresponding to major white matter tracts spanning the entire brain were selected (i.e., no hemispheric differentiation; Table S4). The previously selected SIGMA ROI named "corpus callosum and associated subcortical white matter" was subdivided into 6 ROIs manually delineated based on the Paxinos & Watson Rat Brain Atlas (Paxinos & Watson, 2006): forceps minor (fmi), cingulum (cg), corpus callosum (cc), external capsule (ec), septofimbrial nucleus (sf) and dorsal hippocampal commissure (dhc) (Figure 5). This subdivision enabled a more fine-grained characterization of the distinct fiber tracts that connect cortical and subcortical regions, including interhemispheric, limbic, and hippocampus-associated pathways. We additionally analyzed the anterior part of the anterior commissure (aca), an interhemispheric pathway connecting anterior cortical and limbic regions (Fenlon et al., 2021; Horel & Stelzner, 1981), the fimbria of the hippocampus (fi), a major pathway containing hippocampal efferent projections to subcortical and limbic regions (Meibach & Siegel, 1977a) and the internal capsule (ic), another major pathway connecting cortical and subcortical regions (Coizet et al., 2017; Meibach & Siegel, 1977b) (Figure 5). Additional ROIs were not defined, as manual delineation of regions constrained the level of anatomical granularity that could be achieved while maintaining reliable identification of distinct fiber tracts.

7. Corticosterone assay

Trunk blood for measurement of corticosterone levels was collected immediately after completion of the functional scan. Blood samples were collected in ice-cold lithium heparin-coated tubes (Greiner Bio One), and kept on ice until centrifugation (10,000 rpm for 4 min at 4 °C). Then, plasma was isolated into separate tubes and stored at -20 °C until analysis. Corticosterone ELISA immunoassay (sensitivity: 27.0 pg/ml, ADI-900-097, Enzo Life Sciences) was performed according to the manual. Samples were diluted

1:40 in Assay Buffer without the addition of steroid displacement reagent solution. All samples were measured in duplicate. Corticosterone levels were calculated using linear fitting and logarithmic plotting software (Enzo Life Sciences) for standard curve and data extrapolation.

8. Statistical analysis

To approximate normal distributions, corticosterone levels were logarithmically transformed and correlation coefficients were Fisher's z -transformed. Normality was verified by visually inspecting the Q-Q plots, and/or by statistical tests (D'Agostino-Pearson and Shapiro-Wilk).

For comparisons of two groups (e.g., corticosterone measurements, segregation index), two-tailed independent sample t -tests were used. DMN connectivity was analyzed using a mixed-model two-way ANOVA, with rat line as between-subject factor, and connectivity as within-subject factor. Factor effect sizes are reported as partial eta squared (η^2_p). For mass univariate testing of functional connectivity, volumetry and fractional anisotropy, two-tailed independent sample t -tests were performed with Welch's correction applied by default to account for any violations of equality of variances. For all analyses, P -values were corrected for multiple comparisons using the Benjamini–Hochberg False Discovery Rate (FDR) method.

Pearson correlation coefficients between corticosterone levels, segregation index, and DMN connectivity measures were computed separately for each rat line. Linearity was assessed by Run's test, and bivariate normality was verified by visual inspection of scatterplots and the Shapiro-Wilk test. Correlation coefficients were tested for significance using Pearson's correlation test.

Effect sizes of mean differences between the two rat lines were computed using Hedges' g to account for small sample bias. Effect sizes whose 95% CIs did not cross zero were prioritized for in-text reporting and discussion, as these provide clearer evidence regarding the direction of group differences, with particular attention to consistency of effects across hemispheres. All effect sizes and their 95% CIs are reported in Supplementary Tables S2, S3 and S5. Gardner-Altman estimation plots are used to illustrate group means, mean differences between rat lines, effect sizes, 95% CIs and effect size sampling distributions derived via bootstrap resampling (Ho et al., 2019).

Mass univariate testing of functional connectivity, correlation and effect size analyses were run in Python (3.12.0). All other analyses were conducted in GraphPad Prism (Dotmatics, version 10.1.2.). The significance threshold was set at $P < 0.05$.

RESULTS

Functional connectivity analysis

To assess whether the two rat lines differed in resting-state functional connectivity, rats from both the Low and Control lines underwent functional MRI under light

sedation (Figure 1A). Immediately after the scan, trunk blood was collected to measure circulating levels of plasma corticosterone. An independent sample *t*-test indicated that plasma corticosterone levels did not differ between the two lines at this time point ($t_{22} = 0.57$, $P = 0.57$, Figure 1B), consistent with previously obtained basal (i.e., non-disturbed) morning measurements in these lines (Chapter 3).

DMN analysis

Given the involvement of the DMN in internally directed cognition (Mason et al., 2007) and its reported alterations in PTSD (Akiki et al., 2017), we assessed whether the two rat lines differed in DMN functional connectivity. Twelve bilateral ROIs were selected to cover five major nodes of the rodent DMN, namely the prelimbic system (including orbital cortex; PrL), cingulate cortex (Cg), retrosplenial cortex (RSC), posterior parietal cortex (PPC) and auditory/temporal association cortices (Au/TeA) (Gozzi & Schwarz, 2016; Lu et al., 2012) (Figure 1C, Table S1).

Considering previous reports of reduced mean connectivity within the DMN in PTSD (Doucet et al., 2020; Miller, Hayes, et al., 2017), we first examined whether the Low and Control lines differed in summary measures of network activity, specifically interactions between all DMN nodes (i.e., DMN-DMN connectivity, Figure S1A), and interactions between all DMN nodes and the rest of the brain (i.e., DMN-Other connectivity). These two measurements constituted the within-subject factor “connectivity”. A mixed-model ANOVA revealed a significant main effect of connectivity ($F_{1,22} = 119.10$, $P < 0.01$, $\eta^2_p = 0.84$), showing greater DMN-DMN connectivity than DMN-Other connectivity in both lines (Low line: $t_{22} = 8.85$, $P < 0.01$, $g = -2.30$, 95% CI = [-3.35, -1.25]; Control line: $t_{22} = 8.85$, $P < 0.01$, $g = -2.74$, 95% CI = [-3.88, -1.6]; Figure 1D), consistent with the presence of coherent DMN-like activity. In contrast, the main effect of rat line ($F_{1,22} = 1.70$, $P = 0.21$, $\eta^2_p = 0.14$) or rat line $\times$ connectivity interaction ($F_{1,22} = 2.56$, $P = 0.12$, $\eta^2_p = 0.10$) was not significant. Importantly, there was no significant difference in average whole brain connectivity between lines ($t_{22} = 0.15$, $P = 0.88$, Figure S1A). Together, these results indicate that DMN coherence and interaction with the rest of the brain is not different between the Low and Control lines.

Next, as previously studies reported an increased DMN segregation in PTSD (Akiki et al., 2018; Lei et al., 2015; Sartin-Tarm et al., 2018), we assessed DMN segregation in both lines, computed from DMN-DMN and DMN-Other connectivity measures. An independent sample *t*-test showed that DMN segregation did not differ between the Low and Control line ($t_{22} = 1.39$, $P = 0.18$, $g = 0.55$, 95% CI = [-0.27, 1.37], Figure 1E), indicating similar functional isolation of the DMN between lines.

As stress and glucocorticoid levels have been linked to modulation of DMN connectivity (van Oort et al., 2017; W. Zhang et al., 2019), we determined whether the DMN segregation index was related to individual circulating corticosterone levels within each line, by computing Pearson correlation coefficients between these factors (Figure 1F). The DMN segregation index was negatively correlated with circulating corticosterone levels in the Control line ($r_{12} = -0.62$, 95% CI = [-0.88, -0.07], $P < 0.05$),

consistent with reports of reduced DMN engagement as glucocorticoid levels rise (Y. Zhang et al., 2020). In contrast, there was no significant correlation between the DMN segregation index and circulating corticosterone levels in the Low line ($r_{12} = -0.08$, 95% CI = [-0.62, 0.52], $P = 0.81$), suggesting a dissociation between network engagement and glucocorticoids.

Other network-level summary measures did not significantly correlate with circulating corticosterone levels in either line, namely DMN-DMN connectivity (Low line: $r_{12} = -0.12$, 95% CI = [-0.65, 0.49], $P = 0.72$; Control line: $r_{12} = 0.04$, 95% CI = [-0.55, 0.60], $P = 0.90$, Figure S1B) and DMN-Other connectivity (Low line: $r_{12} = 0.13$, 95% CI = [-0.48, 0.66], $P = 0.69$; Control line: $r_{12} = 0.42$, 95% CI = [-0.20, 0.80], $P = 0.17$, Figure S1C).

Finally, to assess local differences in DMN-DMN connections between the two rat lines (Figure S1D), a FDR-corrected mass univariate analysis was performed across all 276 unique ROI pairs classified as DMN-DMN connectivity. No P -value survived correction ($t_{22} \leq 3.62$, all $q_s > 0.05$, Figure 1G, all Hedges' g s and 95% CIs in Table S2), indicating that DMN connectivity patterns at the level of individual ROI-to-ROI interactions were comparable between lines. Of note, among all DMN-DMN ROI pairs, 95% CIs excluding zero were observed only for connections involving the Au/TeA node of the DMN (g range = [0.88, 1.17]).

Together, these results do not support resting-state differences in DMN engagement or connectivity between lines under basal conditions. However, they point to impaired glucocorticoid modulation of DMN engagement in the Low line.

Whole-brain analysis

Next, we explored differences in functional connectivity between the two rat lines at the whole-brain level in a hypothesis-free manner. To this end, we report effect sizes (Hedges' g) for mean difference in functional connectivity between rat lines per ROI pair to emphasize the magnitude and direction of group differences, together with their 95% CIs to characterize the precision of observed effects. Effect sizes were plotted as a function of their precision, illustrating both the magnitude and uncertainty of estimates across ROI pairs (Figure 2A). All ROI pair effect sizes and their corresponding 95% CIs are listed in Table S2. Here, we highlight selected ROIs with large effect sizes and note where such effects were observed in the corresponding intra- and inter-hemispheric connections.

Among connections showing greater functional connectivity in the Low line relative to the Control line (i.e., Low > Control), two connections exhibited particularly large effect sizes. Specifically, the Low line exhibited greater functional connectivity between the right lateral entorhinal cortex external part (LEntE_R) and right subiculum (S_R) ($g = 2.14$, 95% CI = [1.12, 3.16], Figure 2B). In the left hemisphere, the homologous connection (LEntE_L-S_L) displayed a similarly directed large effect ($g = 0.82$, 95% CI = [-0.01, 1.56], Figure S2A). In contrast, interhemispheric connections showed small effect sizes, including one in the opposite direction (i.e., Low line < Control line; LEntE_R-S_L: $g = -0.38$, 95% CI = [-1.19, 0.40]; LEntE_L-S_R: $g = 0.10$, 95% CI = [-0.74, 0.90],

Figures S2B-C).

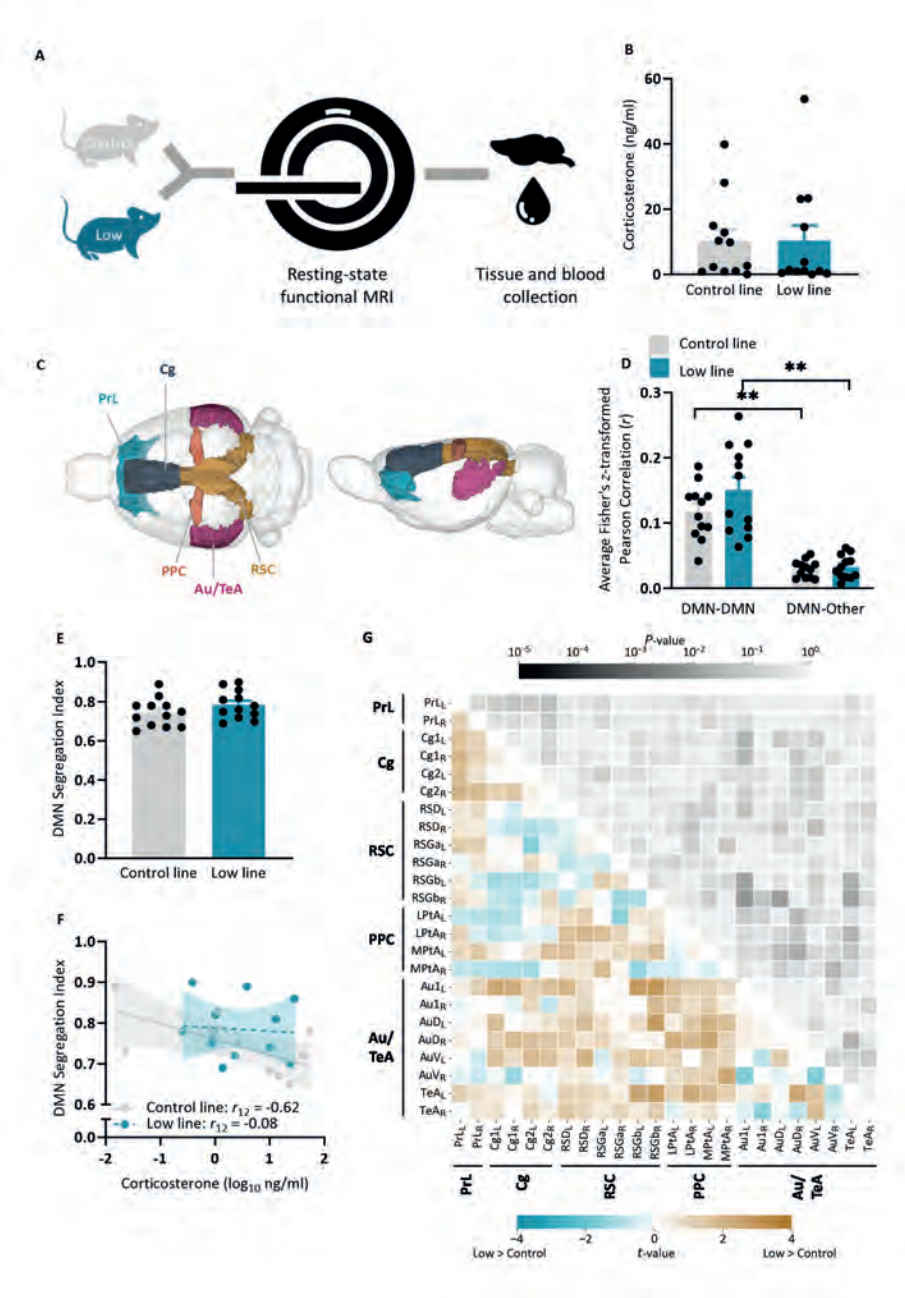


Figure 1 – DMN functional connectivity analysis.

A. Experimental protocol for multimodal MRI. Rats from both lines underwent resting-state functional MRI under light sedation. Immediately after the scan, trunk blood was collected for corticosterone measurements, and rats were perfused for extraction of

fixated heads used in subsequent structural MRI analyses. **B.** Plasma corticosterone levels (mean $\pm$ s.e.m.) of the two rat lines immediately after the functional scan were not significantly different ($n = 12$ rats per line). **C.** Axial and sagittal views of the anatomical definition of the DMN, comprising bilateral ROIs across five major nodes: the prelimbic system (including orbital cortex; PrL), cingulate cortex (Cg), retrosplenial cortex (RSC), posterior parietal cortex (PPC) and auditory/temporal association cortex (Au/TeA). See Table S1 for a complete list of DMN ROIs. **D.** DMN-DMN connectivity (mean $\pm$ s.e.m.) was significantly greater than DMN-Other connectivity in both rat lines, but neither measure significantly differed between groups ($n = 12$ rats per line, $** P < 0.01$). **E.** DMN segregation (mean $\pm$ s.e.m.) did not differ between the Low and Control lines ($n = 12$ rats per line). **F.** DMN segregation presents significant negative correlation with circulating (log-transformed) corticosterone levels in the Control line ($P < 0.05$), but not in the Low line ($P = 0.81$). Dashed regression line represents a non-significant correlation, and a solid line represents a significant correlation. **G.** Matrix of DMN-DMN functional connectivity differences between the two rat lines with t -values in the lower left triangle and corresponding uncorrected P -values in the upper right triangle. No P -values survived FDR correction ($n = 12$ rats per line, $3.62 \geq t_{22} \geq -3.00$, all $qs > 0.05$). Dots represent individual values. The brain illustrations were generated using BrainNet Viewer (Xia et al., 2013).

Similarly, the Low line exhibited greater functional connectivity between the right ectorhinal cortex (Ect_R) and right retrosplenial granular cortex part A ($RSGa_R$) relative to the Control line ($g = 2.21$, 95% CI = [1.18, 3.25], Figure 2C). The homologous connection ($Ect_L - RSGa_L$) and interhemispheric connections ($Ect_R - RSGa_L$ and $Ect_L - RSGa_R$) also displayed the same direction of effect ($Ect_L - RSGa_L$: $g = 0.47$, 95% CI = [-0.41, 1.32]; $Ect_R - RSGa_L$: $g = 1.12$, 95% CI = [0.29, 1.93]; $Ect_L - RSGa_R$: $g = 0.17$, 95% CI = [-0.67, 1.00]; Figure S2D-F).

