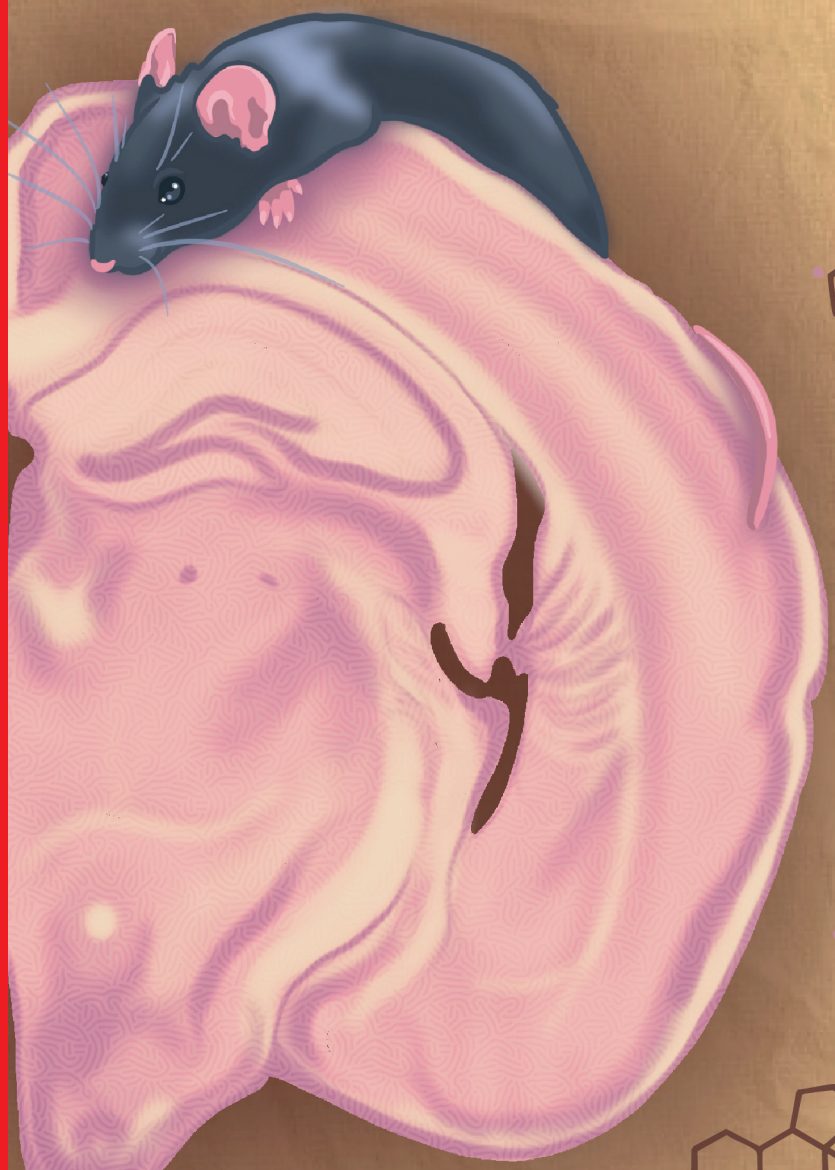
Stress Hormone Effects on Episodic-like Memory: Focusing on Memory Specificity and Strength

Sevgi Bahtiyar



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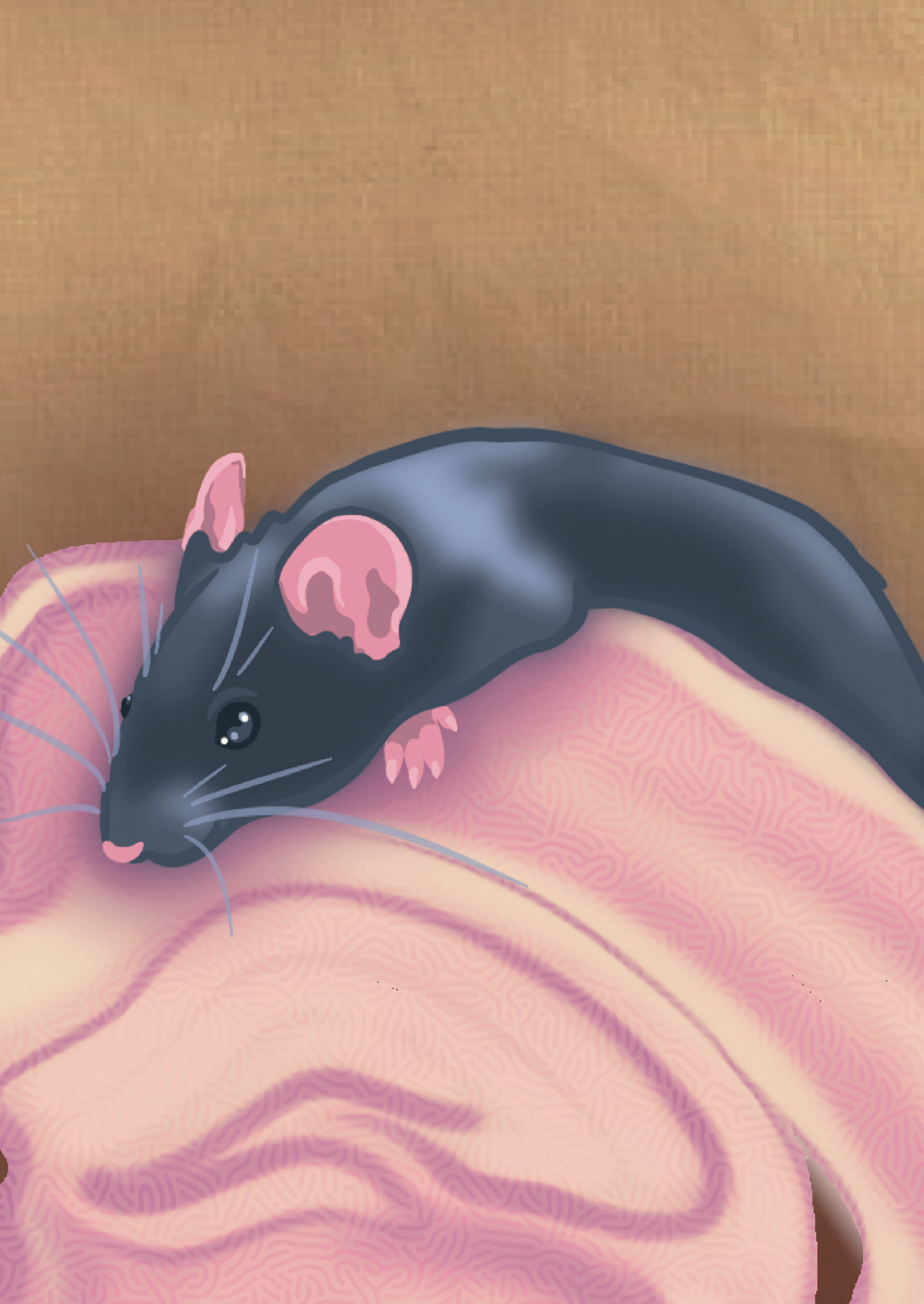


CHAPTER 1

General Introduction

Adapted from: Sevgi Bahtiyar, Kübra Gülmez-Karaca, Marloes J.A.G. Henckens, Benno Roozendaal (2020). Norepinephrine and glucocorticoid effects on the brain mechanisms underlying memory accuracy and generalization. Molecular and Cellular Neuroscience, 108: 103537.