In contrast, the Low line exhibited weaker connectivity between the left substantia nigra (SN_L) and right dysgranular insular cortex (DI_R) relative to the Control line ($g = -1.73$, 95% CI = [-2.68, -0.78], Low < Control, Figure 2D). The same direction of effect was observed for the left intra-hemispheric connection ($SN_L - DI_L$: $g = -0.83$, 95% CI = [-1.62, 0.02], Figure S2G), whereas the remaining connections exhibited effect sizes equal to or near 0 ($SN_R - DI_L$: $g = 0.02$, 95% CI = [-0.77, 0.92]; $SN_R - DI_R$: $g = 0.00$, 95% CI = [-0.84, 0.81], Figure S2H-I).

Volumetry analysis

To assess whether there were volumetric differences between the two rat lines, *ex vivo* high resolution anatomical scans were made and subregional brain volumes were extracted. Paralleling the functional connectivity analysis, first we assessed volume differences between the two rat lines in DMN regions, followed by a whole-brain analysis.

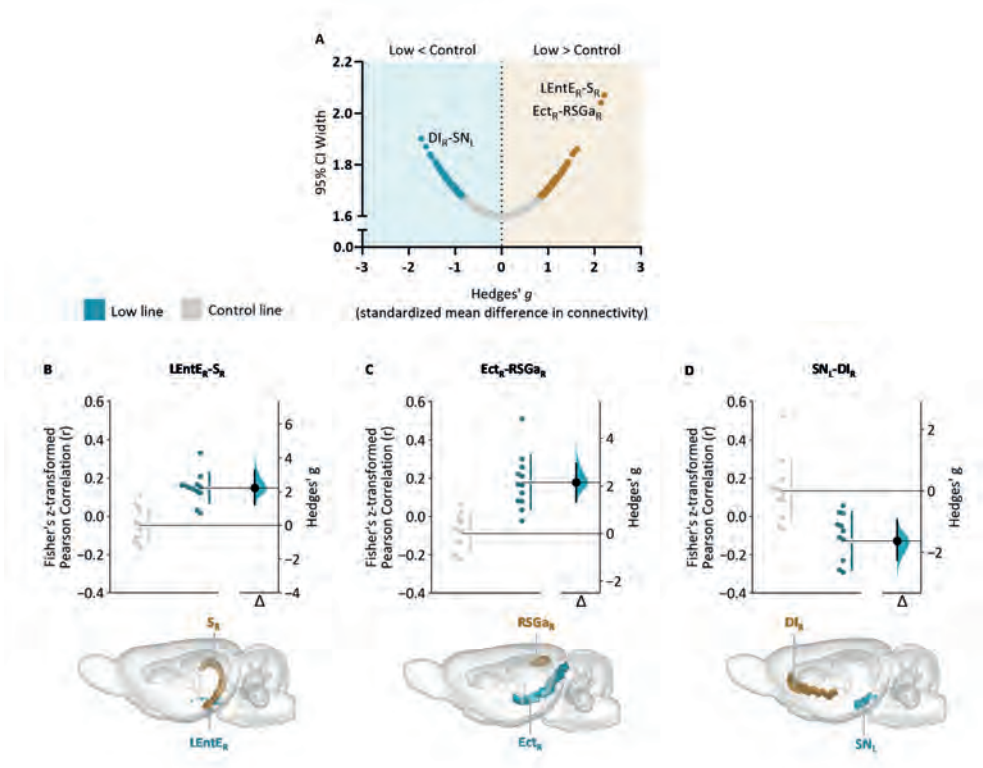


Figure 2 – Whole-brain functional connectivity analysis.

A. Volcano plot of ROI pair functional connectivity differences between rat lines, with Hedges' g on the x-axis and 95% CI width on the y-axis. Each dot represents a single ROI pair and those whose 95% CIs did not cross zero (3.48% of ROI pairs) are color-coded according to direction of effect ($n = 12$ rats per line). **B.** The Low line presented greater functional connectivity relative to the Control line between the right lateral entorhinal cortex external part (LEnt_R) and right subiculum (S_R) ($g = 2.14$, 95% CI = [1.12, 3.16], $n = 12$ rats per line). **C.** The Low line presented greater functional connectivity relative to the Control line between the right entorhinal cortex (Ect_R) and right retrosplenial granular cortex part A (RSGa_R) ($g = 2.21$, 95% CI = [1.18, 3.25], $n = 12$ rats per line). **D.** The Low line presented reduced functional connectivity relative to the Control line between the right dysgranular insular cortex (DI_R) and left substantia nigra (SN_L) ($n = 12$ rats per line). Panels B-D show the Hedges' g for the difference between rat lines in Gardner-Altman estimation plots (Ho et al., 2019). Both groups are plotted on the left axes and the mean difference is plotted on a floating axes on the right as a bootstrap sampling distribution. The effect size is depicted as a dot and the bootstrap-derived 95% CIs are indicated by the ends of the vertical error bar. Dots represent individual values.

DMN analysis

As our resting-state analysis did not reveal functional differences in the DMN between the Low and Control lines, we next examined whether the two lines differed in more stable structural measures, such as the volume of DMN regions. For each DMN ROI, the volumes of the left and right hemispheres were summed to compute total subregion volume (Figure 3A), totaling 12 ROIs. FDR-corrected mass univariate testing revealed a significantly smaller temporal association cortex (TeA) volume in the Low line compared to the Control line ($t_{18} = 3.43$, $q < 0.05$, $g = 1.47$, 95% CI = [0.47, 2.47]). This finding aligns with the observation that, within the DMN, only functional connections involving the Au/TeA node showed 95% CIs excluding zero. In addition, a trend towards a smaller dysgranular retrosplenial cortex (RSD) in the Low line compared to the Control line was observed ($t_{18} = 2.87$, $q = 0.07$, $g = 1.23$, 95% CI = [0.26, 2.19]). Other DMN regions did not show significant differences between lines ($t_{28} \leq 1.23$, all $qs > 0.05$, all Hedges' gs and 95% CIs in Table S3).

Total brain volume did not differ between the two rat lines ($t_{18} = 1.56$, $P = 0.13$, $g = 1.10$, 95% CI = [0.15, 2.04], Figure 3B), indicating that subregional differences between lines were not driven by global brain volume differences.

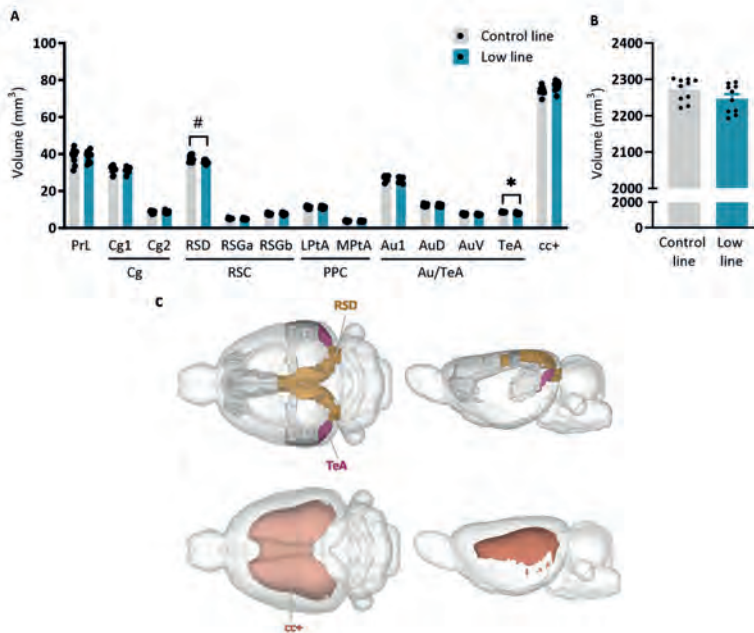


Figure 3 – DMN volumetry analysis.

A. The Low line presents significantly smaller temporal association cortex (TeA) volume (mean $\pm$ s.e.m.) and a trend towards smaller retrosplenial dysgranular cortex (RSD) volume compared to the Control line ($n = 10$ rats per line, * $q < 0.05$, # $q = 0.06$).
B. No difference in total brain volume (mean $\pm$ s.e.m.) between lines ($n = 10$ rats per

line). **C.** Axial and sagittal views of the RSD and TeA (upper), and corpus callosum and associated white matter (cc+, lower).

Whole-brain analysis

Next, we explored differences in white and grey matter volume at the whole-brain level between the two rat lines in a hypothesis-free manner by estimating effect sizes and their corresponding 95% CIs. Effect sizes were plotted as a function of their precision, illustrating both the magnitude and uncertainty of estimates across ROIs (Figure 4A). All ROI effect sizes and their corresponding 95% CIs are listed in Table S3. Here, we highlight selected ROIs with large effect sizes where similar effects were observed in both hemispheres. Volumes of ROIs with consistent effects in both hemispheres were recomputed to assess total volume effect size (Figure 4B-F).

Within grey matter areas, the Low line displayed smaller volume of hippocampal and temporal subregions relative to the Control line. In the hippocampus, the Low line exhibited smaller left and right volume of the presubiculum (PreS_L: $g = -1.46$, 95% CI = [-2.46, -0.46]; PreS_R: $g = -1.79$, 95% CI = [-2.84, -0.74]; total PreS: $g = -1.96$, 95% CI = [-3.07, -1.00], Figure 4C) and CA3 relative to the Control line (CA3_L: $g = -0.95$, 95% CI = [-1.88, -0.02]; CA3_R: $g = -1.25$, 95% CI = [-2.21, -0.28]; total CA3: $g = -1.12$, 95% CI = [-2.21, 0.11], Figure 4D). In the temporal cortex, the Low line exhibited a smaller volume of both left and right entorhinal cortex (Ect) relative to the Control line (Ect_L: $g = -1.15$, 95% CI = [-2.11, -0.20]; Ect_R: $g = -1.15$, 95% CI = [-2.10, -0.20]; total Ect: $g = -1.26$, 95% CI = [-2.32, -0.05], Figure 4E).

In contrast, the Low line presented larger volume of both the left and right bed nucleus of the stria terminalis (BNST) relative to the Control line (BNST_L: $g = -1.42$, 95% CI = [0.28, 2.22]; BNST_R: $g = -1.36$, 95% CI = [0.01, 1.86]; total BNST: $g = 1.11$, 95% CI = [0.30, 1.83], Figure 4F), an extended amygdala subregion that has been implicated in anxiety responses to temporally distant threats and fear generalization (Duvarci et al., 2009; Goode et al., 2020; Radke, 2009).

Within white matter areas, the Low line displayed a larger volume of the left and right fimbria of the hippocampus (fi) relative to the Control line (fi_L: $g = 1.44$, 95% CI = [0.45, 2.44]; fi_R: $g = 1.42$, 95% CI = [0.43, 2.41]; total fi: $g = 1.42$, 95% CI = [0.46, 2.22]; Figure 4G).

Notably, among ROIs where the 95% CIs excluded zero, grey matter regions were predominantly smaller (85.19%), whereas white matter regions were predominantly larger (83.33%) in the Low line relative to the Control line ($\chi^2_1 = 20.78$, $P < 0.01$, odds ratio = 28.75, Figure 4H), suggesting that grey and white matter type may be differentially affected in the Low line.

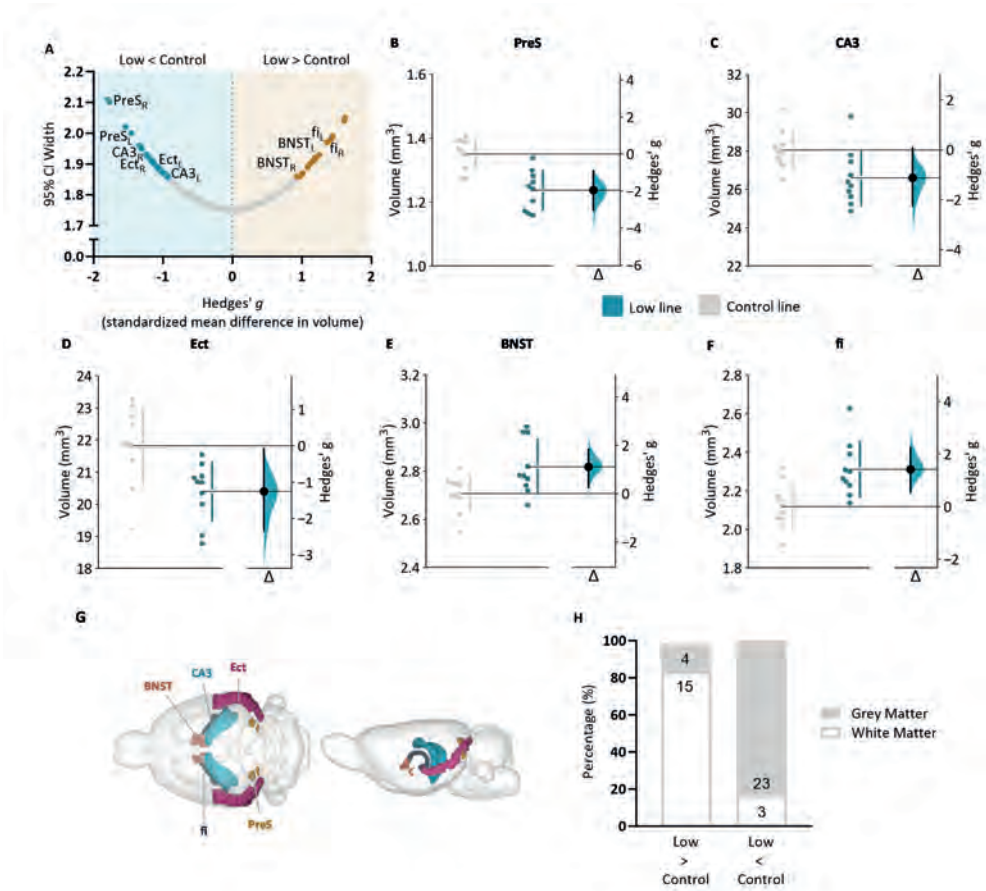


Figure 4 – Whole-brain volumetry analysis.

A. Volcano plot of ROI volume differences between rat lines, with Hedges' *g* on the x-axis and 95% CI width on the y-axis. Each dot represents a single ROI and those whose 95% CIs did not cross zero (19.40% of ROIs) are color-coded according to the direction of effect ($n = 10$ rats per line). **B.** Low line presents smaller total presubiculum (PreS) relative to the Control line ($n = 10$ rats per line). **C.** Low line presents smaller total CA3 relative to the Control line ($n = 10$ rats per line). **D.** Low line presents smaller total ectorhinal cortex (Ect) relative to the Control line ($n = 10$ rats per line). **E.** Low line presents larger total bed nucleus of the stria terminalis (BNST) volume relative to the Control line ($n = 10$ rats per line). **F.** Low line presents larger total fimbria of the hippocampus (fi) volume relative to the Control line ($n = 10$ rats per line). **G.** Axial and sagittal views of the PreS, CA3, Ect, BNST and fi subregions. **H.** Low line presents larger volume mainly in white matter, and smaller volume mainly in grey matter regions, relative to the Control line. Pannels B-F show the Hedges' *g* and 95% CIs for the mean difference between rat lines in Gardner-Altman estimation plots (Ho et al., 2019).

DTI analysis

After the anatomical scan, a series of DTI scans were acquired to derive FA maps. ROI analyses were performed on white matter tracts (Figure 5A), whose selection was based on major white-matter pathways, including those supporting intra-DMN communication (Hsu et al., 2016). FDR-corrected mass univariate testing on FA values did not show significant differences between the two rat lines in any tract ($t_{18} \leq 0.53$, all $q_s > 0.05$, Figure 5B, all Hedges' g s and 95% CIs in Table S5).

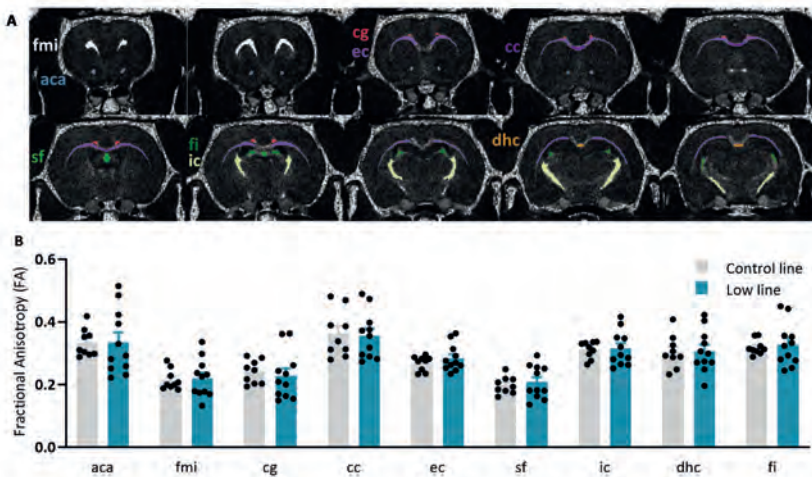


Figure 5 – DTI analysis.

A. ROI analysis of the FA values was performed on selected white matter fibers: anterior commissure anterior part (aca, blue), forceps minor (fmi, white), cingulum (cg, red), external capsule (ec, pink), corpus callosum (cc, purple), septofimbrial nucleus (sf, light green), internal capsule (ic, yellow), fimbria of the hippocampus (fi, dark green), and dorsal hippocampal commissure (dhc, orange). **B.** No statistically significant differences in FA values were found between the two rat lines (mean $\pm$ s.e.m.) ($n = 9-11$ rats per line). Black dots represent individual values.

DISCUSSION

To better understand the contribution of interindividual differences glucocorticoid signaling to vulnerability to PTSD, it is essential to examine how neuroendocrine predispositions, such as stronger HPA-axis negative feedback, influence brain structure and functional connectivity in the absence of prior stress exposure. For this purpose, we conducted both a targeted analysis of the DMN as well as an exploratory whole-brain analysis in two rat lines with differential HPA-axis negative feedback regulation, under basal (i.e., non-stressed, non-treated) conditions. Despite smaller subregional volumes in retrosplenial and temporal nodes, DMN connectivity measures did not differ significantly between the two lines. However, while individual glucocorticoid

levels negatively correlated with the DMN segregation index in the Control line, this effect was absent in the Low line. At the whole-brain level, the Low line showed increased functional connectivity between cortical and hippocampal regions, and retrosplenial and temporal regions relative to the Control line. Structurally, the Low line exhibited smaller PreS, CA3 and Ect volumes, larger BNST and fi volume, and no significant differences in white matter microstructure.

Disturbances in the DMN and altered glucocorticoid signaling are thought to contribute to development and maintenance of symptoms in PTSD (Galatzer-Levy et al., 2014; Lanius et al., 2010; Szeszko et al., 2018). Contrary to expectations, we found no differences in DMN connectivity between these rat lines with differential HPA-axis negative feedback regulation under basal conditions. However, consistent with stress-induced DMN downregulation (W. Zhang et al., 2019; Y. Zhang et al., 2015, 2020), circulating glucocorticoid levels were negatively correlated with DMN segregation index in the Control line. This relationship is thought to reflect adaptive reallocation of attentional resources promoted by stress, as DMN regions typically deactivate during externally directed attention (Raichle et al., 2001).

In contrast, despite comparable basal glucocorticoid levels, this relationship was absent in the Low line, suggesting reduced sensitivity of DMN connectivity to glucocorticoids. Consistent with this finding, under basal conditions the Low line showed correlational evidence of altered MR-dependent *Fkbp5* expression (Chapter 3), a mechanism that may alter GR sensitivity (Hartmann et al., 2021). Although baseline behavior was comparable between the two lines, the Low line was resistant to glucocorticoid administration and exhibited divergent neural activity patterns relative to the Control line (Chapter 3). Accordingly, differences in DMN engagement may only emerge under challenge conditions, such as stress or glucocorticoid administration. This interpretation is supported by reduced volumes in retrosplenial and temporal DMN subregions (RSD, TeA), which may constrain network responsiveness to acute hormonal changes (Hall et al., 2015). Notably, impaired downregulation of the DMN in response to stress has been proposed as a vulnerability marker for stress-related disorders, including PTSD (van Oort et al., 2020). Future studies combining stress or glucocorticoid manipulations with *in vivo* functional imaging will be essential to determine whether this HPA-axis phenotype leads to divergent DMN responses under challenge.

It is important to note that resting-state data were acquired under light sedation with a noradrenergic receptor agonist. As central noradrenergic activity may modulate DMN activity (Mittner et al., 2016; Oyarzabal et al., 2022), its influence cannot be excluded. Nevertheless, using a highly standardized protocol (Grandjean et al., 2023), we detected robust DMN-like activity in both lines.

Exploratory whole-brain effect size analyses further revealed distributed differences in functional connectivity and volumetry between lines. Although interpretation of functional relevance is limited by the basal, undisturbed resting-state condition, it is interesting to note that the largest differences in mean connectivity were distributed across connections relevant for contextual memory processing (lateral entorhinal–

hippocampal–retrosplenial circuitry) (Hargreaves et al., 2005; Smith et al., 2012; Todd & Bucci, 2015), and salience and interoceptive networks (substantia nigra–insula) (Fermin et al., 2023; Y. Zhang et al., 2017). Structural differences, including reduced PreS and CA3 as well as increased BNST and fi volumes in the Low line, align with alterations in mnemonic processing associated with PTSD, such as fear generalization and impaired safety learning. Although these effects do not form a single coherent network, their distribution overlaps with regions known to express MR and GRs (Morimoto et al., 1996; Reul & de Kloet, 1985, 1986). However, it is unclear why specific subregions like the BSNT and fi are most affected when others with similar receptor abundance are not affected to the same extent. Together, these findings support that endocrine predisposition is associated with subtle but consistent differences in the functional and structural organization of circuits relevant to stress, salience and memory.

In conclusion, predisposition for strong HPA-axis negative feedback regulation and low post-stressor glucocorticoid levels can influence neural organization independently of stress exposure. These results support the view that neurobiological features observed in stress-related disorders may reflect pre-existing vulnerability factors that influence individual responses to future stressors, rather than solely the consequences of trauma.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

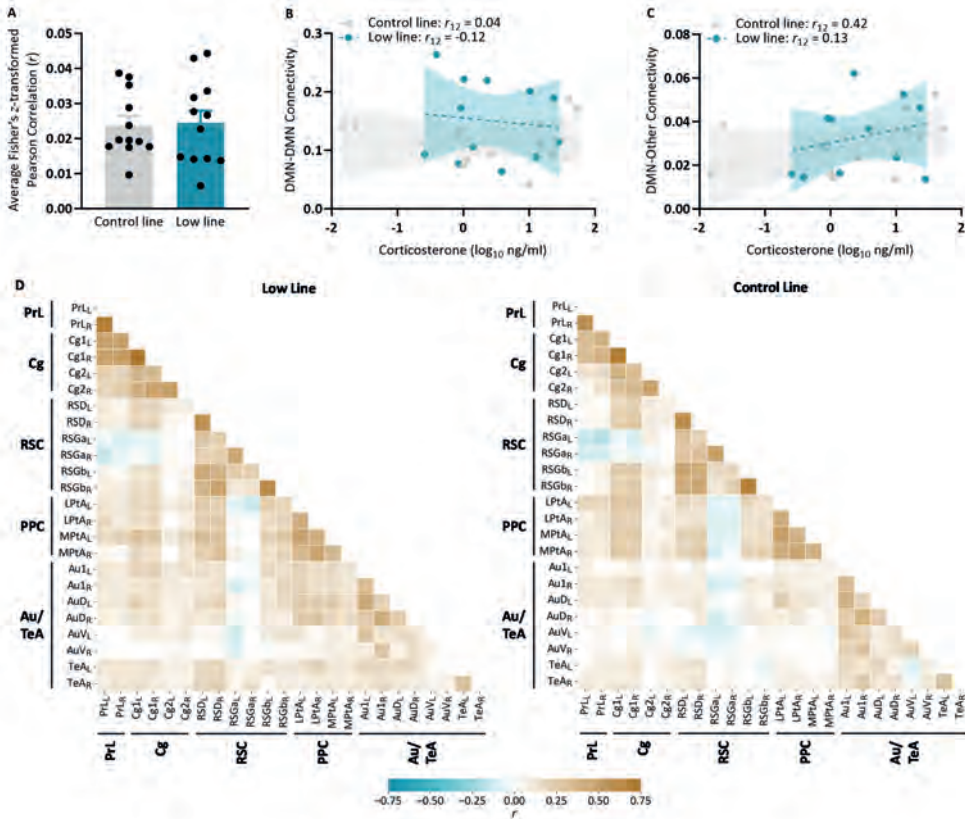


Figure S1 – DMN functional connectivity analysis.

A. No significant difference in average whole brain connectivity between lines ($n = 12$ rats per line, $t_{22} = 0.15$, $P = 0.88$). **B.** DMN-DMN connectivity did not significantly correlate with circulating (log-transformed) corticosterone levels in either rat line ($n = 12$ rats per line, Low line: $r_{12} = -0.12$, 95% CI = [-0.65, 0.49], $P = 0.72$; Control line: $r_{12} = 0.04$, 95% CI = [-0.55, 0.60], $P = 0.90$). **C.** DMN-Other connectivity did not significantly correlate with circulating (log-transformed) corticosterone levels in either rat line ($n = 12$ rats per line, Low line: $r_{12} = 0.13$, 95% CI = [-0.48, 0.66], $P = 0.69$; Control line: $r_{12} = 0.42$, 95% CI = [-0.20, 0.80], $P = 0.17$). **D.** Group average correlation (r) matrices from 12 bilateral DMN ROIs for each rat line ($n = 12$ rats per line). Dashed regression line represents non-significant correlation. Dots represent individual values.

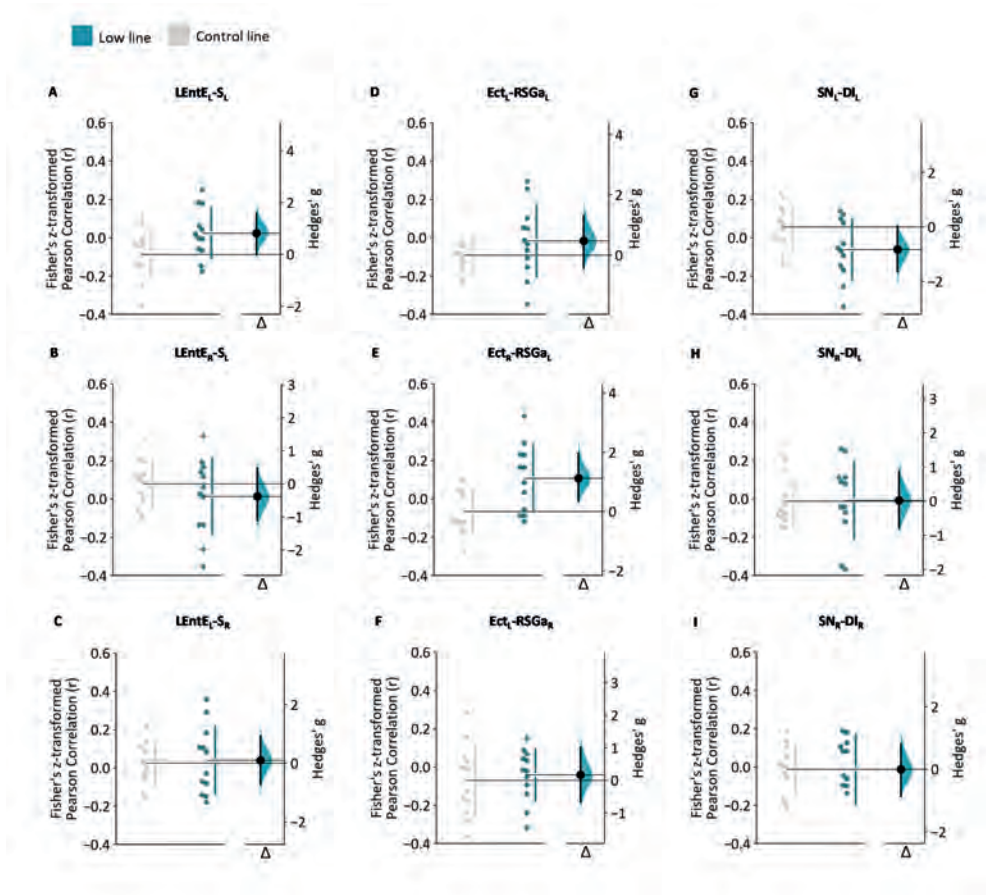


Figure S2 – Whole-brain functional connectivity analysis.

A. LEnt_L-S_L functional connectivity ($g = 0.82$, 95% CI = [-0.01, 1.56], $n = 10$ rats per line). **B.** LEnt_R-S_L functional connectivity ($g = -0.38$, 95% CI = [-1.19, 0.40], $n = 10$ rats per line). **C.** LEnt_L-S_R functional connectivity ($g = 0.10$, 95% CI = [-0.74, 0.90], $n = 10$ rats per line). **D.** Ect_L-RSGa_L functional connectivity ($g = 0.47$, 95% CI = [-0.41, 1.32], $n = 10$ rats per line). **E.** Ect_R-RSGa_L functional connectivity ($g = 1.12$, 95% CI = [0.29, 1.93], $n = 10$ rats per line). **F.** Ect_L-RSGa_R functional connectivity ($g = 0.17$, 95% CI = [-0.67, 1.00], $n = 10$ rats per line). **G.** SN_L-DI_L functional connectivity ($g = -0.83$, 95% CI = [-1.62, 0.02], $n = 10$ rats per line). **H.** SN_R-DI_L functional connectivity ($g = 0.02$, 95% CI = [-0.77, 0.92], $n = 10$ rats per line). **I.** SN_R-DI_R functional connectivity ($g = 0.00$, 95% CI = [-0.84, 0.81], $n = 10$ rats per line). Pannels show the Hedges' g and 95% CIs for the difference between rat lines in Gardner-Altman estimation plots (Ho et al., 2019). Dots represent individual values.

Table S1 – Anatomical definitions of DMN nodes.

DMN Node	ROI #	SIGMA #	ROI	Abbrev.
Prelimbic/Orbital Cortex (PrL)	1	461, 462	Prelimbic System	PrL
Cingulate Cortex (Cing)	2	501, 502	Primary Cingular Cortex	Cg1
	3	701, 702	Secondary Cingular Cortex	Cg2
Retrosplenial Cortex (RSC)	4	651, 652	Retrosplenial Dysgranular Cortex	RSD
	5	661, 662	Retrosplenial Granular Cortex Part A	RSGa
	6	671, 672	Retrosplenial Granular Cortex Part B	RSGb
Posterior Parietal Cortex (PPC)	7	281, 282	Lateral Parietal Association Cortex	LPtA
	8	311, 312	Medial Parietal Association Cortex	MPtA
Auditory/Temporal Association Cortex (Au/TeA)	9	491, 492	Primary Auditory Cortex	Au1
	10	681, 682	Secondary Auditory Cortex Dorsal Part	AuD
	11	691, 692	Secondary Auditory Cortex Ventral Part	AuV
	12	771, 772	Temporal Associative Cortex	TeA

Table S2 – Whole-brain functional connectivity analysis. Online access: <https://doi.org/10.5281/zenodo.20081480>.

Table S3 – Whole-brain volumetry analysis. Online access: <https://doi.org/10.5281/zenodo.20081480>.

Table S4 – Selected ROIs for DTI analysis.

ROI #	ROI	Abbreviation
1	Anterior commissure anterior part	aca
2	Forceps minor	fmi
3	Cingulum	cg
4	Corpus callosum	cc
5	External capsule	ec
6	Septofimbrial nucleus	sf
7	Internal capsule	ic
8	Dorsal hippocampal commissure	dhc
9	Fimbria of the hippocampus	fi

Table S5 – Fractional anisotropy analysis. Online access: <https://doi.org/10.5281/zenodo.20081480>.



CHAPTER 5

General discussion

While much is known about the effects of norepinephrine and glucocorticoids on memory consolidation, how these stress hormones influence more subtle aspects of memory such as specificity and generalization remains poorly understood. Across molecular, neural, and behavioral levels, this thesis demonstrates that norepinephrine biases the brain toward memory specificity, while glucocorticoids bias toward memory generalization. Building on this, the thesis further shows that interindividual differences in endogenous stress hormone signaling shape neurocircuitry and determine response to pharmacological treatment. Given that aberrations in memory specificity and generalization are hallmark features of stress-related disorders such as PTSD, these findings provide a framework for understanding how such interindividual differences may contribute to mnemonic processing affected by emotional arousal and stress, with implications towards PTSD susceptibility and variability in treatment response.

SUMMARY OF MAIN FINDINGS

In **Chapter 2**, I examined the neural and molecular mechanisms through which norepinephrine enhances memory specificity. Using the dual-event inhibitory avoidance task, I first showed that norepinephrine infusions into the basolateral amygdala (BLA) during consolidation not only strengthened memory of the footshock, but also facilitated the separation of the two training events experienced close in time. Specifically, retention latencies were selectively enhanced in the shock box whereas latencies in the non-shock context became shorter. At a molecular level, norepinephrine administration into the BLA reduced miR-134 levels and increased CREB and BDNF mRNA expression in the dorsal blade of the dentate gyrus (dDG). I then demonstrated that this specificity-enhancing effect of norepinephrine is mediated by a miR-134–regulated mechanism in the dDG, as targeted downregulation of miR-134 using a specific antagomir reproduced the enhancement of memory specificity, whereas upregulation via a miR-134 mimic blocked norepinephrine-induced memory specificity. Importantly, both manipulations selectively altered memory specificity, leaving memory of the footshock *per se* unaffected. To determine whether this mechanism is engaged under conditions in which memory specificity naturally occurs, I increased the interval between the two training events and infused either propranolol into the BLA or a miR-134 mimic into the dorsal hippocampus. Both manipulations blocked memory specificity; however, contrary to propranolol, miR-134 upregulation did not affect memory of the footshock. Consistent with this, training on a single event failed to engage the hippocampal miR-134 mechanism, confirming that it is selectively recruited when discrimination between similar experiences is required. This chapter identifies a miR-134–dependent consolidation mechanism in the dDG that selectively regulates memory specificity. Importantly, it demonstrates that noradrenergic activation in the BLA produces two independent effects: it enhances the strength of the memory, and facilitates memory specificity by promoting the separation of memories of two events experienced close in time. Together, these two processes can ensure that only correct associations are being

strengthened.