1. Introduction

Emotionally arousing or stressful experiences induce strong and lasting memories (McGaugh, 2013). A large body of literature shows that this strengthening of memory involves synergistic actions of both norepinephrine (NE) and glucocorticoid hormones (corticosterone in rodents, cortisol in humans (CORT)) (McGaugh and Roozendaal, 2002; Joëls et al., 2011; Roozendaal and McGaugh, 2011; de Quervain et al., 2017; Schwabe et al., 2022). These two major stress hormone systems not only enable an individual to acutely respond to a dangerous or threatening situation, but also to prepare it for future exposures by inducing long-term behavioral changes, including effects on learning and memory (McGaugh, 2013). The tight regulation of emotional memories is normally a highly adaptive phenomenon (Dunsmoor and Paz, 2015). However, memories can be subject to multiple types of modifications beyond mere strengthening (Schacter, 1999), and understanding the mnemonic modifications produced by stress and emotional arousal might provide important insight into the operational constraints of our episodic, autobiographical memory system under stressful circumstances (Loftus, 1979; Payne et al., 2007; Kensinger et al., 2007b; Segal et al., 2012; Hoscheidt et al., 2014). Moreover, it might enhance insight into the neurobiological mechanisms underlying stress-related psychopathology, as aberrant memory processing of stressful events can have a maladaptive outcome. Particularly, strong, yet poorly contextually embedded, memories are believed to be a major risk factor for the development of psychopathological conditions such as posttraumatic stress disorder (PTSD) and phobias (Lissek et al., 2005; de Quervain et al., 2017; Lis et al., 2020).

Although it is now well accepted that our memory for stressful or emotionally arousing experiences is stronger, it is still debated to what extent the emotional impact of an experience is also associated with changes in memory specificity, fidelity and susceptibility to incorporation of misinformation (Morgan et al., 2004; Porter et al., 2008; Hoscheidt et al., 2014). Human studies show contradictory results. On the one hand, stress exposure has been shown to result in strengthened, generalized, gist-based mnemonic processing of the central theme of the episode, accompanied by reduced specificity, impaired context dependency, overconfidence of later recollection or even increased false memory (Loftus, 1979; Payne et al., 2002; Talarico and Rubin, 2003; Sharot et al., 2004; Schwabe and Wolf, 2009; Rimmele et al., 2011). On the other hand, stress effects on memory have been reported to lead to enhanced memory for details (Kensinger et al., 2007a; Kensinger et al., 2007b; Segal et al., 2012; Wiemers et al., 2013; Qi et al.,2021). In contrast to human research, this topic has received little attention in animal research, mainly

due to constraints of existing behavioral tasks in assessing the quality of memory. Recently, new behavioral tasks for rats have been designed that made it possible to investigate stress and stress hormone effects on episodic-like specificity of memory. Interestingly, these rat studies have indicated that the two stress hormones NE and CORT exert opposing effects on memory specificity (Atucha et al., 2017; Roozendaal and Mirone, 2020), which could, at least in part, provide a possible explanation for the contradictory findings in humans.

However, relatively little is known about the neurobiological underpinnings of the opposing effects of NE and CORT on this episodic-like specificity of memory. Particularly, the specific role of distinct subregions of the hippocampus, a brain region that is critically involved in episodic-like specificity of memory, in these modulatory effects and the potential involvement of other brain regions remains unknown. In this thesis, a series of experiments with mice will be presented, utilizing two different episodic-like memory tasks, i.e., the dual-event inhibitory avoidance task and object-in-context (OiC) task, to investigate the effects of NE and CORT on both the consolidation and retrieval of episodic-like specificity of memory and their task-dependent effects on hippocampal activity as well as brain-wide neuronal activity.

In the following sections, I will first give a short overview of the literature that has examined the role of NE and CORT in modulating the consolidation and retrieval of memory of different types of experiences, followed by recent advances in the understanding of NE and CORT effects on regulating episodic-like specificity of memory in rodents. Then, I will describe the neural circuitry of interacting brain regions that mediates these modulatory effects of stress hormones on memory, with a specific focus on the role of the hippocampus in episodic-like specificity of memory. Lastly, I will describe how stress hormone effects on modulating neural processes underlying memory processes depend on mitochondrial function and energy mobilization within the brain. Finally, I will present the scope of this thesis and give a brief description of each of the experimental chapters.

2. Stress hormone effects on memory

Stress and emotional arousal rapidly activate the sympathetic nervous system, which triggers the release of catecholamines such as NE and epinephrine from the adrenal medulla and sympathetic nerve endings (Mason, 1968). Peripherally released catecholamines cannot directly enter the brain (Weil-Malherbe et al.,

1959), but can bind to adrenoceptors on the ascending vagus nerve, which affects brain function by activating noradrenergic cell groups within the nucleus of the solitary tract (NTS) and locus coeruleus (LC) (Williams et al., 1998). NE is also directly released in the brain upon stress exposure by the activation of noradrenergic cells in the NTS and LC (Aston-Jones and Cohen, 2005; Valentino and Van Bockstaele, 2008; Sara, 2009). NE affects brain function by binding to locally expressed α-adrenergic and β-adrenergic G-protein coupled receptors, and is associated with a rapid and widespread reconfiguration of brain network activity to increase vigilance and attention (Aston-Jones and Cohen, 2005; Seeley et al., 2007; Zerbi et al., 2019).

Concurrently, the hypothalamic-pituitary-adrenocortical (HPA) axis is activated, which culminates in the release of CORT from the adrenal cortex in a delayed fashion after stressor onset (i.e., 15–30 min) (Ulrich-Lai and Herman, 2009). Since CORT crosses the blood-brain barrier, it can readily access the brain and modulate neural processing by activating glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) (Reul and de Kloet, 1985). The GR is widely expressed throughout the brain, whereas MR expression is mainly restricted to limbic areas. Intracellular MRs have a high affinity for CORT and are mostly saturated during basal conditions, whereas GRs have a low affinity for CORT and are only significantly occupied by higher levels of circulating CORT, e.g., in situations of stress (de Kloet et al., 2005).

GR activation has been linked to processes such as learning and memory and negative feedback of the HPA axis, whereas MR-mediated effects have been associated with the appraisal and responsiveness to stressful stimuli (de Kloet et al., 2005). Typically, MRs and GRs reside in the cytoplasm, but upon ligand binding translocate to the nucleus, where they influence gene transcription (Datson et al., 2001). More recent work has indicated that stress levels of CORT also activate low-affinity MRs and GRs residing in or near the plasma membrane, and thereby affect stress responding and neuronal function in a rapid, non-genomic fashion (Karst et al., 2002; Dallman, 2005; de Kloet et al., 2008; Roozendaal et al., 2010).

Several decades of research have identified that both NE and CORT, in a predominantly synergistic manner, can influence different memory processes, ranging from its initial encoding and consolidation to retrieval mechanisms (de Quervain et al., 1998; Roozendaal, 2002; Roozendaal and McGaugh, 2011; Schwabe et al., 2012; de Quervain et al., 2017; Schwabe et al., 2022). In the next sections, I will particularly focus on the acute effects of NE and CORT on memory consolidation and retrieval since those are of main relevance to my thesis.