In **Chapter 3**, I investigated whether stronger HPA-axis feedback regulation and low post-stressor glucocorticoid levels influence the strength and generalization of extinction memory, as well as their interaction with glucocorticoid treatment. I first established that a rat line selectively bred for blunted post-stressor corticosterone levels (i.e., Low line) also exhibits stronger HPA-axis negative feedback regulation compared to a Control line, along with increased expression of MR and FKBP5 genes in blood. I then assessed whether these two lines differ in strength and generalization of extinction memory using a fear conditioning and extinction paradigm, in which extinction memory was tested in both the conditioning and extinction contexts enabling the assessment of memory strength and generalization, respectively. Under non-treated conditions, the two lines did not differ in strength or generalization of extinction memory. However, glucocorticoid administration immediately after the extinction training enhanced both the strength and generalization of extinction memory in the Control line. In contrast, glucocorticoid administration to the Low line did not affect extinction memory, suggesting a resistance to glucocorticoid treatment. Consistent with the behavioral results, at the neural level, glucocorticoid treatment in the Control line was associated with reduced DG activity, whereas in the Low line this reduction was absent and accompanied by increased DG–prelimbic cortex connectivity. Interestingly, I did not find evidence for a difference in phosphorylation of the glucocorticoid receptor (GR) in the dorsal hippocampus in response to glucocorticoid treatment between lines. This chapter shows that glucocorticoid treatment effects on memory strength and generalization diverges between rat lines with differential HPA-axis negative feedback regulation.

In **Chapter 4**, I assessed whether stronger HPA-axis negative feedback regulation influences brain structure and connectivity. To this end, I performed an *a priori* confirmatory analysis of the Default Mode Network (DMN) as well as exploratory whole-brain analysis, using a multimodal magnetic resonance imaging (MRI) approach. While no differences were found in DMN connectivity between lines, the Low line exhibited a dissociation between DMN engagement and circulating glucocorticoid levels, suggesting altered network sensitivity to glucocorticoid modulation. In addition, the Low line exhibited a smaller volume of the temporal association cortex, a node of the DMN. Beyond the DMN, I identified a range of structural and connectivity differences between the two lines, primarily in hippocampal and temporal regions. Notably, the bed nucleus of the stria terminalis was larger in the Low line. No evidence of differences in white matter microstructure was found between the two lines. These findings suggest that stronger HPA-axis negative feedback regulation is associated with changes in brain structure and functional connectivity.

Together, these studies demonstrate that norepinephrine, glucocorticoids, as well as interindividual differences in their endogenous signaling, do not merely influence memory strength, but systematically shape memory specificity and generalization. In the following sections, I first discuss how stress hormones differentially modulate these fine-grained aspects of memory, along with their underlying molecular

and neural mechanisms. I then examine how interindividual differences in stress hormone systems contribute to variability in memory processing. Finally, I consider the translational implications of these findings towards human research on the development and treatment of stress-related disorders, with particular focus on their relevance for developing personalized treatment approaches for PTSD.

STRESS HORMONES AS MODULATORS OF MEMORY SPECIFICITY AND GENERALIZATION

Stress Hormone Modulation of Behavior

The ability to generalize previous experiences to new situations, or in contrast to recall context-specific and detail-rich memories, is essential for adaptive behavior. Whereas generalization supports the transfer of threat-related knowledge across contexts, memory specificity is critical for keeping experiences distinct, thereby limiting the spread of fear to safe or irrelevant environments. Extensive research of the stress hormones norepinephrine and glucocorticoids has established that they dose-dependently enhance memory consolidation, but how these stress hormones may modulate memory specificity and generalization remained relatively unexplored until recent years.

Converging evidence from recent studies (Atucha et al., 2017; Bahtiyar et al., 2025; Gulmez Karaca et al., 2025; Guo, 2024; Roozendaal & Mirone, 2020; Song, 2021), together with our findings in **Chapters 2 and 3**, demonstrate that memory expression is not necessarily determined at retrieval, but can be influenced during consolidation by physiological conditions that bias later retrieval toward either memory specificity or generalization. Specifically, local norepinephrine infusions into the BLA immediately after training promoted memory specificity in **Chapter 2**, whereas systemic glucocorticoid administration immediately after extinction training promoted generalization of extinction memory in rats of the Control line in **Chapter 3**. These effects point to a critical role of consolidation, as post-training manipulations do not interfere with the learning experience itself and thus exclude influences on attentional, sensory, or motivational processes. This interpretation is further supported by the finding in **Chapter 2** showing that memory tested 30 min after norepinephrine administration did not show enhanced specificity, indicating that the norepinephrine effect does not necessarily emerge at retrieval but requires consolidation processes to unfold. These findings indicate that consolidation represents a critical window during which stress hormones bias memory specificity and generalization.

The term “generalization” is often used as an umbrella label for similar behavioral outcomes, yet such outcomes may arise from more selective processes such as memory linking (see Box 1) (Cai et al., 2016; Choucry et al., 2024), whereby representations of distinct events become associated such that retrieval of one can trigger recall of the other. Within this framework, the effects of stress hormones observed in **Chapters 2 and 3** are more consistent with the modulation of memory linking rather than with a strict definition of generalization (i.e., context independence). For example,

Box 1 – The Specificity-Generalization Axis

Memory specificity usually refers to a detailed memory of an event bound to a specific context. By contrast, memory generalization usually refers to a memory that is context-independent, presenting as more gist-like. While these terms are widely used, growing interest in stress-related disorders and their mnemonic aberrations increasingly calls for more fine-grained definitions of the processes that occupy the space of context-dependent memory expression between these extremes.

Recent work has begun to define part of this intermediate space: the ability to link distinct yet related memories (Choucry et al., 2024). For example, if a safe context and a shock-associated context become neurally linked (i.e., co-activated), an animal may fear the safe context by association. Events occurring close in time are more likely to become linked (i.e., prospective memory linking) (Cai et al., 2016; Sehgal et al., 2025), whereas events well separated in time may also become linked through offline processes such as replay during sleep (i.e., retrospective memory linking) (Zaki et al., 2025). Importantly, linked memories may still retain detail (i.e., identity) even as they become associated (Choucry et al., 2024). Linking is thought to rely both on overlap between neuronal ensembles allocated to each memory, as well as on synapse- and dendrite-specific plasticity mechanisms (Choucry et al., 2024).

At the same time, it is important to note that generalization can also arise from stimulus similarity, whereby the memory of an auditory cue may be expressed when another similar cue is presented (Asok et al., 2019). Further, generalization may occur for a specific stimulus (e.g., auditory cue), but not necessarily for the context in which it was encountered (Desmedt et al., 2003; Rescorla, 1976). Thus, at the behavioral level, what may be broadly classified as “generalization” could reflect some degree of stimulus generalization (i.e., patterns of similarity in contextual, auditory or other cues), or memory linking (i.e., active association between memories), or even a combination of both.

These processes are likely interconnected rather than fully independent. For instance, retrospective linking likely favors memories that share features, and similarity between experiences could facilitate their offline integration (Zaki et al., 2025). Conversely, strong discrimination between similar inputs might prevent both linking and stimulus generalization by keeping neural representations distinct. Together, these observations temper the specificity–generalization dichotomy, instead framing it as a multidimensional space.

in **Chapter 2**, post-training propranolol administration to attenuate endogenous noradrenergic signaling similarly enhanced retention latencies in the shock and non-shock boxes, while responses to a novel box remained unaffected, suggesting linking rather than context independence. In **Chapter 3**, it is unclear whether glucocorticoid administration induced memory linking or context independence, as memory was

only tested in the training contexts and not in a novel context. Evidence from the dual-event inhibitory avoidance task suggests that, in paradigms with two training events, glucocorticoid administration may induce memory linking, as it similarly enhanced retention latencies in the shock and non-shock boxes, with no significant change in response in a novel box (Roozendaal & Mirone, 2020). In line with this interpretation, Xu et al. (2023) showed that generalized extinction memories may depend on whether synaptic plasticity associated with both fear and extinction memories co-exists on individual dendritic branches, a mechanism thought to support memory linking (Choucry et al., 2024). The distinction between linking and context independence is crucial, as it implies that glucocorticoid signaling may not indiscriminately broaden memory representations, but instead regulate how experiences become selectively integrated across episodes.

These findings contrast with studies in which rodents were exposed to a single training context and generalization was assessed in a novel context. Under these conditions, glucocorticoid administration has been shown to promote fear generalization to novel contexts (dos Santos Corrêa et al., 2019; Lesuis et al., 2021). This suggests that glucocorticoids may promote either complete context independence or memory linking, depending on the structure of the learning experience. Specifically, in paradigms involving multiple training events, glucocorticoid effects may favor linking between similar or temporally proximal experiences, whereas in single-event paradigms they may promote generalization to novel contexts. In contrast, as demonstrated in **Chapter 2**, norepinephrine administration following a single training event enhances only memory strength, consistent with the absence of a requirement for discrimination. Together, these observations indicate that the effects of stress hormones on memory specificity and generalization may critically depend on the representations engaged during learning. In particular, prior experience and the requirement to discriminate between similar contexts or cues may determine whether glucocorticoid administration promotes linking or context independence.

Extensive research has established that glucocorticoid effects on memory consolidation depend on arousal-induced noradrenergic activation (Okuda et al., 2004; Quirarte et al., 1997; Roozendaal et al., 2006). Although stress hormone interaction has not yet been directly examined in the context of memory specificity and generalization, it is possible that glucocorticoid-induced generalization may also be constrained by the magnitude and pattern of noradrenergic engagement elicited by the learning experience. Human research has shown that training in multiple events induces rapid noradrenergic activation that facilitates event segmentation (Dunsmoor et al., 2018). Using a genetically encoded sensor, a rodent study has supported that event boundaries drive phasic norepinephrine release and the formation of distinct neural representations (McKenzie et al., 2025). Accordingly, tasks that require discrimination between events may recruit greater phasic noradrenergic signaling, thereby enabling glucocorticoids to promote generalization, whereas this effect may be reduced or absent in conditions with reduced requirement for discrimination. Consistent with this idea, glucocorticoid administration promoted memory generalization when

the object-in-context task included training contexts that were different from the habituation contexts, increasing discrimination demands (Guo, 2024). In contrast, glucocorticoids impaired generalization when the contexts were identical, reducing such demands (Guo, 2024). In line with this, familiarity with a context accelerated the rate in which phasic norepinephrine decayed to basal levels (McKenzie et al., 2025). Notably, elevated tonic noradrenergic activation (i.e., hyperarousal) may disrupt event segmentation during encoding (Clewett et al., 2025), potentially facilitating memory generalization. Together, these observations support the idea that the magnitude and pattern of noradrenergic activation during encoding may determine the effect of subsequent glucocorticoid manipulation (Roosendaal & Hermans, 2017). In this way, variability in discrimination demands and arousal levels may partially explain why glucocorticoid administration produces divergent effects on memory specificity and generalization across experimental paradigms.

Together, these findings highlight that task characteristics critically determine how effects of stress hormones on memory specificity and generalization manifest. First, these effects may vary with the level of intrinsic noradrenergic activation induced by the task. Second, modulation of specificity and generalization can only be observed in paradigms that place demands on discrimination, such as those involving multiple training contexts, distinct experiences with shared features, or the inclusion of a novel context in a recall test. Critically, these task features may better reflect daily life, as individuals are constantly exposed to thousands of contexts that share both similar and distinct features. Such task-dependent effects of glucocorticoid administration are likely accompanied by corresponding differences in underlying neural processing.

Stress Hormone Modulation of Neural Circuits

Both **Chapter 2 and 3** converge on the DG as a key locus through which norepinephrine and glucocorticoids bias memory toward specificity or generalization. Within pattern separation and completion frameworks, the DG is thought to rapidly form distinct representations from similar sensory input provided by the entorhinal cortex (Yassa & Stark, 2011), which are relayed to the CA3 to retrieve previously stored representations using these partial cues. By regulating the degree of representational overlap between inputs, DG computations are therefore well positioned to determine whether memories remain specific or generalize across contexts (Asok et al., 2019).

In **Chapter 2**, noradrenergic activation of the BLA promoted memory specificity, an effect associated with downregulation of miR-134 in the dDG. While this chapter focused on a molecular mechanism within the dDG, converging evidence suggests that norepinephrine also modulates DG-dependent circuit interactions to support pattern separation. For instance, post-training norepinephrine administration in the object-in-context task with a different habituation condition (i.e., requiring discrimination) enhances dDG–CA3 connectivity (Guo, 2024), a pathway that has been associated with pattern separation and memory specificity (Lee et al., 2020; Leutgeb et al., 2007). In addition, increased CA1 activity was also observed (Guo, 2024), potentially reflecting

enhanced storage of memory of the temporal order of the two training events into segregated, non-overlapping populations of hippocampal principal CA1 neurons (Tronson et al., 2009). Moreover, human research has shown that locus coeruleus (LC) activation may facilitate processing of novel events via interactions with the DG and CA3 (Clewett et al., 2025). Importantly, in **Chapter 2**, local infusion of norepinephrine into the dorsal hippocampus did not induce memory specificity, suggesting that the specificity-enhancing effects of norepinephrine may be specifically driven by interaction of noradrenergic activation in the BLA with the DG, likely via modulation of entorhinal inputs. Together, these findings indicate that noradrenergic activation in the BLA promotes memory specificity not only through local DG plasticity, but also by strengthening DG-driven hippocampal network dynamics.

In **Chapter 3**, glucocorticoid-induced generalization of extinction memory was accompanied by a reduced recruitment of active DG neurons in rats of the Control line. Given the importance of contextual discrimination in the expression of extinction memory (Maren et al., 2013), recent work has emphasized a role for both pattern separation and completion processes in extinction memory (Laing & Dunsmoor, 2023; Neudert et al., 2023). Fear and extinction memories are encoded in partially distinct neuronal ensembles that can coexist and compete for behavioral expression (Lacagnina et al., 2019). Within this framework, in **Chapter 3**, reduced DG activity following glucocorticoid administration is consistent with decreased segregation between fear- and extinction-related representations, thereby reducing contextual discrimination and promoting generalization. This interpretation aligns with evidence that linking of fear and extinction memories depends on structural plasticity in neurons shared across neuronal ensembles (Xu et al., 2023). Consistent with reduced DG activity, in the object-in-context task with different habituation, glucocorticoid-treated mice displayed increased GABAergic activity in the ventral blade of the DG, as well as in the CA1 *stratum radiatum* (Guo, 2024). Importantly, in **Chapter 3**, no evidence was found of direct glucocorticoid action in the hippocampus, as GR phosphorylation levels (i.e., markers of activation and inactivation) did not differ between vehicle- and glucocorticoid-treated Control line rats. This raises the question of whether the generalization-enhancing effects of glucocorticoids are also mediated by the BLA, similarly to specificity-enhancing effects of norepinephrine.

Importantly, the effects of glucocorticoids on DG activity might depend on task demands. In a paradigm involving a single training event, glucocorticoids have been associated with increased DG activity alongside enhanced generalization (Lesuis et al., 2021), in contrast to the reduced DG activity observed in **Chapter 3**. One critical difference is that such paradigms do not seem to require discrimination between competing memory representations. In the absence of competing engrams, increased DG activity may reflect enhanced encoding strength rather than improved pattern separation. Consistent with this interpretation, stress hormone-induced changes in dDG–CA3 connectivity are not observed in single-event tasks that do not require discrimination (Guo, 2024). As pattern separation is thought to emerge from cellular processes within the DG, the differential DG recruitment and connectivity by stress

hormones likely reflect distinct molecular and cellular mechanisms.

Stress Hormone Modulation of Cellular and Molecular Mechanisms

The DG receives nonspatial contextual information from the lateral entorhinal cortex via the lateral perforant path (LPP) (Hargreaves et al., 2005). In response to LPP input, adult-born immature granule cells have been reported to exert monosynaptic inhibition onto mature granule cells within the DG (Drew et al., 2016; Luna et al., 2019). This inhibitory control may contribute to the sparse neural activity that supports pattern separation and, consequently, memory specificity and generalization. With the exception of Guo (2024), studies on stress hormone effects on these processes have not distinguished between excitatory and inhibitory cell populations, including those in **Chapters 2 and 3**. Therefore, any account of how norepinephrine and glucocorticoids influence this proposed DG mechanism remains speculative.

As demonstrated in **Chapter 2**, noradrenergic activation of the BLA may promote decay and subsequent downregulation of miR-134 in the dDG. miR-134 exerts an inhibitory influence on *Creb* and *Bdnf* expression, thus, its downregulation leads to increased expression of these plasticity-related molecules (Numakawa et al., 2011). Notably, BDNF activity is associated with dendritic spine formation (Zagrebelsky et al., 2020) and adult neurogenesis (Choi et al., 2009). As such, reduced miR-134 and increased *Bdnf* levels could facilitate these forms of long-term plasticity in LPP–dDG cell synapses during the consolidation period (Figure 1 inset) (Gao et al., 2010; Numakawa et al., 2011; Schratt et al., 2006). Such changes may strengthen the inhibitory control exerted by immature granule cells over mature granule cells, thereby facilitating pattern separation, and ultimately improving discrimination during retention testing.

Given the opposing behavioral effects of glucocorticoids and norepinephrine, glucocorticoids may exert opposing effects at the molecular level as well. Through both genomic and non-genomic mechanisms, glucocorticoids influence a broad range of intracellular pathways (Karst & Joëls, 2005; Krueger et al., 2020; Olijslagers et al., 2008), including reduction in BDNF expression within the DG (Chao & McEwen, 1994; Cosi et al., 1993). miR-134 expression appears to be regulated by SIRT1, a deacetylase involved in histone acetylation and transcriptional control (Gao et al., 2010). Functional interactions between SIRT1 and GR signaling have been described (Amat et al., 2007; Pasquereau et al., 2021), although direct GR binding to the SIRT1 promoter remains uncertain. In line with this, prenatal dexamethasone exposure has been shown to induce long-lasting upregulation of hippocampal miR-134 through GR-dependent modulation of SIRT1, leading to reduced neural progenitor proliferation (Jiang et al., 2022). These observations suggest that glucocorticoids may reduce BDNF-related plasticity both directly, via GR-mediated transcriptional regulation, and indirectly, via SIRT1-dependent upregulation of miR-134 (Figure 1 inset). In line with this, glucocorticoids have been shown to reduce hippocampal neurogenesis (Schoenfeld & Gould, 2012) and modulate dendritic spine formation (Gulyaeva, 2023). Such effects could modulate inhibitory regulation within the DG, biasing the system

away from pattern separation and toward greater memory generalization.

Notably, in **Chapter 2**, artificial downregulation and upregulation of miR-134 in the DG via antagomir and mimic infusion, respectively, selectively modulated memory specificity but not strength, suggesting that these two processes can be dissociated into distinct mechanisms. However, it is yet unclear what molecular mechanisms might specifically regulate memory strength.

Extended model of memory modulation theory for discrimination of episodic-like memories

The memory modulation theory posits that the BLA serves as a central amplifier, where arousal- and stress-induced noradrenergic activation coupled with glucocorticoid signaling modulate neuroplasticity in other brain regions (McGaugh, 2000). In this theory, norepinephrine and glucocorticoids serve similar, but complementary, roles by enabling different types of memories to be stored more robustly and persistently (Roozendaal and McGaugh, 2011). Here, I extend this theory by proposing that, when endogenously released, not only do these stress hormones synergistically enhance memory consolidation via the BLA, but they serve opposing roles in determining discrimination of episodic-like memories in the hippocampus (Figure 1). That is, norepinephrine biases memory representations in the DG towards memory specificity, whereas glucocorticoid interaction with noradrenergic activation biases towards generalization. Notably, the degree of generalization promoted by glucocorticoids may depend on prior experience and the level of noradrenergic activation elicited by the learning experience.

From an adaptive standpoint, a functional dissociation between noradrenergic and glucocorticoid signaling in memory discrimination may be advantageous. An emotionally arousing, but not stressful experience (e.g., a salient yet manageable event, like eating delicious blueberries) may primarily elicit central noradrenergic activation, thereby promoting the encoding of detailed and specific representations of the episode. Such specificity would support accurate discrimination of similar future experiences without broadly altering interpretative frameworks (i.e., eating all berries, including poisonous red ones). In contrast, a stressful experience (e.g., one characterized by threat, like encountering an aggressive wolf) would lead to the release of systemic glucocorticoids together with catecholamines, thereby directing processing toward generalization. Situations are typically appraised as stressful when (perceived) demands exceed coping capacity, signaling heightened uncertainty, uncontrollability and potential threat (McEwen, 1998). Under such conditions, it may be advantageous not only to consolidate the memory strongly, but also to increase the likelihood that it will be retrieved across novel yet similar contexts (i.e., encountering a large enthusiastic dog). By promoting generalization, glucocorticoid signaling may therefore facilitate the application of past experience to future uncertain situations, ultimately enhancing survival.

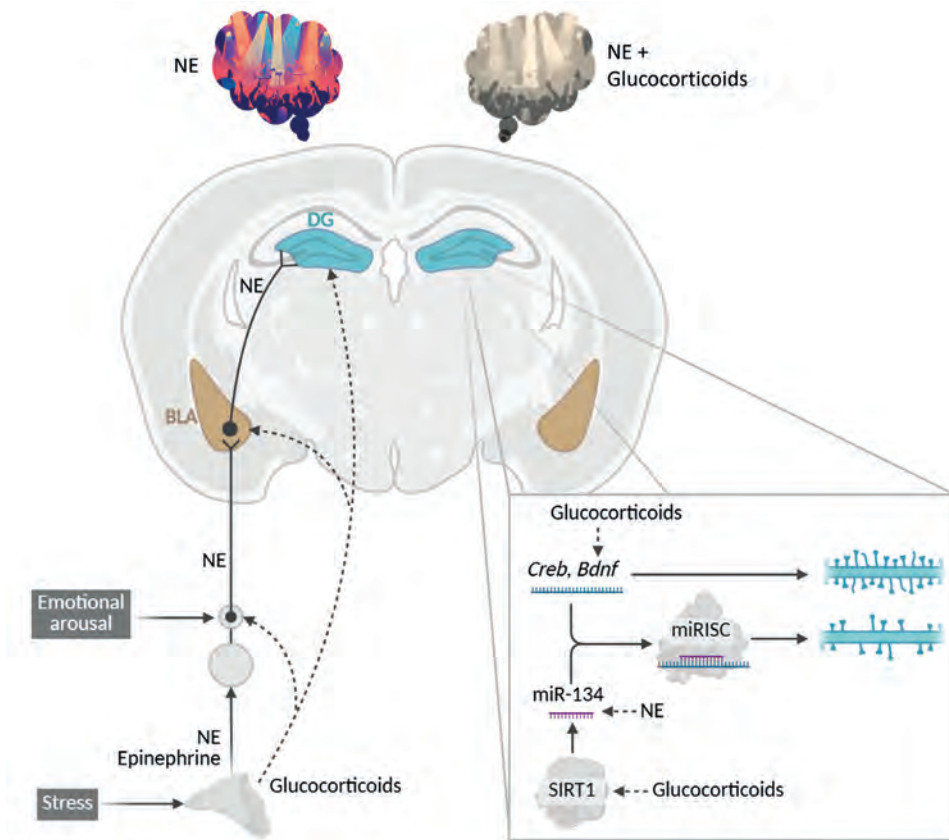


Figure 1 – Extended model of memory modulation theory for discrimination of episodic-like memories. Arousal-induced noradrenergic activation (NE) of the basolateral amygdala (BLA) promotes memory specificity, whereas stress-induced glucocorticoids together with catecholamines promote generalization, by modulating pattern separation in the dentate gyrus (DG). Within the DG (inset), glucocorticoids and NE may act at multiple points in the BDNF and miR-134 pathway to regulate spine formation and neurogenesis. miRISC: miR-induced silencing complex. Created in BioRender.com.

Importantly, this framework supports a more nuanced view of glucocorticoid-induced generalization. Although such effects are sometimes characterized as maladaptive, particularly in the context of PTSD research (Desmedt et al., 2015), generalization is not inherently dysfunctional. Rather, its adaptiveness likely depends on the degree of generalization, the demands of the environment, and the extent to which generalized responses remain proportional to actual threat. Critically, the degree or tendency towards memory specificity and generalization may also vary across individuals, as both the noradrenergic and glucocorticoid signaling systems present significant interindividual variability.

INTERINDIVIDUAL DIFFERENCES IN NORADRENERGIC AND GLUCOCORTICOID SIGNALING

Variability in stress regulation and mnemonic processing should not be regarded as statistical noise or experimental confounds, but rather as a biologically meaningful dimension along which vulnerability and resilience to stress-related disorders may emerge (de Boer et al., 2017; Franklin et al., 2012; Koolhaas et al., 2010; Rab & Admon, 2021). As central regulators of stress and memory, noradrenergic and glucocorticoid systems are likely important sources of such interindividual variability. Importantly, these systems may not directly determine behavior under basal conditions, but instead shape how individuals respond to stress, arousal, and pharmacological modulation.

Interindividual Differences as Moderators of Neural Activity and Behavior

In **Chapters 3 and 4**, I used two rat lines that differed in their HPA-axis negative feedback regulation to examine whether such variation influenced memory and underlying neurocircuitry. Under basal (i.e. non-stressed, non-treated) conditions, no overt behavioral and neural differences were observed in the Low line compared to the Control line. Specifically, in **Chapter 3**, no differences were observed in the strength or generalization of fear and extinction memories. Likewise, **Chapter 4** revealed no significant differences in DMN connectivity. Note that these assessments were conducted under limited conditions, and stronger HPA-axis regulation may influence other behaviors and neural circuits not examined here. Consistent with this, studies using the same rat lines reported differences in extinction memory strength when using a less emotionally-arousing extinction protocol (Monari et al., 2024), but did not find differences in anxiety-related behavior or stress-induced aggression (Walker et al., 2017; Walker & Sandi, 2018).

At first glance, these null findings might suggest limited functional relevance. However, the absence of overt differences does not preclude underlying alterations in brain function and structure. Low line rats presented elevated CA3 activity independent of treatment condition in **Chapter 3**, as well as localized changes in temporal subregions of the DMN and distributed changes in whole-brain functional connectivity and volumetry mainly across hippocampal and temporal regions in **Chapter 4**. Further, in **Chapter 4**, despite no differences in DMN connectivity between lines, correlational analysis showed that in the Control line increased glucocorticoid levels are associated with reduced DMN engagement, whereas this relationship was absent in the Low line. This pattern was mirrored in **Chapter 3** where *Fkbp5* expression, a marker of corticosteroid receptor activation, correlated with circulating glucocorticoid levels in the Control line, but not in the Low line. These findings suggest that stronger HPA-axis negative feedback regulation is accompanied by a disruption in glucocorticoid modulation of molecular and neural mechanisms and a reorganization of stress- and memory-related circuitry that remains behaviorally silent under basal conditions.

One possibility is that compensatory processes, such as noradrenergic signaling,

maintain stable behavior despite underlying circuit differences. Endogenous conditions may simply not push the system into a range in which DG-dependent computations diverge sufficiently to influence behavior. Correlational analyses in **Chapter 3** suggest a disruption in MR-dependent *Fkbp5* expression in the Low line under basal conditions, which can alter GR responsivity to increased glucocorticoid levels (Hartmann et al., 2021). In this manner, latent neurobiological differences may only become functionally relevant under conditions of glucocorticoid challenge. This became apparent following exogenous glucocorticoid administration after extinction training. In **Chapter 3**, glucocorticoids dose-dependently enhanced extinction strength and generalization in Control line rats. In contrast, Low line rats did not respond to glucocorticoid treatment. Thus, pre-existing differences in stress hormone regulation did not manifest under basal conditions, but emerged when the system was pharmacologically challenged.

Neural findings paralleled this behavioral pattern. In **Chapter 3**, in Control line rats, glucocorticoid administration reduced DG recruitment, whereas this effect was absent in Low line rats. Instead, glucocorticoid administration in these animals was associated with increased connectivity between the DG and prelimbic cortex (PrL), even in the absence of a change in extinction memory strength and generalization. It has been shown that dorsal hippocampus-PrL connections support context-dependent extinction expression (Mourão et al., 2026), and that activation of direct projections between these regions strengthens fear memories and suppresses extinction memories (Ye et al., 2017). As such, this shift in network dynamics suggests that glucocorticoids, rather than facilitating extinction memory, may promote the maintenance of established fear representations in the Low line. These findings support that HPA-axis regulation, or more broadly, the individual physiological context, may determine mnemonic “strategy” upon glucocorticoid exposure: stronger HPA-axis regulation may bias the system toward stability and rigid maintenance of established fear responses, rather than flexibly acquiring new knowledge. This interpretation is consistent with evidence that glucocorticoids can shift behavior away from flexible hippocampal processing and toward striatum-based habitual response strategies (Giovanniello et al., 2025; Schwabe & Wolf, 2009; Siller-Pérez et al., 2017). Critically, such a shift in mnemonic strategy has been attributed to MR signaling (Vogel et al., 2016).

In this sense, HPA-axis regulation may be better conceptualized not as a direct driver of differential mnemonic processing, but as a moderator of responsivity to neuromodulatory signals. Variability in molecular and neurotransmitter systems may define the operating range, thereby determining how strongly and in which direction a system reacts when challenged.

Origins of Interindividual Differences

Evidence shows that both pre-existing vulnerability and stress-induced changes contribute to interindividual differences in noradrenergic and glucocorticoid signaling. In **Chapter 3 and 4**, I used a rat model with pre-existing differences in glucocorticoid signaling. Even in the absence of prior stress, Low line rats presented higher blood mRNA levels of *Nr3c2* (encoding MR) and *Fkbp5* (encoding corticosteroid receptor co-chaperone) in **Chapter 3**, alongside gene expression alterations in GABAergic, vasopressinergic, and adrenal signaling pathways reported previously (Walker et al., 2017). Owing to the selective breeding process and based on the high heritability of glucocorticoid responsiveness in these rat lines (Walker et al., 2017), HPA-axis regulation status is most likely transmitted intergenerationally in this rat model via genetic variations, potentially in the MR gene.

Even when exposed to the same stressor, some individuals show robust glucocorticoid increases, whereas others exhibit minimal or no response (Henckens et al., 2016; Man et al., 2023). This interindividual variability appears relatively stable across repeated mild-to-moderate stress exposures (Cohen & Hamrick, 2003). However, repeated or severe stress, particularly during early neurodevelopmental periods (Lupien et al., 2009), may alter glucocorticoid-related signaling, especially via interactions with genetic risk variants (Zannas et al., 2016). In a rat model of early life stress, glucocorticoid administration failed to suppress fear recall, mirroring the resistance observed in the Low line in **Chapter 3** (Atsak et al., 2018). This resistance was linked to an uncoupling between glucocorticoid and endocannabinoid signaling. These findings suggest that stronger HPA-axis negative feedback regulation may, to some extent, recapitulate key effects of early life stress by disrupting the integration of glucocorticoid signaling with downstream molecular pathways.

This pattern also highlights how interindividual differences may arise from interactions with other neuromodulatory and hormonal systems. For instance, early life stress can alter MR expression in a sex-dependent manner, and estrogens are known to modulate both noradrenergic and glucocorticoid signaling, contributing to sex differences in memory, fear expression, extinction, and generalization (Stockhorst & Antov, 2016). More broadly, mnemonic processing depends on interactions between glucocorticoids, norepinephrine, glutamate, dopamine, endocannabinoids, and GABA (Aston-Jones & Cohen, 2005; Atsak et al., 2012; McIntyre et al., 2012). Alterations may arise at multiple levels—from hormone release and receptor expression to intracellular signaling, synaptic plasticity, and large-scale circuit organization. As a result, distinct biological pathways may converge on similar behavioral outcomes, helping to explain the limited success of reductionist “one molecule, one behavior” disease models in psychiatry.

TRANSLATIONAL IMPLICATIONS TOWARDS STRESS-RELATED DISORDERS

Rodent Models and Translational Relevance

The fundamental research described in this thesis aims to understand how stress hormones, whether endogenously regulated or exogenously administered, influence memory and neurocircuitry. Rat models were used in all experimental chapters, as fear and memory are highly conserved processes across species. Indeed, stress and memory research is one of the few areas in neuroscience in which findings from basic animal studies have translated relatively directly into clinical applications (De Quervain et al., 2019).

In **Chapters 3 and 4**, I used a rat model characterized by stronger HPA-axis negative feedback regulation (as measured by the low-dose dexamethasone suppression test (DST)) and lower post-stressor glucocorticoid levels relative to a Control line. Similar endocrine features have been described in subsets of PTSD patients, for example stronger HPA-axis suppression following dexamethasone administration (Yehuda et al., 1993). In addition, blood measurements revealed increased *Fkbp5* and *Nr3c2* (MR) expression, but unchanged *Nr3c1* (GR) expression in the Low line rats. Interestingly, decreased FKBP5 methylation and altered FKBP5 expression have also been reported in PTSD populations (Klengel et al., 2013). Importantly, this is a rat model of differential HPA-axis regulation rather than PTSD *per se*, enabling the study of glucocorticoid signaling in the absence of trauma-associated neuroplasticity. In humans, such endocrine and molecular alterations are difficult to dissociate from the effects of trauma itself. This approach therefore allows assessment of the extent to which these biological characteristics contribute to vulnerability prior to trauma exposure and to symptoms commonly observed in PTSD.