2.1. Memory consolidation

2.1.1. NE effects on memory consolidation

Extensive evidence indicates that noradrenergic activation is critically involved in mediating stress and emotional arousal effects on memory consolidation. Memory consolidation refers to a process by which a short-term memory trace is transferred into stable long-term memory (McGaugh, 2000). Studies have delineated that NE acts within many different, but highly interconnected, brain regions (McGaugh, 2000; Roozendaal and McGaugh, 2011). Previous studies have indicated a particularly important role for the basolateral amygdala (BLA) in orchestrating the memory-enhancing effect of NE by modulating neuroplasticity and memory processes in different memory storage sites (Ikegaya et al., 1997; McGaugh, 2004; McIntyre et al., 2005; Lovitz and Thompson, 2015; Chen et al., 2018). NE or β-adrenoceptor agonists administered posttraining into the BLA, or other brain regions such as the hippocampus or medial prefrontal cortex (mPFC), have been shown to enhance the consolidation of memory of many different types of emotionally arousing training experiences commonly used in experiments with rats and mice, including inhibitory avoidance (Liang et al., 1986; Izquierdo et al., 1992; Bevilaqua et al., 1997; Ferry and McGaugh, 1999), contextual fear conditioning (LaLumiere et al., 2003), water-maze spatial learning (Hatfield and McGaugh, 1999), appetitive conditioning (Salinas et al., 1997) and object recognition (Roozendaal et al., 2008; Barsegyan et al., 2014). Conversely, posttraining infusions of β-adrenoceptor antagonists impair memory retention and block the memory-enhancing effects of co-administered NE (Liang et al., 1995; Hatfield and McGaugh, 1999; Roozendaal et al., 2008; Barsegyan et al., 2014). In addition to β-adrenoceptor influences, NE effects on memory consolidation also involve the activation of α1- and α2-adrenoceptors (Ferry et al., 1999a). The activation of α1-adrenoceptors by NE generally exerts excitatory effects, while activation of α2-adrenoceptors induces neuronal inhibition (Berridge and Waterhouse, 2003; Jones, 2004), both likely act via an interaction with β-adrenoceptors (Ferry et al., 1999a). The memory-enhancing effects of NE follow an inverted U-shaped dose–response relationship. Moderate doses of NE enhance memory consolidation, whereas both lower and higher doses are less effective or even impair memory (Gold and van Buskirk, 1978; Roozendaal et al., 2008; Barsegyan et al., 2014). It is possible that the complex dose-response effects of NE on memory consolidation are caused, in part, by a dose-dependent activation of these different adrenoceptor types (Ferry et al., 1999b; Gilbert et al., 2001; Arnsten, 2009). However, this biphasic response pattern is characteristic for almost every drug tested, and a more universal explanation for this pattern is not available.

2.1.2. CORT effects on memory consolidation

There is also extensive evidence that CORT is involved in regulating the consolidation of memory processes (Cottrell and Nakajima, 1977; Oitzl and de Kloet, 1992; Sandi and Rose, 1994; Bohus, 1994; McEwen and Sapolsky, 1995; Roozendaal and McGaugh, 1996; Lupien and McEwen, 1997; Roozendaal, 2000; de Quervain et al., 2009; Joëls et al., 2011; de Quervain et al., 2017). Administration of the glucocorticoid-synthesis inhibitor metyrapone prevents the enhancing effects of stress and emotional arousal on memory consolidation (Roozendaal et al., 1996), whereas systemic posttraining injections of CORT or the synthetic glucocorticoid dexamethasone enhance memory for many different types of emotionally arousing training experiences (Kovács et al., 1977; Flood et al., 1978; Roozendaal and McGaugh, 1996; Sandi et al., 1997; Cordero and Sandi, 1998; Hui et al., 2004; Okuda et al., 2004; Roozendaal et al., 2006). As with NE, posttraining injections of CORT produce dose- and time-dependent enhancement of memory (Cottrell and Nakajima, 1977; Sandi and Rose, 1994; Roozendaal and McGaugh, 1996; Zorawski and Killcross, 2002; Okuda et al., 2004; Zoladz and Diamond, 2009). The memory-modulating effects of CORT appear to involve the selective activation of the low-affinity GR (Oitzl and de Kloet, 1992; Roozendaal et al., 1996; Lupien and McEwen, 1997; Finsterwald and Alberini, 2014) as blockade of GRs, but not MRs, shortly before or immediately after training impairs long-term memory. Further supporting a role for the GR in mediating these effects is the observation that local administration of a GR agonist infused into the BLA, hippocampus or mPFC enhances memory consolidation of inhibitory avoidance and other emotionally arousing training experiences (Roozendaal and McGaugh, 1997a; Roozendaal et al., 1999a; Roozendaal et al., 2009; Barsegyan et al., 2010, Barsegyan et al., 2019). Noteworthy, as with NE, these memory-modulatory influences critically depend on BLA interactions with other brain regions (for a review see: Roozendaal and McGaugh, 2011). The findings of several studies have indicated that lesions or functional inactivation of the BLA prevent the enhancing or impairing effects of GR agonist or antagonist administration systemically or into other brain regions on memory consolidation (Quirarte et al., 1997; Roozendaal and McGaugh, 1997b; Barsegyan et al., 2019; Roozendaal et al., 1999b, Roozendaal et al., 2009).

2.1.3. Role of CORT-NE interactions in modulating memory consolidation

Evidence from several studies indicates that interactions between CORT and NE may be essential in mediating stress effects on memory consolidation. For example, CORT administration after footshock delivery in an inhibitory avoidance task rapidly augments NE levels within the BLA (McReynolds et al., 2010). On the other hand, attenuation of NE signaling with a β-adrenoceptor antagonist administered systemically or directly into the BLA, or into several other brain regions, blocks glucocorticoid effects on memory consolidation for emotionally arousing events

(Quirarte et al., 1997; Roozendaal et al., 2002, Roozendaal et al., 2006; Barsegyan et al., 2010). Findings of studies investigating the mechanism of CORT interactions with the noradrenergic system suggest that CORT facilitates memory consolidation, in a permissive fashion, by potentiating the NE-signaling cascade (Roozendaal et al., 2002). Several experimental findings have shown that CORT effects on increasing NE signaling have an onset that is too fast to be mediated via transcriptional regulation in the nucleus and likely involve a rapid, non-genomic mode of action (Roozendaal et al., 2010; Zhou et al., 2012; Chen et al., 2012; Karst and Joëls, 2016). Several studies have suggested that such rapid glucocorticoid effects on the noradrenergic system might involve an intermediate increase in endocannabinoid signaling (Campolongo et al., 2009; Atsak et al., 2015). Importantly, the release of NE and CORT must occur in a precisely timed manner for their synergistic interactions; mistiming of CORT elevations with respect to NE release might suppress the effects of NE, preventing CORT–NE synergy (Joëls et al., 2011; Chen et al., 2022).

Studies using the object recognition task further investigated whether noradrenergic activation induced by emotional arousal is essential in enabling CORT effects on memory consolidation. Prior work modulated endogenous arousal and thus NE levels in the brain, by giving rats either extensive habituation (twice a day for seven days) or no prior habituation to the training context, after which they were allowed to explore objects in the apparatus (Figure 1A). CORT administered systemically immediately after object recognition training enhanced the consolidation of memory of this experience of rats that were not previously habituated to the context, and therefore were emotionally aroused by the novelty of the experience (Okuda et al., 2004; Roozendaal et al., 2006) (Figure 1B). In contrast, posttraining administration of CORT did not enhance the consolidation of object recognition memory of habituated rats, which showed an attenuated arousal response during the training (Figure 1C). In non-habituated (i.e., emotionally aroused) rats, the β-adrenoceptor antagonist propranolol blocked the CORT-induced memory enhancement (Roozendaal et al., 2006). To determine whether the failure of CORT to enhance memory consolidation under low-arousing conditions was due to insufficient training-induced noradrenergic activation, a low dose of the α2-adrenoceptor antagonist yohimbine (YOH), which increases NE levels in the brain, was co-administered with the CORT to well-habituated rats immediately after object recognition training. The crucial finding of this study was that such an augmented NE tone was sufficient to mimic the effects of emotional arousal in that simultaneously administered CORT enhanced memory consolidation (Roozendaal et al., 2006). Collectively, these findings indicate that CORT interacts with the NE system to selectively enhance long-term memory for emotional material

(de Quervain et al., 2017). By contrast, CORT seems to have little effect on emotionally neutral information.

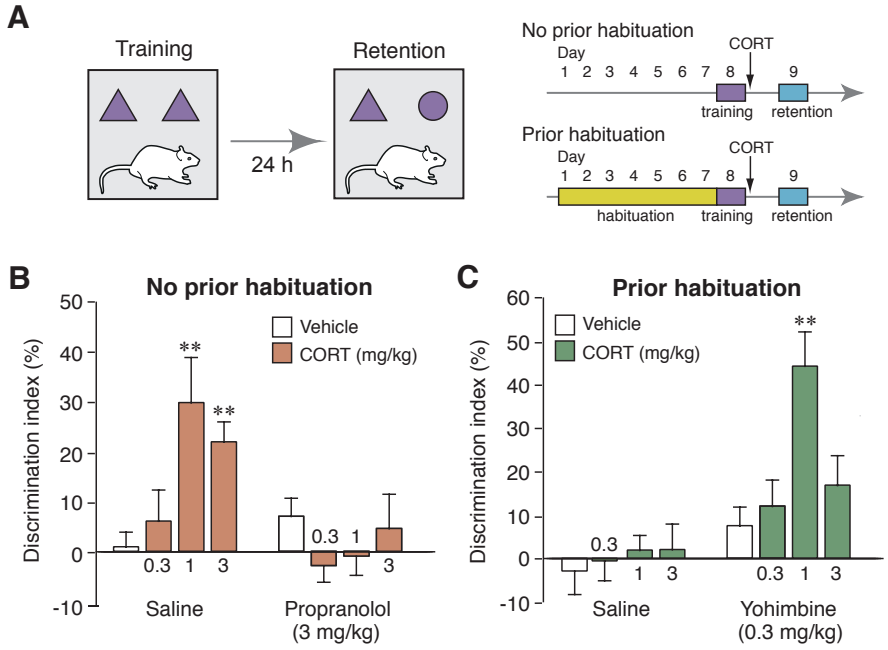


Figure 1. Glucocorticoids interact with arousal-associated noradrenergic activation in enhancing memory consolidation for object recognition training. A. Experimental design of the object recognition task. Rats are either habituated to the training context twice per day for 7 days (prior habituation) or not habituated (no prior habituation). On day 8, they are given a 3-min training trial during which they can freely explore two identical objects, followed by systemic drug administration. Retention is tested 24 h later by placing the rats back into the apparatus for 3 min. On the retention trial, one of the objects is replaced by a novel object. Data represent discrimination index (%) on the 24-h retention trial, expressed as mean ± SEM. The discrimination index is calculated as the difference in time spent exploring the novel and the familiar object, expressed as the ratio of the total time spent exploring both objects. **B.** Corticosterone (CORT 0.3, 1 or 3 mg/kg, s.c.) dose-dependently enhances object recognition memory in non-habituated (emotionally aroused) rats, and co-administration of the β-adrenoceptor antagonist propranolol (3 mg/kg, s.c.) blocks this CORT-induced memory enhancement. **C.** CORT does not enhance object recognition memory in rats that were previously habituated to the training context (emotionally non-aroused rats), but co-administration of the noradrenergic stimulant yohimbine (YOH 0.3 mg/kg, s.c.) enables the CORT effect on object recognition memory. $^{**}P < 0.01$ vs vehicle.
Adapted from Roozendaal et al., 2006.

2.2. Memory retrieval

2.2.1. CORT effects on memory retrieval

In contrast to its enhancing effects on memory consolidation, stress and stress hormones generally impair the retrieval of memory (Roozendaal, 2002). In 1998, de Quervain and colleagues conducted a comprehensive investigation on the impact of stress exposure on memory retrieval in rats (de Quervain et al., 1998). For this, rats were initially trained on a water-maze spatial task and then on the next day exposed to stress of footshock shortly prior to the retention test. They showed that footshock exposure 30 min before retention testing impaired task performance, but that footshock exposure either 2 min or 4 h before testing was ineffective. This time-dependent effect was associated with elevated circulating CORT levels at the time of retention testing, which were maximally enhanced 30 min after stress exposure. To further examine whether the elevated CORT levels play a role in mediating this stress effect on memory retrieval, CORT was directly administered to the animals 30 min before the retention test and it was found that this dose-dependently impaired task performance (de Quervain et al., 1998). Several subsequent studies have replicated these initial findings and showed that the administration of CORT or GR agonists to rats or mice shortly before memory retention testing impairs retrieval of previously acquired spatial or contextual memory (de Quervain et al., 1998; Roozendaal et al., 2004b; Cai et al., 2006; Schutsky et al., 2011; Atsak et al., 2012). In human studies, similar outcomes have been observed: glucocorticoid administration or an exposure to psychosocial stressors in controlled laboratory settings was found to impair memory retrieval (de Quervain et al., 2000; de Quervain et al., 2003; Buss et al., 2004; Het et al., 2005; Kuhlmann et al., 2005). Neuroimaging studies in humans have revealed that CORT administration reduced activity in the hippocampus and adjacent cortical regions during the retrieval of declarative memory (de Quervain et al., 2003; Oei et al., 2007), whereas animal studies have shown that CORT or GR agonist administration directly into the hippocampus impairs retrieval of spatial memory in a water maze (Roozendaal et al., 2004a).

2.2.2. Role of CORT-NE interactions in modulating memory retrieval

Several studies have shown that the effects of glucocorticoids in impairing memory retrieval also require the activation of noradrenergic mechanisms. For example, it was shown that systemic administration of a β-adrenoceptor antagonist prevented the memory retrieval impairment caused by simultaneous injections of CORT (Roozendaal et al., 2004a; Roozendaal et al., 2004b). Similarly, human work indicated that the detrimental effect of CORT on memory retrieval could be counteracted by administering a β-blocker (de Quervain et al., 2007). In addition,

CORT administration was observed to exclusively hinder the retrieval of emotionally arousing information or during test conditions characterized by heightened arousal, supposedly linked to increases in endogenous NE levels (Kuhlmann and Wolf, 2006). The question of whether NE itself can influence memory retrieval received little attention, but several studies found that blockade of NE signaling with a β-adrenoceptor antagonist alone is insufficient to affect memory retrieval (Roozendaal et al., 2004a; Roozendaal et al., 2004b; de Quervain et al., 2007).