Such a model also allows for the identification of new targets, biomarkers and important players in a specific endocrine phenotype. For example, as discussed in previous sections, evidence suggests an important role of MR signaling for stronger HPA-axis negative feedback regulation, and related neural and behavioral alterations. Although MRs have also been implicated in memory processes (Atucha et al., 2015; Vogel et al., 2016), they have received little attention in PTSD research compared to GRs.

Importantly, these rat lines were generated via selective breeding. Selective breeding and the consequent enrichment for genetic variants related to a specific glucocorticoid signaling phenotype is congruent with the polygenic nature of PTSD (Waszczuk et al., 2022). On the other hand, this method also entails the possibility of co-selection for changes in neurobiological systems other than the one targeted. For example, it is interesting to note that elevated noradrenergic activation has been described in the Low line, despite not being a direct target of the breeding selection process. Specifically, Low line rats have been shown to exhibit elevated norepinephrine levels in the DG during REM sleep, reduced vagal tone and elevated heart rate in response to stressors compared to the Control line (Huzard et al., 2019; Monari et al., 2024). Given

that chronic hyperarousal states are commonly observed in PTSD, these observations raise questions on how changes in the glucocorticoid system may interact with the noradrenergic system to contribute towards the pathophysiology of PTSD.

Together, these characteristics of the rat model support a phenotype consistent with that described in many patients with PTSD. However, PTSD is a highly heterogeneous disorder comprising multiple subtypes (e.g., dissociative, complex), which are likely underpinned by distinct pathophysiological mechanisms. As such, it is important to keep in mind that rodent psychiatric models can only be as informative as the clinical phenotypes they are designed to mimic, underscoring the need for continued refinement of human disorder characterization.

Bridging Rodent and Human Research

Despite the translational success of the stress and memory field, translating findings between rodent and human research in neuroscience remains challenging. Rodent and human studies each provide distinct advantages, and translation is likely to improve most when these strengths are deliberately combined. In contrast to human studies, rodent research allows for more invasive procedures, such as the local drug infusions performed in **Chapter 2**, as well as the assessment of molecular markers within the brain, such as measurement of hippocampal GR phosphorylation levels in **Chapter 3**. Additionally, the possibility of selective breeding or genetic manipulations enables the generation of models with stable endocrine phenotypes and experimentally controlled interindividual variability, such as our stronger HPA-axis negative feedback regulation rat model in **Chapters 3 and 4**.

On the other hand, human research provides clear advantages in terms of ecological validity, clinical relevance, and subjective phenomenology. Rather than viewing these approaches separately, there is a need to align rodent and human research within a bidirectional translational framework. **Chapters 3 and 4** were embedded within such an approach, comprising parallel rodent and human research branches. The patient project first aims to examine whether trauma history (e.g., early life stress, single or multiple traumatic events) was associated with individual differences in HPA-axis regulation in PTSD patients, and further whether they were associated with treatment outcomes (de Noij et al., 2024). The second aim is to test whether glucocorticoid administration would differentially enhance extinction memory, and extinction-related neural activity, as a function of HPA-axis regulation in PTSD patients. As such, the human branch aimed to validate a stratification and personalized treatment approach based on HPA-axis regulation, while the rodent branch provided mechanistic evidence at the molecular, neural, and behavioral levels without the confounding influence of trauma, past negative experiences or PTSD pathology. These complementary approaches strengthen the proof-of-concept for targeting stress-hormone regulation to optimize extinction-based interventions.

To increase translatability, methodological alignment between species was prioritized. Both branches employ the low-dose DST as a measure of HPA-axis negative feedback

regulation (**Chapter 3**), use an acute stress challenge to assess stress-induced glucocorticoid response (**Chapter 3**), use MRI-based imaging to assess structural and functional brain alterations (**Chapter 4**), quantify circulating molecular markers of glucocorticoid signaling (**Chapter 3**), and implement homologous fear conditioning and extinction paradigms (**Chapter 3**). This cross-species harmonization enhances interpretability and reduces the conceptual gap that often limits translational neuroscience.

Rethinking Stress Hormone Administration for Prevention and Treatment of PTSD

Several studies have examined stress hormone administration for prevention and as adjuncts for exposure-based therapies for stress-related disorders, such as PTSD. Initial findings suggested that stress hormone augmentation could enhance therapeutic outcomes, consistent with their well-established effects on memory consolidation (Brueckner et al., 2019; De Quervain et al., 2017, 2019; Meir Drexler et al., 2019; Merz et al., 2018), however, results have not been uniformly positive (Argolo et al., 2015; Inslicht et al., 2022; Lehrner et al., 2021; Yehuda, Bierer, et al., 2015). A key limitation of these approaches is their primary focus on memory strength—namely, the enhancement or impairment of consolidation—as the principal determinant of therapeutic success (De Quervain et al., 2017, 2019). Yet, the high incidence of relapse in PTSD highlights that reducing fear expression alone is insufficient. Rather, the ability of extinction memories to generalize across contexts appears to be critical, as PTSD is not merely characterized by strong aversive memories, but also by the overgeneralization of fear responses to safe contexts and cues (Lissek, 2012). In this framework, the findings presented in **Chapters 2 and 3** suggest that the suitability of stress hormones as treatment adjuvants depends not only on their effects on consolidation, but also on how they modulate memory specificity and generalization. Importantly, these questions have not yet been systematically tested in human research and clinical settings. However, if similar effects on memory specificity and generalization are observed in humans, they could have major implications for the selection of pharmacological targets for each intervention stage (Figure 2).

In predictable high-risk settings, pharmacological approaches to prevent the development of PTSD rely primarily on the idea that this risk could be attenuated by disrupting the consolidation or reconsolidation of aversive memories (Schelling et al., 2004). It has been proposed that noradrenergic and glucocorticoid signaling could be harnessed for this purpose via receptor antagonists (De Quervain et al., 2017). The findings in **Chapter 2** suggest that this idea may be incomplete, as pharmacological blockade of noradrenergic signaling during consolidation (e.g., via propranolol) may not only reduce memory strength, but could also inadvertently lead to reduced contextualization and more generalized fear memory, potentially undermining therapeutic goals (Figure 2). In line with this, repeated propranolol administration starting 6h after a traumatic event did not prevent PTSD development in emergency department patients (Hoge et al., 2012; Pitman et al., 2002). In contrast, GR antagonists may both reduce memory strength and generalization, therefore

showing greater promise for effective prevention of PTSD (Figure 2). Notably, the miR-134–dependent mechanism identified in **Chapter 2** suggests that memory specificity can be modulated without necessarily impairing consolidation, as they may be underlined by (partially) distinct mechanisms. Harnessing such a specificity-enhancing mechanism could therefore, in principle, reduce the indiscriminate retrieval of traumatic memories while preserving the integrity of the memory itself, thereby limiting the development of PTSD-like symptomatology. Although the translational feasibility of targeting this mechanism remains to be established, these findings highlight the potential value of shifting prevention strategies from memory suppression toward the regulation of memory specificity.

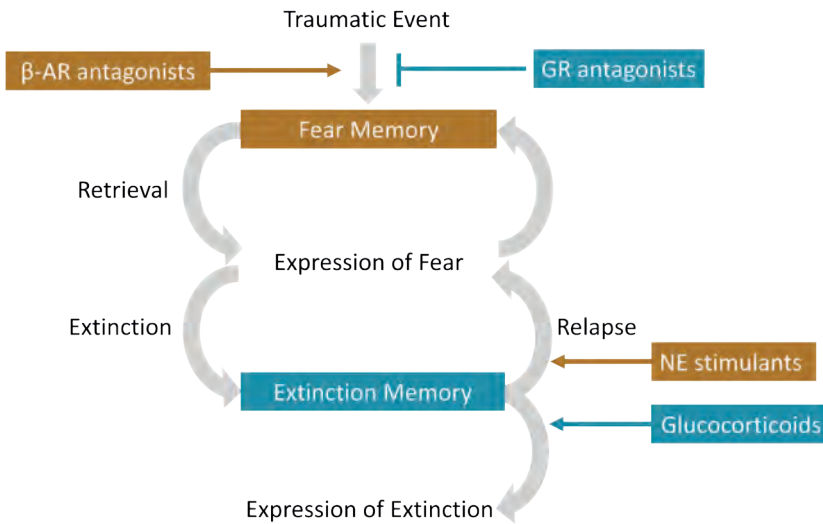


Figure 2 – Rethinking stress hormone administration for the prevention and treatment of PTSD. Adapted from De Quervain et al. (2017). Stress hormone interventions that may undermine therapeutic goals are denoted in yellow, whereas interventions that may prevent PTSD or promote treatment success are denoted in blue.

During extinction-based treatments such as exposure therapy, stress hormone administration has been proposed as a means to enhance extinction consolidation. However, effective treatment requires not only the formation of extinction memories, but also their generalization beyond the therapeutic context. The opposite regulation of memory specificity and generalization by stress hormone signaling indicates that the choice of which hormone to use in treatment should be carefully considered. Findings from **Chapter 2** support that, while noradrenergic stimulation could facilitate extinction through enhanced consolidation, it might simultaneously increase memory specificity, thereby potentially strengthening the context dependency of extinction memories and increasing the likelihood of relapse (Figure 2). In contrast, as demonstrated in **Chapter 3**, post-extinction glucocorticoid administration can

enhance both extinction memory consolidation and generalization across contexts (Figure 2). These results suggest that glucocorticoids may represent a particularly promising target for augmenting exposure-based therapies. Glucocorticoid-based treatments may be especially effective when combined with novel extinction protocols designed to promote generalization, such as exposure across multiple contexts or variable cues (de Jong et al., 2019; Gold, 2008; Hoppen et al., 2024).

Considerations for Translation into Clinical Research

Several factors should be considered when translating these findings to clinical research. First, the generalization-enhancing effects of glucocorticoids observed in **Chapter 3** were demonstrated in the context of a recent fear memory (i.e., extinction training was performed 1 day after fear conditioning). However, clinically relevant traumatic memories often refer to events that may have occurred years before treatment, and have therefore undergone systems consolidation. In contrast to recent memories, which rely more strongly on hippocampal processing, systems-consolidated remote memories are supported by distributed cortical networks (Frankland & Bontempi, 2005) and may therefore be less sensitive to hippocampal modulation. As demonstrated in **Chapters 2 and 3**, the effects of stress hormones on memory specificity and generalization appear to rely on hippocampal mechanisms. Thus, it is unclear to which extent these findings may be applicable to the treatment of remote traumatic memories. Future preclinical work will be necessary to determine whether glucocorticoid-based interventions can similarly enhance generalization when applied to systems-consolidated remote fear memories, which more closely resemble the conditions commonly observed in PTSD patients.

Second, it is critical to consider the timing of stress hormone administration within the treatment procedure. Based on their known effects on memory retrieval, it has been proposed that administering stress hormones prior to extinction training may produce a dual effect: impairing retrieval of the original aversive memory while simultaneously enhancing extinction learning (De Quervain et al., 2017). Some studies have further supported that such pre-training administration may promote greater context independence compared to post-extinction administration (Meir Drexler et al., 2019). Importantly, as discussed in previous sections, the effects of glucocorticoids on generalization appear to depend on the specific memory representations that are engaged during the learning experience (Guo, 2024). As such, previous experience, as well as the requirement to discriminate between similar contexts or cues, may influence how glucocorticoid administration modulates memory generalization, either expanding dependency to additional contexts or rendering memory fully context-independent. Therefore, careful consideration of environmental cues, timing and treatment structure may be essential to optimize the therapeutic effects of glucocorticoid administration.

Third, evidence from both clinical and preclinical studies suggests that a single administration of stress hormones may be insufficient to produce long-lasting

therapeutic effects (Putman & Roelofs, 2011). In line with this, post-extinction glucocorticoid administration in **Chapter 3** did not prevent renewal at a 14-day extinction recall test, supporting that repeated administrations may be necessary to sustain treatment benefits. At the same time, the potential side effects associated with repeated glucocorticoid administration require careful consideration of dosing schedules. Thus, it may be advantageous to target specific treatment sessions in which stress hormone augmentation is most likely to enhance long-term generalization, for example, during exposure to multiple contexts. An additional, less invasive approach may involve leveraging the endogenous glucocorticoid circadian rhythm. For example, aligning treatment sessions with the cortisol awakening response or early morning peaks in endogenous glucocorticoid levels has been shown to enhance extinction memory consolidation (Meuret et al., 2015, 2016). Given the findings in **Chapter 3** that exogenous glucocorticoids may also facilitate generalization, it would be important to determine whether scheduling treatment sessions during circadian peaks in glucocorticoid levels similarly enhances the generalization of extinction memory. Such timing-based strategies may provide a means to minimize both the chance of relapse as well as pharmacological burden.

Finally, while the inverted-U dose-dependent effects of stress hormone administration have been extensively established on memory consolidation, it is still unclear whether their effects on memory specificity and generalization follow the same pattern. Such a relationship implies that treatment efficacy depends on endogenous hormone levels, such that even modest glucocorticoid administration may be detrimental when baseline levels are already elevated (Akirav et al., 2004). As such, establishing the dose-effect relationship will be paramount to clarifying how endogenous and exogenous levels of stress hormones may interact to modulate memory specificity and generalization. Importantly, despite the mechanistic promise of glucocorticoid interventions on extinction-based treatment, some studies have been unable to show an uniformly beneficial role of glucocorticoids across patients, hinting at an important role of interindividual variability in determining treatment outcome.

Vulnerability & Prevention

Endogenous differences in stress hormone regulation may not only influence mnemonic processing, but also provide a window into vulnerability for stress-related psychopathology. In **Chapter 4**, enhanced HPA-axis negative feedback regulation was associated with localized alterations in brain structure and functional connectivity, despite the absence of overt differences in DMN connectivity under basal conditions. Importantly, the dissociation between glucocorticoid levels and DMN engagement suggests that such differences may only become apparent under stress or glucocorticoid challenge. In line with this, previous work has suggested that an inability to appropriately modulate the DMN in response to stress may represent a biomarker of vulnerability to stress-related psychopathology, including PTSD (van Oort et al., 2020). In line with this, Miller et al. (2017) showed that reduced DMN connectivity was associated with greater PTSD symptom severity, but only in

individuals with elevated polygenic risk for psychopathology, pointing to a gene-by-environment interaction. Together, these findings suggest that alterations in DMN connectivity may reflect a genetically and endocrinologically mediated vulnerability that becomes functionally relevant following trauma exposure. Identifying such genetic and endocrine risk profiles may therefore have important implications for prevention, as individuals with altered HPA-axis responsivity or increased genetic risk may benefit from early monitoring or targeted preventive interventions.

Stratification & Personalized Treatment

Recent randomized controlled trials have not shown uniformly beneficial effects of stress hormone augmentation in PTSD treatment. Whereas some patients showed marked improvement, others showed little benefit (De Quervain et al., 2017; Lehrner et al., 2021; Yehuda, Bierer, et al., 2015). Such findings highlight the high heterogeneity across the PTSD patient population, and raise the possibility that specific subgroups of patients may respond differently to stress hormone-based interventions.

In line with this idea and results from previous studies (Monari et al., 2024), the initial hypothesis underlying **Chapter 3** was that stronger HPA-axis negative feedback regulation and lower glucocorticoid levels might contribute to memory-related impairments, and that such impairments caused by presumed glucocorticoid signaling insufficiency could therefore be corrected by exogenous glucocorticoid administration, as previously observed in adrenalectomy studies (Borrell et al., 1983, 1984; McCormick et al., 1997). It was thus expected that animals with stronger HPA-axis negative feedback regulation would show greater benefit from glucocorticoid treatment. However, the findings in **Chapter 3** did not support this prediction. In contrast to Control line rats, glucocorticoid administration did not alter extinction memory strength and generalization in Low line rats. These findings therefore suggest that glucocorticoid treatment may not be universally beneficial, particularly in individuals characterized by stronger HPA-axis negative feedback regulation.

Critically, the neural findings in Low line rats raise the possibility that glucocorticoid treatment could even have adverse effects in some subgroups. In **Chapter 3**, glucocorticoid administration increased DG–prelimbic cortex connectivity in Low line rats, a pathway that may strengthen fear memories and suppress extinction memories (Ye et al., 2017). Because the experimental design did not include a novel context, it remains unclear whether in our paradigm this neural pattern reflects a neutral effect or a shift toward greater fear memory generalization at the expense of the extinction memory. If the latter is true, glucocorticoid administration could be harmful rather than merely ineffective in individuals with stronger HPA-axis negative feedback regulation. These findings therefore make patient stratification even more important: rather than simply identifying individuals most likely to benefit from glucocorticoid treatment, it may also be necessary to identify those who are at greater risk of adverse outcomes.