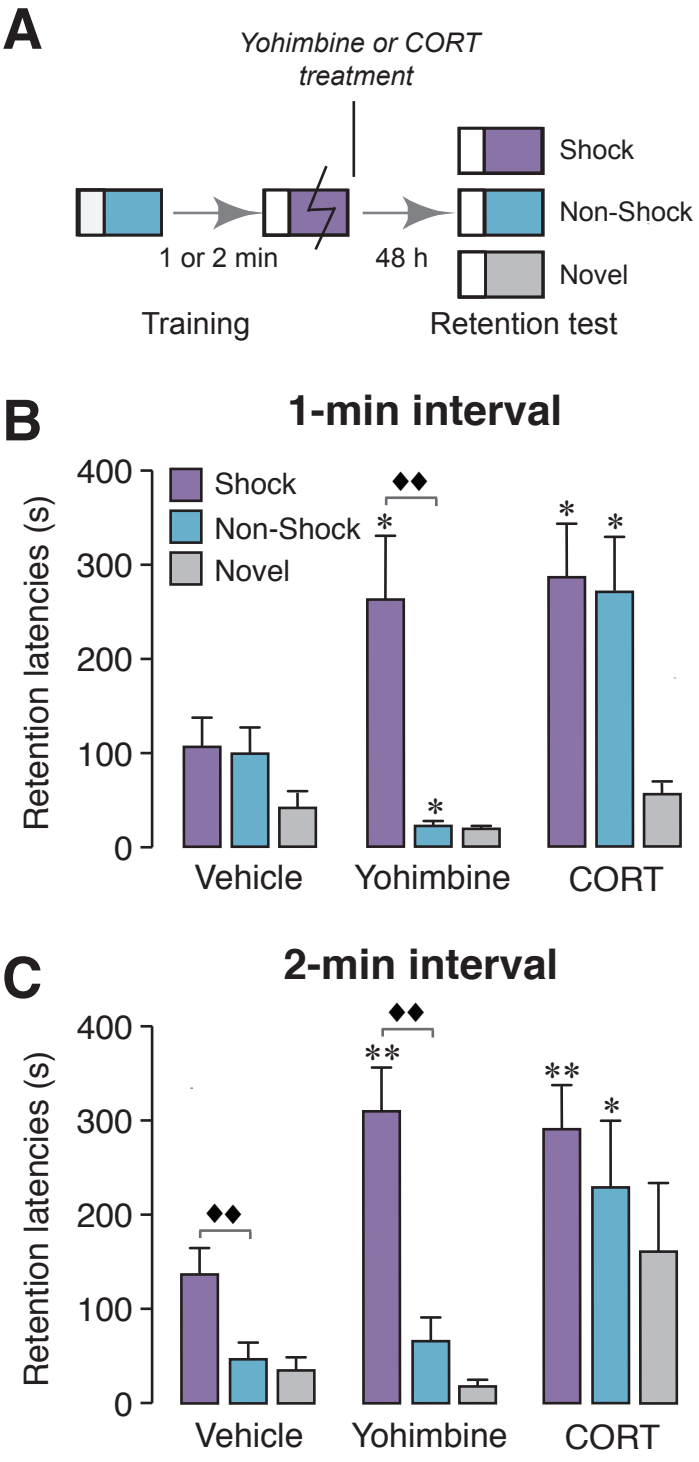
3. Stress hormone effects on memory specificity

The above-mentioned findings clearly indicate that both NE and CORT, in a predominantly synergistic manner, regulate the strength of memory. However, these experimental paradigms typically do not allow for the investigation of potential modulatory effects on qualitative aspects of the memory. Inhibitory avoidance is one such behavioral task commonly used to investigate learning and memory processes in rodents (Gold, 1986; McGaugh and Roozendaal, 2009). In this task, the rodent is initially placed in a safe light compartment in a straight alley and receives a single aversive footshock after stepping from this starting compartment into a dark compartment within the same apparatus. Retention of the training is tested mostly 1 or 2 days later by measuring the animal's latency to enter the dark compartment upon re-exposure to the training context. However, good memory performance, i.e., long retention latencies, on the inhibitory avoidance task does not necessarily indicate an accurate memory of the shock-context association.

To be able to assess the quality of both contextual and episodic-like aspects of inhibitory avoidance memory, Atucha and Roozendaal (2015) developed a modified version of this task; the dual-event inhibitory avoidance task, also termed inhibitory avoidance discrimination task, for rats (Atucha and Roozendaal, 2015). During training, the rat visits two distinct inhibitory avoidance apparatuses with a short interval, but only receives a footshock upon entering the dark compartment of the latter context, i.e., the shock box (Figure 2A). On the retention test, retention latencies to enter the dark compartment of these two previously encountered training contexts (shock box and non-shock box) as well as of a novel box are recorded. Longer retention latencies in the shock box reflect an enhanced strength of the memory, whereas longer latencies in the shock box relative to the non-shock box indicate episodic-like specificity of the association of footshock with the correct training context. Latencies in the novel box can be used to determine whether changes in memory specificity are restricted to the contexts (or events)

encountered during training, or whether they also generalize to not previously seen contexts (Atucha and Roozendaal, 2015; Colucci et al., 2019). The length of the interval between the two training episodes determines the difficulty of the task (Atucha and Roozendaal, 2015; Atucha et al., 2017; Roozendaal and Mirone, 2020). As shown in Figure 2B, rats trained with a short 1-min interval between the two training episodes, likely causing a temporal overlap of the two memory traces, show similar retention latencies in the shock box and non-shock box, indicating that they are unable to associate the shock experience with the correct training context. In contrast, rats trained with a longer interval of 2 min exhibit retention latencies in the shock box that are longer than those in both the non-shock box and novel box (Figure 2C), indicating a specific memory of the shock-context association.

To investigate whether the stress hormones NE and CORT affect the episodic-like specificity of memory, Roozendaal and Mirone (2020) trained rats on the dual-event inhibitory avoidance task, followed immediately by a systemic administration of the noradrenergic stimulant YOH or CORT. In accordance with the above-described effects, both YOH and CORT enhanced retention latencies in the shock box (Figure 2B-C). Most importantly, YOH induced specificity of an otherwise unspecific memory, as shown by longer retention latencies in the shock box but shorter retention latencies in the non-shock box (Figure 2B). In contrast, CORT promoted generalization across the two training episodes by increasing retention latencies in both the shock box and non-shock box (Figure 2C). YOH or CORT did not affect retention latencies in the novel box, suggesting that their modulatory effects did not extend beyond the two training contexts. Taken together, these findings indicate that NE and CORT, while both strengthening memory of the shock experience per se, produce opposite effects on the specificity of the shock-context association (Roozendaal and Mirone, 2020). The finding that systemic YOH treatment reinforces episodic-like specificity is consistent with other findings indicating that posttraining NE administration into the BLA also enhances the specificity of the association of an object with its specific training context in an object-in-context recognition task (Barsegyan et al., 2014), and maintains long-term specificity of the shock-context association on the dual-event inhibitory avoidance task (Atucha et al., 2017). The finding that CORT induced a non-specific strengthening of memory across training episodes is in agreement with other evidence indicating that posttraining CORT administration also induced a non-specific fear memory to an auditory stimulus that was temporally disconnected from footshock delivery (Kaouane et al., 2012), as well as enhanced contextual generalization of fear memory (Lesuis et al., 2021).


A
Yohimbine or CORT
treatment
Shock
Non-Shock
Novel
1 or 2 min
48 h
Training
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B
1-min interval
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< Figure 2. Opposite effects of YOH and CORT on episodic-like specificity of memory. A. Experimental design of the dual-event inhibitory avoidance task. During training, the rat visits two distinct inhibitory avoidance apparatuses with either a 1 or 2-min interval, but only receives a footshock upon entering the dark compartment of the latter context, i.e., the shock box. The noradrenergic stimulant YOH (1 mg/kg, s.c.) or CORT (3 mg/kg, s.c.) is administered immediately after the training. Forty-eight hours later, retention latencies to enter the dark compartment of these two previously encountered training contexts (shock box and non-shock box) as well as of a novel box are tested in a counterbalanced order. Each apparatus has the same geometry, but the non-shock box and novel box have distinct visual contextual features (i.e., stripes and circles taped to the wall) as well as distinct tactile features by tape placed on the floor (Atucha and Roozendaal, 2015). **B.** Step-through latencies (mean + SEM) in seconds on the 48-h retention test of rats trained on the dual-event inhibitory avoidance task with a 1-min interval ('difficult version') between the two training episodes. Vehicle-treated rats do not show a specific shock-context association as indicated by similar retention latencies in the shock box and non-shock box. YOH enhances both specificity and strength of memory as shown by specific long latencies in the shock box. By contrast, CORT enhances latencies in both the shock box and non-shock box, indicating lack of specificity. **C.** Step-through latencies (mean + SEM) in seconds on the 48-h retention test of rats trained on the dual-event inhibitory avoidance task with a 2-min interval ('easy version') between the two training episodes. Vehicle-treated rats show specific memory of the shock-context association. YOH strengthens memory while maintaining memory specificity. In contrast, CORT induces reduced specificity by enhancing 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. Adapted from Roozendaal and Mirone, 2020.

4. Stress hormone effects on neural systems regulating memory processing

4.1. Neural circuitry involved in mediating stress hormone-induced enhancement of memory consolidation

Stress hormones exert widespread effects on the brain, engaging numerous brain regions involved in memory processing. Prior research has implicated a critical modulatory role for noradrenergic signaling within the BLA in mediating stress hormone effects on memory (McGaugh, 2000). Many previous studies have shown that either neurotoxic lesions of the BLA or the administration of a β-adrenoceptor antagonist into the BLA prevent the effects of posttraining systemic stress hormone administration on the consolidation of long-term memory (Liang et al., 1986; Roozendaal and McGaugh, 1996; Quirarte et al., 1997). Moreover, posttraining administration of NE or CORT directly into the BLA has been found sufficient to enhance the consolidation of memory of emotionally arousing training experiences (Roozendaal and McGaugh, 1997; Roozendaal et al., 2002; Roozendaal, 2003, Roozendaal et al., 2004). More recent studies have shown that optogenetic manipulation of BLA activity posttraining is similarly effective in modulating memory consolidation (Huff et al., 2013).

The BLA exerts these effects by regulating neural plasticity and information storage processes within a wide range of memory-related brain regions, including the hippocampus, mPFC, caudate nucleus, and nucleus accumbens. For example, noradrenergic activation of the BLA (intra-BLA infusions of a β-adrenoreceptor agonist) immediately after inhibitory avoidance training was found to increase the expression of the neuroplasticity protein Arc (activity-regulated cytoskeleton) within the hippocampus (McIntyre et al., 2005), whereas inactivation of the BLA (lesions of the BLA) was found to prevent the augmenting effect of stress hormone administration on neuronal activity in the hippocampus, mPFC or other brain regions on memory consolidation (Roozendaal and McGaugh, 1997; Roozendaal et al., 1999a).

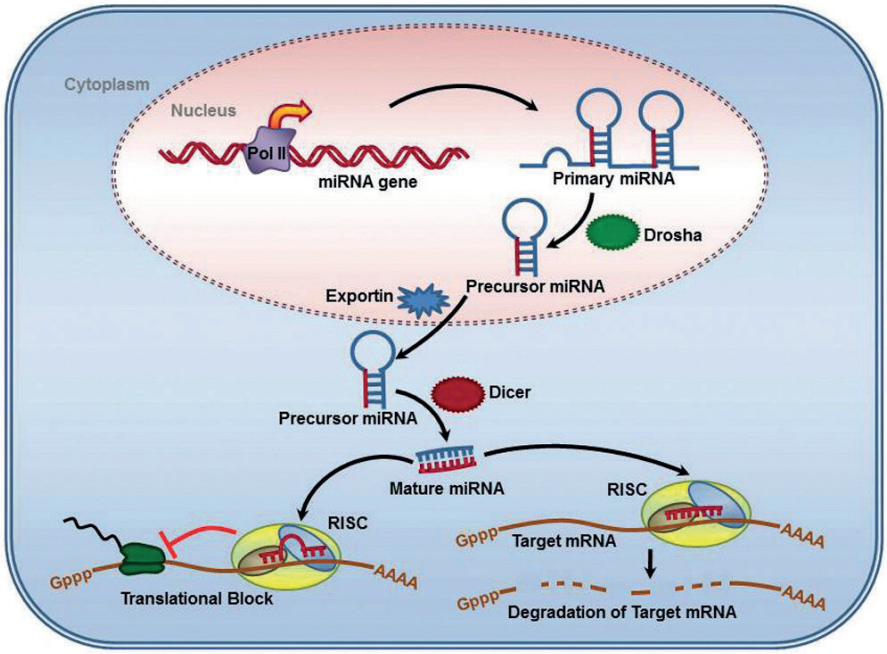
The exact neural circuit affected by stress hormones likely depends on the type of memory being processed, and thus target regions of the BLA projections activated (Roozendaal and McGaugh, 2011). For example, previous research has demonstrated that the administration of a GR agonist into the dorsal striatum enhanced memory on a cued water maze task, while local administration in the hippocampus was found to improve spatial memory in the water maze task (Quirarte et al., 2009). Additionally, it has been found that GR agonist administration into the insular cortex enhanced object recognition memory, whereas hippocampal administration enhanced object location memory (Roozendaal et al., 2010). The effect of glucocorticoid administration into these different brain regions in enhancing the consolidation of these different types of memories were all found to depend on a functional BLA and specifically on noradrenergic signaling within the BLA (Roozendaal and McGaugh, 2011). Further, posttraining activation of the BLA (with stress hormones or other neurostimulant drugs) was found to enhance memory on all these different memory tasks (Packard and Teather, 1998; Roozendaal and McGaugh, 2011), indicating that the BLA plays a pivotal role within a broader memory-modulatory network that coordinates stress hormone effects on memory in other brain regions (Roozendaal, 2003).

4.2. Neural circuitry regulating stress hormone effects on memory specificity

Recent findings from our research group have indicated that noradrenergic activation enhances episodic-like specificity of memory on the dual-event inhibitory avoidance task by promoting the separation of memory of the two training events into two distinct memories (Atucha et al., 2024). The ability to separate similar input patterns into distinct memory representations is known as "pattern separation", and findings suggest that this function is localized in the dorsal blade of the dentate gyrus (dDG) of the hippocampus (Leutgeb et al., 2007; Yassa and Stark, 2011). NE

administration into the BLA after training on the dual-event inhibitory avoidance task was found to enhance the consolidation of pattern-separated memories within the dDG via post-transcriptional regulation of gene expression by microRNAs (miRs). miRs are small non-coding RNAs that are capable of RNA silencing by binding to complementary sequences of their messenger RNA (mRNA) targets (See Box 1 for further information). NE administration into the BLA after training induced a down-regulation of miR-134 within the dDG, and this down-regulation was found sufficient to facilitate the separation of memory of the two training events. Down-regulation of miR-134 was found to increase mRNA levels of its target genes cAMP response element-binding (*Creb*) and brain-derived neurotrophic factor (*Bdnf*). *Creb* and *Bdnf* are canonical memory mechanisms that play critical roles in memory consolidation (Silva et al., 1998; Mizuno et al., 2000). Moreover, *de novo* synthesis of *Bdnf* within the dDG has been implicated in the consolidation of separated memories (Bekinschtein et al., 2013). Importantly, this miR-134-mediated mechanism within the dDG did not affect general strength of the memory and was not recruited following training on a single event. These findings highlight that the BLA recruits a distinct neural mechanism dependent on memory requirements, facilitating the separation of overlapping memory representations and enabling the selective strengthening of specific associations into long-term memory.