Together, the findings from **Chapter 3** argue strongly against uniform treatment

approaches across PTSD patient populations and instead support biomarker-guided stratification. For example, it has been shown that low glucocorticoid levels after a motor vehicle accident may predict development of PTSD (Delahanty et al., 2000). However, it is important to note that: 1) basal measurements are not necessarily predictive of stress-induced levels (Ryan et al., 2016), owing to the partially distinct mechanisms, thereby limiting predictive functional relevance; and 2) as demonstrated in **Chapters 3 and 4**, similar basal glucocorticoid levels may conceal important physiological differences in HPA-axis regulation that critically influence treatment response. Functional biomarkers may therefore be more informative than static measurements. Such biomarkers could include variations of the DST as performed in **Chapter 3**, or measures of DMN responsivity to glucocorticoids (Doucet et al., 2020; van Oort et al., 2020). Combining these approaches with genetic and state-dependent epigenetic markers, such as FKBP5 polymorphisms and gene expression, may provide a more accurate characterization of biologically distinct PTSD subtypes.

It is also important to recognize that HPA-axis alterations may not always represent stable trait markers. Some evidence suggests that successful treatment can normalize aspects of HPA-axis negative feedback regulation (Yehuda, Bierer, et al., 2015), particularly if changes occurred in adulthood (Lupien et al., 2009), indicating that such endocrine differences may reflect current symptom state rather than enduring vulnerability. In contrast, physiological alterations linked to early life stress, or prolonged trauma exposure may be more persistent (Lupien et al., 2009). As such, distinguishing between state- and trait-like biomarkers may be essential for biomarker-guided personalized treatment approaches. More broadly, mnemonic processing depends on the interaction between many different neuromodulatory systems. As such, beyond the HPA axis, interindividual variability in other neuromodulatory systems, such as the endocannabinoid system (Campolongo & Trezza, 2012; Hill & McEwen, 2009), may further influence extinction learning, stress reactivity, symptomatology and pharmacological response in PTSD populations. Therefore, a comprehensive personalized approach will likely require multi-system profiling rather than single-marker prediction.

Finally, the findings in **Chapter 3** highlight a central limitation in both animal and human research: the implicit assumption of homogeneous physiological backgrounds. Even when patient studies apply strict inclusion and exclusion criteria, substantial pathophysiological variability may remain within the included sample. Ignoring this variability may obscure subgroup-specific effects and contribute to inconsistent findings across studies and clinical trials (Kent et al., 2010; Malone et al., 2014). As such, it is paramount to include relevant physiological measurements when evaluating a drug for potential treatment. In addition, it is crucial to continue efforts towards characterizing biologically distinct PTSD subgroups, for example, by using clustering approaches (Guðmundsdóttir et al., 2025) that integrate genetic, endocrine, neural, behavioral and psychological measures (Hellhammer et al., 2018). Such approaches may not only improve patient stratification and treatment selection, but also guide the development of more translationally relevant animal models that

model distinct PTSD subtypes rather than the disorder as a whole.

CONCLUSION

In this thesis, I demonstrate that stress hormones do not merely strengthen memories during consolidation, but also influence more subtle aspects of memory: norepinephrine biases memory toward specificity and glucocorticoids toward generalization. These effects are expressed across molecular, circuit, and behavioral levels, with the DG emerging as a key substrate through which stress hormones influence pattern separation and consequently memory specificity and generalization. Together, these findings extend memory modulation theory by incorporating memory specificity and generalization as core dimensions of stress hormone action. Importantly, these processes are not uniform across individuals: latent differences in endogenous stress hormone regulation reshape neural circuitry and determine responsiveness to glucocorticoid modulation, revealing that variability in physiological state critically influences memory processing. As such, this work further provides a mechanistic framework linking stress hormone signaling to variability in fear- and -extinction related memory processing, offering new insight into vulnerability, resilience, and treatment response in stress-related disorders such as PTSD. More broadly, these findings underscore the need to move beyond “one-size-fits-all” approaches toward biologically informed, individualized models of psychiatric intervention in which treatments are tailored to the biological characteristics of each patient rather than to the diagnostic label alone.



APPENDIX

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ENGLISH SUMMARY

Emotionally arousing and stressful experiences tend to be better remembered than neutral ones. Emotional arousal and stress influence memory via the activation of different stress hormone systems, such as norepinephrine and glucocorticoids (corticosterone in rodents, cortisol in humans). While early research emphasized the role of stress hormones in strengthening the consolidation of memory, accumulating evidence indicates that they may also modulate more fine-grained aspects, such as memory specificity and generalization. From an adaptive perspective, prioritizing emotionally salient information is essential for survival; however, the use of previously acquired knowledge must remain flexible to be appropriately applied to future novel situations. Such characteristics are particularly relevant in stress-related psychopathologies, including post-traumatic stress disorder (PTSD), where fear overgeneralization and impaired safety learning (i.e., extinction) are thought to contribute to the persistence of symptoms.

Crucially, individuals differ markedly in their physiological response to emotional arousal and stress, as well as in the underlying noradrenergic and glucocorticoid signaling. Given the influence of these neuromodulators on memory, interindividual variability in endogenous stress hormone signaling may lead to differential memory processing of emotional events. Such differential processing, resulting in either more specific or more generalized memories, may therefore contribute to individual differences in vulnerability and treatment response in PTSD. In this thesis, I examine, in rats, how the stress hormones norepinephrine and glucocorticoids modulate memory specificity and generalization, and associated neural circuitry, with particular attention to how endogenous signaling differences influence these processes.

In **Chapter 2**, I examined the neural and molecular mechanisms through which norepinephrine enhances memory specificity. In real life, our experiences are often composed of highly similar events which can be confused and thus prone to incorrect associations being formed. Recent work in rats has demonstrated that systemic norepinephrine administration enhances memory specificity in a dual-event inhibitory avoidance task, an episodic-like learning task in which animals are subsequently exposed to two similarly looking boxes, but receive footshock in only one box. It had not been investigated whether this specificity-enhancing effect is mediated by noradrenergic signaling within the basolateral amygdala and its downstream influence on plasticity in other brain regions. Using this dual-event inhibitory avoidance task, I first showed that norepinephrine infusions into the basolateral amygdala after the training experience not only strengthened memory of the footshock, but also facilitated the separation of the two training events experienced close in time. As a consequence, norepinephrine-treated rats selectively avoided the box in which they had received shock. I then demonstrated that this memory specificity-enhancing effect of norepinephrine is mediated by a miR-134-regulated consolidation mechanism in the dentate gyrus (DG) of the hippocampus, whereby downregulation of miR-134 induces memory specificity. To determine whether this mechanism is engaged under conditions in which memory specificity

naturally occurs, I increased the interval between the two training events and either blocked noradrenergic activation in the basolateral amygdala or upregulated miR-134 levels in the dorsal hippocampus. Both manipulations prevented memory specificity; however, miR-134 upregulation did not affect memory of the footshock. Consistent with this, training on a single event failed to engage the hippocampal miR-134 mechanism, confirming that it is selectively recruited when discrimination between similar experiences is required. This chapter identifies a miR-134-dependent consolidation mechanism in the hippocampus that selectively regulates memory specificity. Importantly, it demonstrates that noradrenergic activation of the basolateral amygdala produces two independent effects: it enhances the strength of the memory, and facilitates memory specificity by promoting the separation of memories of two events experienced close in time. Together, these two processes can ensure that only correct associations are strengthened.

In **Chapter 3**, I investigated whether individual differences in glucocorticoid regulation influence the strength and generalization of safety memory, as well as their interaction with glucocorticoid treatment. Glucocorticoids have been explored as pharmacological adjuvants to extinction-based therapies in PTSD patients, although findings have been mixed, pointing to subgroup-dependent effects. Glucocorticoids are released by the hypothalamic-pituitary-adrenal (HPA) axis into the blood stream, which in turn inhibit HPA-axis activity to ensure that glucocorticoid effects do not last too long. Stronger HPA-axis negative feedback regulation has been reported in some groups of PTSD patients; however, it remains unclear whether interindividual differences in endogenous glucocorticoid regulation influence responsiveness to extinction-based treatment and glucocorticoid augmentation. For this purpose, I used a rodent model, which allows for the study of underlying neural and molecular mechanisms. I first established that a rat line selectively bred for blunted post-stressor corticosterone levels (i.e., Low line) also exhibits stronger HPA-axis negative feedback regulation compared to a Control line, along with increased expression of HPA-axis-related genes in blood. I then assessed whether these two lines differ in extinction memory using a fear conditioning and extinction paradigm, in which extinction memory was tested in both the conditioning and extinction contexts enabling the assessment of its strength and generalization, respectively. Under non-treated conditions, the two lines did not differ in strength or generalization of extinction memory. However, glucocorticoid administration immediately after the extinction training enhanced both the strength and generalization of extinction memory in the Control line. In contrast, glucocorticoid administration to the Low line did not affect extinction memory, suggesting a resistance to glucocorticoid treatment. Consistent with the behavioral results, at the neural level, glucocorticoid treatment in the Control line was associated with reduced DG activity, whereas in the Low line this reduction was absent and accompanied by increased DG–prefrontal cortex connectivity. This chapter shows that glucocorticoid treatment effects on memory strength and generalization diverges between rat lines with differential HPA-axis negative feedback regulation, indicating that endogenous HPA-axis regulation moderates

responsiveness to glucocorticoid treatment.

In **Chapter 4**, I used the same two rat lines to assess whether stronger HPA-axis negative feedback regulation also influences brain structure and connectivity. Stress and glucocorticoids are known to influence neural plasticity through mechanisms such as dendritic remodeling, neurogenesis and myelination. They have also been shown to modulate large-scale brain networks, such as the default mode network, which is involved in internally directed thought and shows altered connectivity in PTSD. However, it is unclear to what extent stable interindividual differences in endogenous glucocorticoids and their regulation by the HPA axis may influence brain connectivity and structure, and contribute towards PTSD vulnerability. To this end, I performed an *a priori* confirmatory analysis of the default mode network as well as exploratory whole-brain analysis in the same rat lines, using a multimodal magnetic resonance imaging (MRI) approach. No differences were found in default mode network connectivity between the two lines. In the Control line, increasing glucocorticoid levels were associated with decreased default mode network engagement. However, in the Low line this relationship was absent, suggesting altered network sensitivity to glucocorticoid modulation. In addition, the Low line exhibited a larger volume of the temporal association cortex, a node of the default mode network. Beyond this network, I identified a range of structural and connectivity differences between the two lines, primarily in hippocampal and temporal regions. Notably, the bed nucleus of the stria terminalis, a brain region that is importantly involved in fear generalization, was larger in the Low line. No evidence of differences in white matter microstructure was found between the two lines. These findings show that stronger HPA-axis negative feedback regulation is associated with changes in brain structure and functional connectivity, which may comprise a pre-existing vulnerability toward PTSD.

Across experiments, norepinephrine and glucocorticoids exerted opposing effects, with noradrenergic activation promoting memory specificity and glucocorticoid signaling promoting generalization, particularly under conditions requiring discrimination between experiences. These effects were not attributable to changes in encoding or retrieval. Instead, they reflect consolidation-dependent processes, likely mediated through interactions between the basolateral amygdala and DG of the hippocampus. At the molecular level, opposing regulation of plasticity-related pathways, including miR-134-dependent mechanisms in the DG, may underlie these divergent outcomes. My findings extend the memory modulation theory by showing that stress hormones not only enhance consolidation, but they serve opposing roles in determining discrimination of episodic-like memories in the hippocampus, biasing memory towards specificity or generalization. These findings also have direct translational relevance, suggesting that pharmacological treatment strategies targeting stress hormone systems should consider their effects on memory specificity and generalization as key factors determining treatment success. Critically, I showed that interindividual differences, such as in the regulation of endogenous glucocorticoid signaling by the HPA axis, influence brain structure and determine

response to exogenous glucocorticoid administration both at the behavioral and neural level. As such, the findings in this thesis underscore the importance of moving away from uniform treatment approaches toward a more personalized model of psychiatric treatment for PTSD, in which interventions are tailored to the biological characteristics of each patient rather than to the diagnostic label alone.

NEDERLANDSE SAMENVATTING

Emotioneel opwindende en stressvolle ervaringen worden doorgaans beter onthouden dan neutrale ervaringen. Emotionele opwindende en stress beïnvloeden het geheugen via de activatie van verschillende stresshormoonssystemen, zoals noradrenaline en glucocorticoïden (corticosteron bij knaagdieren, cortisol bij mensen). Waar vroeger onderzoek vooral de rol van stresshormonen in het versterken van het geheugen benadrukte, is er nu meer onderzoek dat laat zien dat ook meer verfijnde aspecten van het geheugen worden beïnvloed, zoals geheugenspecificiteit en generalisatie. Vanuit een perspectief van adaptatie is het prioriteren van emotioneel relevante informatie essentieel voor overleving; tegelijkertijd moet het gebruik van eerder verworven kennis flexibel blijven om adequaat te kunnen worden toegepast in nieuwe, onbekende situaties. Deze eigenschappen zijn met name relevant bij stressgerelateerde psychopathologieën, waaronder posttraumatische stressstoornis (PTSS), waarbij overgeneralisatie van angst en verstoord veiligheidsleren (d.w.z. extinctie) bijdragen aan het voortbestaan van symptomen.

Cruciaal is dat individuen sterk verschillen in hun fysiologische respons op emotionele opwindende en stress, evenals in de onderliggende noradrenerge en glucocorticoïde signalering. Gezien de invloed van deze neuromodulatoren op het geheugen kan interindividuele variabiliteit in endogene stresshormoonsignalering leiden tot verschillen in de verwerking van emotionele gebeurtenissen. Dergelijke verschillen, resulterend in meer specifieke dan wel meer gegeneraliseerde herinneringen, kunnen bijdragen aan individuele verschillen in kwetsbaarheid en behandelrespons bij PTSS. In dit proefschrift onderzoek ik, in ratten, hoe de stresshormonen noradrenaline en glucocorticoïden geheugenspecificiteit en generalisatie moduleren, evenals de bijbehorende neurale netwerken, met bijzondere aandacht voor hoe verschillen in endogene signalering deze processen beïnvloeden.

In **Hoofdstuk 2** onderzocht ik de neurale en moleculaire mechanismen waarmee noradrenaline de geheugenspecificiteit versterkt. In het dagelijks leven bestaan onze ervaringen vaak uit sterk op elkaar lijkende gebeurtenissen, die gemakkelijk verward kunnen worden en daardoor vatbaar zijn voor het vormen van onjuiste associaties. Recent onderzoek bij ratten heeft aangetoond dat systemische toediening van noradrenaline de geheugenspecificiteit verhoogt in een dual-event inhibitory avoidance taak, een episodisch-achtige leertaak waarin dieren worden blootgesteld aan twee visueel vergelijkbare contexten, maar slechts in één daarvan een korte elektrische schok ontvangen. Het was echter nog niet onderzocht of dit effect wordt gemedieerd door noradrenerge signalering in de basolaterale amygdala en de invloed daarvan op plasticiteit in andere hersengebieden. Met behulp van deze taak toonde ik eerst aan dat toediening van noradrenaline in de basolaterale amygdala na de leerervaring niet alleen de herinnering aan de elektrische schok versterkte, maar ook de scheiding bevorderde van twee gebeurtenissen die kort na elkaar plaatsvonden. Hierdoor vermeden ratten die met noradrenaline waren behandeld selectief de context waarin zij de schok hadden ontvangen. Vervolgens liet ik zien dat dit effect op geheugenspecificiteit wordt gemedieerd door een door miR-134 gereguleerd

consolidatiemechanisme in de dentate gyrus (DG) van de hippocampus, waarbij neerregulatie van miR-134 leidt tot verhoogde geheugenspecificiteit. Om te bepalen of dit mechanisme ook actief is onder omstandigheden waarin geheugenspecificiteit van nature optreedt, verlengde ik het interval tussen de twee leergebeurtenissen en blokkeerde ik ofwel de noradrenerge activatie in de basolaterale amygdala, of verhoogde ik de miR-134-niveaus in de dorsale hippocampus. Beide manipulaties voorkwamen geheugenspecificiteit; verhoging van miR-134 had echter geen effect op de herinnering aan de schok zelf. In overeenstemming hiermee werd dit miR-134-mechanisme niet geactiveerd als de dieren in een enkele context werden getraind, hetgeen bevestigt dat dit mechanisme selectief wordt gerekruteerd wanneer discriminatie tussen vergelijkbare ervaringen vereist is. Dit hoofdstuk identificeert een miR-134-afhankelijk consolidatiemechanisme in de hippocampus dat specifiek geheugenspecificiteit reguleert. Belangrijk is dat het aantoonde dat noradrenerge activatie van de basolaterale amygdala twee onafhankelijke effecten heeft: het versterkt de geheugensterkte en bevordert geheugenspecificiteit door de scheiding van herinneringen aan twee dicht opeenvolgende gebeurtenissen te faciliteren. Samen zorgen deze processen ervoor dat alleen correcte associaties worden versterkt.