Box 1



MicroRNAs (miRs) are short, non-coding RNA molecules that consist of approximately 22 nucleotides. These miRs can be independently regulated by their own promoters or organized into clusters, which allows for potential co-regulation (Ambros, 2004). The biogenesis of miRs involves several essential steps (Joshi et al., 2011). Initially, primary miRs are transcribed from miR genes and then cleaved by the nuclear RNase III enzyme Drosha, forming precursor miRs. These pre-miRs are exported to the cytoplasm and further processed by the enzyme Dicer, which generates a mature miR in the form of an imperfect double-stranded RNA duplex. The duplex is unwound, and the single-stranded mature miR is incorporated into the RNA-induced silencing complex (RISC), along with Argonaute proteins. Within RISC, the mature miR interacts with other protein factors to coordinate downstream gene-silencing processes (Ambros, 2004). MiRs exert their regulatory effects by binding to the 3' untranslated region (3'UTR) of target messenger RNA (mRNA) molecules. The degree of complementarity between the miR and its target mRNA determines the outcome of this binding interaction. Perfect complementarity results in mRNA degradation, while imperfect complementarity leads to translational repression (He and Hannon, 2004; Joshi et al., 2011; Christensen and Schratt, 2009).
Adapted from Joshi et al., 2011.

Another series of experiments in our laboratory indicated that systemic YOH and CORT administration also induce opposite effects on episodic-like specificity of memory in an OiC task in mice (Guo, 2024). In this task, mice are exposed to two consecutive object-context combinations and need to acquire the association between the object and the context in which this object was encountered. Whereas YOH administration was found to enhance (the specificity of) this OiC memory, CORT administration impaired OiC memory (Guo, 2024). These findings are thus highly comparable to the opposite effects of YOH and CORT administration on the episodic-like specificity of memory on the dual-event inhibitory avoidance task (Roozendaal and Mirone, 2020). Moreover, Guo (2024) examined the effect of YOH and CORT on hippocampal activity, assessed by the activity marker c-Fos, during the posttraining consolidation period. Whereas YOH or CORT did not affect the total number of active, i.e., c-Fos-expressing, neurons within the dDG, YOH-treated animals displayed a positive correlation between the number of c-Fos-expressing neurons in the dDG and cornu ammonis 3 (CA3). The dDG-CA3 pathway has an important role in pattern separation (Rolls, 1989; Mizumori et al., 1990; Rolls, 1996; Schaaf et al., 1998; Gilbert et al., 2001; Leutgeb et al., 2007; Dees and Kesner, 2013), and these findings thus are consistent with the view that noradrenergic activation recruits pattern separation (Atucha et al., 2024). In contrast, CORT administration significantly weakened the strength of this dDG-CA3 correlational activity (Guo, 2024), supporting the view that CORT impairs pattern separation resulting in a linking of memory of the two training events.

The findings further showed that YOH increased the total number of c-Fos-expressing cells in the cornu ammonis 1 (CA1) region of the hippocampus (Guo, 2024). A crucial function of the CA1 is to encode and store memory of the temporal order of events, in such a way that individual events can be stored distinctly from one another over time (Skaggs et al., 1996; Gilbert et al., 2001; Fortin et al., 2002; Kesner et al., 2002). Hence, it can be proposed that the YOH-induced increase in the total number of c-Fos-positive cells in the CA1 reflects an enhanced storage of memory of the two training events into separated and distinct populations of principal CA1 neurons (Tronson et al., 2009). In contrast, the impairing effect of CORT on OiC memory was associated with a selective increase in active GABAergic neurons in the CA1 region, indicating that also here the effect of CORT is opposite to that of YOH such that CORT might promote the storage of memory of the two training events into an overlapping neuronal ensemble.

Other work has additionally shown that rather intense fear conditioning, associated with elevated CORT secretion, induced non-specific fear memory, that generalized

to other contexts (Dos Santos Correa et al., 2019). This work implicated increased activity in the BLA, anterior insular cortex and prelimbic cortex during remote retrieval in mediating these behavioral effects (Dos Santos Correa et al., 2022). Other studies related a CORT-induced generalization of contextual fear to a specific and persistent rise in the excitability and quantity of activated DG granule cells. Targeted chemogenetic inhibition of these sparse cells in the DG prevented glucocorticoid-induced fear generalization, and reinstated contextual memory specificity, with no impact on the expression of cued fear memory (Lesuis et al., 2021). These studies provide additional support that a generalized memory can occur as the result of impaired DG function.

4.3. Large-scale networks and time-dependent effect of stress hormones on network activity

Animal studies have mostly investigated individual brain regions or interactions between two brain regions in regulating stress hormone effects on memory, but human neuroimaging studies have provided extensive evidence that the brain is organized as a set of large-scale neural networks, which recruitment and function are subject to the modulatory actions of stress hormones (Hermans et al., 2014a). The first large-scale network, identified by Raichle and colleagues in 2001, was the 'default mode' network (DMN), consisting of midline brain regions that consistently deactivate during tasks requiring goal-directed attention. This network has been identified as a crucial substrate for internal cognition and self-referential functions traditionally deemed unique to humans, such as empathy, recollection, imagination, conceptual processing, and conscious awareness (Buckner et al., 2008). This network interchangeably activates with two neurocognitive systems responsible for externally directed attention: the salience network (SN) and the executive control network (ECN). The SN is a broad-scale network that consolidates cognitive processing linked to significant stimuli, encompassing exogenous attention (Mather et al., 2016). It prominently features the amygdala and encompasses other regions such as the dorsal anterior cingulate/dorsomedial prefrontal cortex, anterior insula, temporoparietal junction, thalamus, striatum, and hypothalamus. The ECN, involving the prefrontal cortex (PFC), limbic and subcortical structures such as the striatum, orchestrates higher-order cognitive functions, including working memory, goal-directed planning, complex decision-making, and endogenous attention (Seeley et al., 2007; Corbetta et al., 2008; Hermans et al., 2011; Menon, 2011).

Stress exposure has been shown to induce a temporary shift in the activity, interactions, and function of these networks (Hermans et al., 2011; 2014b). Following a stressful event, there is an initial shift towards the SN, while ECN

function is suppressed (van Oort et al., 2017); effects that have been linked to the early rise in NE following stress exposure (Hermans et al., 2011). This shift primarily serves to direct attention to pertinent information. As the effects of catecholamines wane and (slow) glucocorticoids actions take effect in later stages, the network reconfiguration reverses, favoring the ECN and DMN to facilitate the consolidation of stress-related information (Hermans et al., 2014b; Schwabe et al., 2022).