In **Hoofdstuk 3** onderzocht ik of individuele verschillen in glucocorticoïdregulatie van invloed zijn op de sterkte en generalisatie van veiligheidsleren, evenals op de interactie met glucocorticoïde behandeling. Glucocorticoïden zijn onderzocht als farmacologische aanvulling op extinctiegebaseerde therapieën bij PTSS-patiënten, hoewel de resultaten gemengd zijn en wijzen op groepafhankelijke effecten. Glucocorticoïden worden door de hypothalamus-hypofyse-bijnieras (HPA-as) in de bloedbaan vrijgegeven en remmen vervolgens de activiteit van deze as om te voorkomen dat hun effecten te lang aanhouden. Een sterkere negatieve terugkoppeling van de HPA-as is gevonden bij sommige groepen PTSS-patiënten; het is echter onduidelijk of interindividuele verschillen in endogene glucocorticoïdregulatie de respons op extinctiebehandeling en glucocorticoïde toediening beïnvloeden. Voor dit doel maakte ik gebruik van een diersysteem, dat het mogelijk maakt onderliggende neurale en moleculaire mechanismen te bestuderen. Ik stelde eerst vast dat een rattenlijn die selectief is gefokt voor afgevlakte corticosteronresponsen na stress (de zogenoemde Low-lijn) ook een sterkere negatieve terugkoppeling van de HPA-as vertoont dan een Controlelijn, samen met verhoogde expressie van HPA-as-gerelateerde genen in het bloed. Vervolgens onderzocht ik of deze lijnen verschillen in extinctiegeheugen met behulp van een angstconditionerings- en extinctieleertaak, waarbij extinctiegeheugen werd getest in zowel de conditionerings- als de extinctiecontext om respectievelijk sterkte en generalisatie van het extinctiegeheugen te testen. Zonder glucocorticoïdtoediening verschilden de twee lijnen niet in extinctiegeheugen. Toediening van glucocorticoïden direct na extinctietraining versterkte echter zowel de sterkte als de generalisatie van extinctiegeheugen in de Controlelijn. In de Low-lijn had deze behandeling geen effect, wat wijst op een resistentie tegen glucocorticoïden. In overeenstemming met deze gedragsresultaten ging glucocorticoïdbehandeling in de Controlelijn

gepaard met verminderde neuronale activiteit in de DG, terwijl deze afname in de Low-lijn ontbrak en gepaard ging met verhoogde connectiviteit tussen de DG en de prefrontale cortex. Dit hoofdstuk laat zien dat de effecten van glucocorticoiden op geheugen afhangen van individuele verschillen in HPA-as-regulatie.

In **Hoofdstuk 4** gebruikte ik dezelfde twee rattenlijnen om te onderzoeken of sterkere negatieve terugkoppeling van de HPA-as ook de hersenstructuur en connectiviteit beïnvloedt. Stress en glucocorticoiden beïnvloeden neurale plasticiteit via processen zoals dendritische remodelering, neurogenese en myelinisatie. Daarnaast is aangetoond dat zij grootschalige hersennetwerken moduleren, zoals het default mode network, dat betrokken is bij intern gerichte cognitieve processen en waarvan de connectiviteit veranderd is bij PTSS. Het is echter onduidelijk in hoeverre stabiele interindividuele verschillen in endogene glucocorticoiden en hun regulatie door de HPA-as de hersenconnectiviteit en -structuur beïnvloeden en bijdragen aan kwetsbaarheid voor PTSS. Hiertoe voerde ik een a priori bevestigende analyse van het default mode network uit, evenals een exploratieve whole-brain analyse in dezelfde rattenlijnen, met behulp van een multimodale MRI-benadering. Er werden geen verschillen gevonden in connectiviteit binnen het default mode network tussen de twee lijnen. In de Controlelijn ging een toename in glucocorticoidniveaus gepaard met verminderde betrokkenheid van het default mode network. In de Low-lijn ontbrak deze relatie, wat wijst op veranderde gevoeligheid van het netwerk voor glucocorticoid modulatie. Daarnaast vertoonde de Low-lijn een groter volume van de temporale associatiecortex, een knooppunt van het default mode network. Buiten dit netwerk identificeerde ik een aantal andere structurele en connectiviteitsverschillen tussen de lijnen, voornamelijk in hippocampale en temporale gebieden. Opvallend was dat de bed nucleus van de stria terminalis, een hersengebied dat een belangrijke rol speelt in angstgeneralisatie, groter was in de Low-lijn. Er werden geen verschillen gevonden in witte-stof microstructuur. Deze bevindingen tonen aan dat sterkere negatieve feedbackregulatie van de HPA-as samenhangt met veranderingen in hersenstructuur en functionele connectiviteit, die mogelijk een reeds bestaande kwetsbaarheid voor PTSS weerspiegelen.

Over alle experimenten heen hadden noradrenaline en glucocorticoiden tegengestelde effecten: noradrenerge activatie bevorderde geheugenspecificiteit, terwijl glucocorticoiden generalisatie bevorderden, met name in situaties waarin onderscheid tussen ervaringen vereist is. Deze effecten zijn niet toe te schrijven aan verschillen in codering of ophalen van herinneringen, maar weerspiegelen processen die afhankelijk zijn van consolidatie, waarschijnlijk gemedieerd door interacties tussen de basolaterale amygdala en de DG van de hippocampus. Op moleculair niveau kunnen tegengestelde regulatie van plasticiteitsmechanismen, waaronder miR-134-afhankelijke processen in de DG, aan deze effecten ten grondslag liggen. Mijn bevindingen breiden bestaande theorieën over geheugenmodulatie uit door te laten zien dat stresshormonen niet alleen de consolidatie versterken, maar ook tegengestelde rollen spelen in het bepalen van hoe specifiek of gegeneraliseerd herinneringen worden opgeslagen. Deze bevindingen hebben mogelijk ook een directe klinische relevantie:

behandelingen die zich richten op stresshormoonssystemen zouden rekening moeten houden met hun effecten op geheugenspecificiteit en generalisatie als belangrijke factoren voor behandeluitkomsten. Cruciaal is dat interindividuele verschillen, zoals in de regulatie van endogene glucocorticoïdsignalering door de HPA-as, zowel de hersenstructuur beïnvloeden als de respons op glucocorticoïdbehandeling bepalen, zowel op gedragsmatig als op neuronaal niveau. Deze bevindingen onderstrepen het belang van een verschuiving van uniforme behandelstrategieën naar een meer gepersonaliseerd model van psychiatrische behandeling bij PTSS, waarbij interventies worden afgestemd op de biologische kenmerken van de individuele patiënt in plaats van uitsluitend op de diagnostische classificatie.

RESEARCH DATA MANAGEMENT

This research adhered to the applicable laws and ethical guidelines. Research data management was performed according to the FAIR principles. The information below details how this was achieved.

Ethics

The results of this thesis are based on rodent research studies which were conducted in accordance with Dutch and European regulations. The Central Authority for Scientific Procedures on Animals (Centrale Commissie Dierproeven (CCD), The Hague, The Netherlands) has given approval to conduct the studies of **Chapter 2** (AVD10300 2021 15634) and **Chapters 3 and 4** (AVD10300 2020 10685). Additionally, the local Animal Welfare Body (Instantie voor Dierenwelzijn (IvD)) and Animal Experiment Committee (Dierexperimentencommissie (DEC)), Groningen and Nijmegen, The Netherlands) have approved all protocols associated with the present studies, most of which are registered in the Radboudumc platform iVentionLES. All efforts were made to minimize animal discomfort and to reduce the number of animals used. This research was funded by the Dutch Medical Research Council (ZonMw TOP grant 91218038), which did not entail any financial conflict of interest.

Findable, Accessible, Interoperable and Reusable

Data from **Chapter 2** are findable and accessible via Zenodo and **Chapters 3 and 4** via the Radboud Data Repository (RDR). See table below for data collection DOIs, corresponding access conditions, and Data Use Agreement (DUA).

The Data Sharing Collections (DSCs) include statistical results and selected analysis pipelines, which will remain available for at least 10 years after termination of the studies. The Data Acquisition Collections (DACs) include large raw experimental data (e.g. behavioral video recordings, microscopy images and MRI scans), and can be made available upon request to the collection managers. MRI data are organized according to the BIDS standards, with concomitant readme files. Long-lived file formats (e.g. .sav, .csv, .tif,) ensure that data remains usable in the future.

All standard operating procedures were stored in LabGuru (<https://radboudumc.labguru.com/knowledge/>) and accessible to all members of the Department of Medical Neuroscience of the Radboud university medical center and Donders Institute.

Chapter	Closed Access (RDR-DAC)	Open Access (Zenodo/RDR-DSC)	DUA
2		DOI: 10.5281/zenodo.15496898	CC-BY-4.0
3 and 4	DOI: 10.34973/xagg-vn74	DOI: 10.34973/0e9j-2e92	CC0-1.0

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ABOUT THE AUTHOR

Mariana Pais, born in 1996 in Coimbra, Portugal, is a biologist specializing in the neuroscience of stress and memory. From 2014 to 2017, she completed a Bachelor's degree in Biology at the University of Coimbra. During this time, she participated in an ERASMUS exchange semester at University College Dublin in Ireland, where she took honors courses in neuroscience and became fascinated by the biological basis of memory. From 2017 to 2019, Mariana pursued the Research Master in Cognitive and Clinical Neuroscience, specializing in Fundamental Neuroscience, at Maastricht University in the Netherlands. For her master's internship, she moved to Groningen, where she conducted research at the GELIFES Institute on the optogenetic manipulation of memory engrams and the effects of sleep deprivation on memory.

In 2019, Mariana joined the Donders Institute for Brain, Cognition and Behaviour at the Radboud university medical center in Nijmegen, The Netherlands, to pursue a PhD under the supervision of Prof. dr. Benno Roozendaal and Prof. dr. Erno Hermans. During her doctoral research, she investigated how stress hormones norepinephrine and glucocorticoids influence memory specificity and generalization, with a particular focus on how interindividual differences in these systems affect memory. Most of the scientific studies she conducted during her PhD are described in this thesis.

LIST OF PUBLICATIONS

de Nooij, L., Wirz, L., Heling, E., **Pais, M.**, Hendriks, G.J., Verkes, R.J., Roozendaal, B., Hermans, E.J. (2024) Exogenous glucocorticoids to improve extinction learning for post-traumatic stress disorder patients with hypothalamic-pituitary-adrenal-axis dysregulation: a study protocol description. *European Journal Psychotraumatology*, 15(1):2364441. doi: 10.1080/20008066.2024.2364441.

Atucha, E., **Pais, M.**, Ronzoni, G., Schoenmaker, C., Atsak, P., Roura, D., Lohkamp, K.J., Schelling, G., McGaugh, J.L., Glennon, J.C., Aschrafi, A., Roozendaal, B. (2025) Noradrenergic activation of the basolateral amygdala facilitates memory specificity for similar events experienced close in time. *Nature Neuroscience*, 28(9):1910-1918. doi: 10.1038/s41593-025-02014-0.

PHD PORTFOLIO

Name of PhD candidate: Mariana Isabel Dos Santos Ribeiro Pais
Graduate School: Donders Graduate School
Department: Medical Neuroscience
Research period: October 2019 – May 2026
Supervisors: Prof. dr. B. Roozendaal, Prof. dr. E.J. Hermans

Courses	Hours	Year
- Scientific Integrity	7	2020
- Achieving your goals and performing more successfully in your PhD	42	2020
- Project management for PhDs	56	2020
- The art of presenting science	36	2020
- Science journalism and communication	84	2020
- Microscopy image analysis with FIJI	14	2020
- Writing a review article	28	2020
- Internship supervision training	14	2020
- Mindfulness-based stress reduction for PhDs	28	2021
- Writing scientific articles	96	2022
- Effective writing strategies	75	2022

Conferences, summer schools and seminars	Location	Year
- Stress NL Meeting	Utrecht, NL	2019
- PhD Day on Science Communication: organizing committee	Online	2021
- Munich Winter Conference on Stress	Munich, DE	2022
- Dutch Neuroscience Meeting	Tiel, NL	2022
- Stress NL Meeting	Amsterdam, NL	2022
- Radboud Research Rounds on Stress: oral presentation	Nijmegen, NL	2022
- International Convention of Psychological Science: oral presentation	Brussels, BE	2023
- International Conference on Learning and Memory: oral and poster presentation	Huntington Beach, USA	2023
- Dutch Neuroscience Meeting: poster presentation	Tiel, NL	2023
- Summer School Stress & Cognition: poster presentation	Nijmegen, NL	2023

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- International Society of Psychoneuroendocrinology: oral presentation	London, UK	2023
- Dutch Neuroscience Meeting	Tiel, NL	2024

Supervision of internships	Study program	Duration	Year
- Julie van Immerseel	Bsc Biomedical Sciences	4 months	2021
- Wija Oosterveld	Msc Cognitive Neuroscience	6 months	2022
- Zehra Sezgin	Bsc Medicine	2 months	2022
- Lara Loermans	Bsc Biomedical Sciences	6 months	2023
- Leonie Loomans	Msc Biomedical Sciences	6 months	2023
- Rohit Sundar	Msc Cognitive Neuroscience	7 months	2024

Academic service and leadership	Duration	Year
- Representative for the PhD Candidates of the Medical Neuroscience Department in the Donders PhD Council	12 months	2020-2021
- Chair of the PhD Organization of Nijmegen	23 months	2020-2022
- Representative for the Behavioral Neuroscience Lab in the Advisory Boards for the Translational Neuroscience Unit and Molecular Laboratories	24 months	2022-2024
- Mentor for internship students	12 months	2023-2024
- Member of the Sustainability Team of the Medical Neuroscience Department	14 months	2023-2024

Teaching	Year
- Object Recognition Workshop in the Neurobiology of Stress course and Translational Neuroscience minor course (Bsc and Msc Biomedical Sciences)	2021-2024

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All the work described in this thesis (plus all the pilot experiments and analyses not described here) could not have been accomplished without the effort and enthusiasm of my students. My deepest thanks to Julie, Wija, Zehra, Lara, Leonie, and Rohit. Your greatest contribution to me, however, has been your own personal development during your internships. Even when results were slow to arrive and nothing seemed to work, watching you grow into increasingly independent researchers brought me immense joy. I wish you great success in whichever path you choose to follow.

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DONDERS GRADUATE SCHOOL

For a successful research Institute, it is vital to train the next generation of scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School in 2009. The mission of the Donders Graduate School is to guide our graduates to become skilled academics who are equipped for a wide range of professions. To achieve this, we do our utmost to ensure that our PhD candidates receive support and supervision of the highest quality.

Since 2009, the Donders Graduate School has grown into a vibrant community of highly talented national and international PhD candidates, with over 500 PhD candidates enrolled. Their backgrounds cover a wide range of disciplines, from physics to psychology, medicine to psycholinguistics, and biology to artificial intelligence. Similarly, their interdisciplinary research covers genetic, molecular, and cellular processes at one end and computational, system-level neuroscience with cognitive and behavioural analysis at the other end. We ask all PhD candidates within the Donders Graduate School to publish their PhD thesis in the Donders Thesis Series. This series currently includes over 600 PhD theses from our PhD graduates and thereby provides a comprehensive overview of the diverse types of research performed at the Donders Institute. A complete overview of the Donders Thesis Series can be found on our website: <https://www.ru.nl/donders/donders-series>.

The Donders Graduate School tracks the careers of our PhD graduates carefully. In general, the PhD graduates end up at high-quality positions in different sectors, for a complete overview see <https://www.ru.nl/donders/destination-our-former-phd>. A large proportion of our PhD alumni continue in academia (>50%). Most of them first work as a postdoc before growing into more senior research positions. They work at top institutes worldwide, such as University of Oxford, University of Cambridge, Stanford University, Princeton University, UCL London, MPI Leipzig, Karolinska Institute, UC Berkeley, EPFL Lausanne, and many others. In addition, a large group of PhD graduates continue in clinical positions, sometimes combining it with academic research. Clinical positions can be divided into medical doctors, for instance, in genetics, geriatrics, psychiatry, or neurology, and in psychologists, for instance as healthcare psychologist, clinical neuropsychologist, or clinical psychologist. Furthermore, there are PhD graduates who continue to work as researchers outside academia, for instance at non-profit or government organizations, or in pharmaceutical companies. There are also PhD graduates who work in education, such as teachers in high school, or as lecturers in higher education. Others continue in a wide range of positions, such as policy advisors, project managers, consultants, data scientists, web- or software developers, business owners, regulatory affairs specialists, engineers, managers, or IT architects. As such, the career paths of Donders PhD graduates span a broad range of sectors and professions, but the common factor is that they almost all have become successful professionals. For more information on the Donders Graduate School, as well as past and upcoming defenses please visit: <http://www.ru.nl/donders/graduate-school/phd/>.