More recent rodent functional neuroimaging studies have revealed that the rodent brain is similarly organized as a set of functional large-scale networks. Similar to humans, rodents exhibit an SN anchored in the insula and extending to the dorsal anterior cingulate cortex, including subareas like the amygdala (Grandjean et al., 2019; Tsai et al., 2020; Mandino et al., 2021), which seems to serve similar functions as in the human brain (Mandino et al., 2021; Tsai et al., 2020). Other work has identified a DMN-like network in rodents, with similar topological features as in humans and primates (Stafford et al. 2014; Zhan et al., 2014; Sforazzini et al., 2014; Liska et al., 2015). Rodent studies into the effects of stress on network function underscore the modulatory actions of stress (hormone) exposure. Activity of the DMN was found increased following chronic stress exposure (Henckens et al. 2015), and direct chemogenetic stimulation of LC neurons (triggering NE release) strongly increased connectivity within the SN (Zerbi et al., 2019). The development of rodent fMRI opens the way for investigating previously unexplored rodent brain network architecture and offers opportunities to elucidate how large scale networks are modulated by stress hormone administration. Another method employed in rodent studies to discern neural network function at the whole-brain level involves the assessment of neuronal activity patterns using whole-brain mapping of immediate early gene (IEG) expression (Jin et al., 2022). These studies apply the assessment of the number of IEG-expressing neurons as a proxy for the extent of neuronal activity in a given region, and within-group correlations in these numbers across brain regions as a proxy for their functional connectivity. A recent study explored the impact of post-learning CORT administration on remote fear memory, and revealed that the CORT-induced impairment of remote memory was associated with an altered connectivity in amygdala regions and reduced connectivity with the dDG (during remote recall), indicative of disrupted network connectivity linked to diminished remote memory retrieval (Brosens et al., 2023). Recent advancements in immunohistochemical methods and optical clearing techniques, along with ex vivo imaging technologies such as light sheet fluorescence microscopy (LSFM), have further advanced these efforts by enabling the visualization of neuronal activity throughout the intact brain (Ertürk et al., 2012; Chung et al., 2013; Renier et al., 2014; Secher et al., 2014; Jensen et al., 2015; Kjaergaard et al., 2019; Rocha et al.,

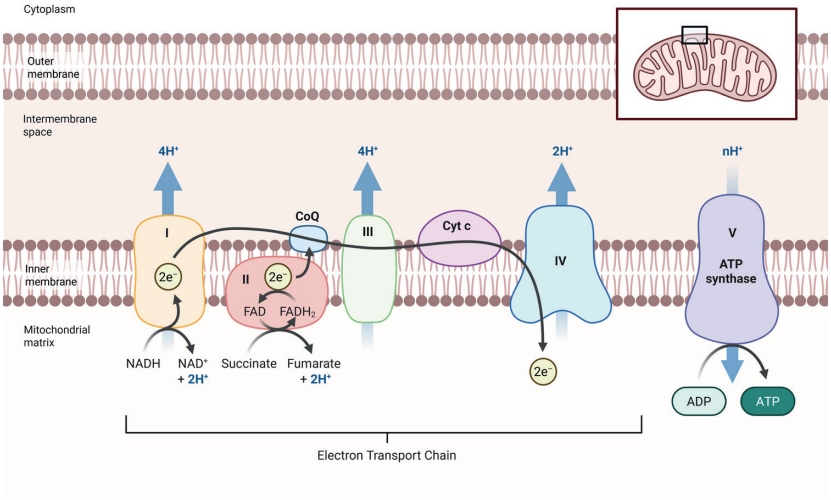
2019). These recent developments now allow for an unbiased assessment of brain-wide activity patterns associated with complex behaviors, and their modulation by stress hormones (Jin et al., 2022).

5. Stress hormones and mitochondria

5.1. Stress and glucocorticoid effects on mitochondrial function

Stress requires an organism to rapidly redirect its energy supply towards supporting an appropriate survival response. As the brain is central for making decisions regarding survival, its ability to reallocate energetic resources in response to stress is of crucial importance (Hunter et al., 2016). Mitochondria are essential for adequate neuronal functioning (Kann and Kovacs, 2007). Extensive evidence indicates that stress has a profound influence on mitochondria, affecting their function, including energy (e.g., ATP) production, which is vital for supporting the resource demands of the stress response (Picard and McEwen, 2018; Daniels et al., 2020). Findings have demonstrated that acute stress can rapidly enhance mitochondrial activity, primarily increasing respiratory chain complex I activity (see Box 2), via non-genomic actions (Hunter et al., 2016; Picard et al., 2018a). Other studies have shown that CORT administration, mimicking acute stress, to isolated mitochondria acutely boosts the effectiveness of the respiratory chain (Picard et al., 2018a). In a more delayed fashion, acute stress or glucocorticoid exposure enhances mitochondrial activity via a genomic regulation of mitochondrial DNA (mtDNA) (Hunter et al., 2016; Picard et al., 2018). Glucocorticoids can directly bind to GR located on mitochondria to stimulate mitochondrial transcription (Psarra, 2011). However, chronic stress or glucocorticoid exposure exerts opposite effects on specific mtDNA transcripts involved in the composition of respiratory chain complex I, impairing their structure, function and gene expression (Du et al., 2009; Hunter et al., 2016; Picard et al., 2018b; Xie et al., 2020). These alterations appear to be GR dependent and could potentially involve epigenetic modifications in mtDNA methylation (Psarra and Sekeris, 2011; Psarra et al., 2006; Du et al., 2009; Hunter et al., 2016). These detrimental effects of chronic stress exposure on mitochondrial function can lead to an insufficient energy supply, compromising subsequent responses to an acute stressor and increasing one's susceptibility to the development of psychopathologies (Morava et al., 2013).

Box 2. Mitochondrial electron transport chain



The main role of mitochondria is oxidative phosphorylation, which generates ATP (adenosine triphosphate) by utilizing the energy released during the oxidation of the resources (food). ATP serves as the main energy source for most biochemical and physiological processes, including growth, movement, and maintaining homeostasis (Brand et al., 2015). Mitochondria's energy is produced by steps of the electron transport chain (ETC), also known as the respiratory chain, consisting of mitochondrial complexes I to IV (NADH: ubiquinone oxidoreductase [CI], succinate dehydrogenase [CII or SDH], ubiquinol: cytochrome c oxidoreductase [CIII], and cytochrome C oxidase [CIV]). In the ETC, high-energy electrons (2e-), derived from earlier metabolic reactions, are transferred along the complexes. At Complex IV, oxygen (O2) acts as the final electron acceptor, resulting in its reduction to water (H2O). Meanwhile, NADH (ubiquinone oxidoreductase) and FADH2 (flavin adenine dinucleotide) facilitate electron transfer between the mitochondrial complexes. In the last step to produce ATP, the membrane potential is used to convert adenosine diphosphate (ADP) and inorganic phosphate (Pi) into the energy-rich molecule adenosine triphosphate (ATP) (Emmerzaal, 2022).

5.2. Effects of altered mitochondrial function on memory

Several studies have indicated that mitochondrial function also plays a critical role in the regulation of memory processes. Mice with genetically altered mtDNA were found to show severe impairments in spatial memory at long retention delays, which was accompanied by reduced levels of Ca2+/calmodulin-dependent kinase II-α (α-CaMKII), a protein crucial for establishing spatial remote memory (Tanaka et al., 2008). Another study by Hara et al. (2014) discovered a correlation between the deterioration of working memory in older monkeys and an increased presence of donut-shaped mitochondria. The formation of such donut-shaped mitochondria is a distinctive feature of mitochondrial stress, a condition that can be experimentally induced under controlled settings (*in vitro*) through the use of respiratory chain poisons, involving oxidative stress (Liu and Hajnóczky, 2011; Ahmad, et al., 2013).

There is also emerging evidence that mitochondrial function is involved in mediating the effects of stress exposure and glucocorticoids on memory. Firstly, mitochondria are implicated in various aspects of the stress response, including glucocorticoid synthesis (Bose et al., 2002; Picard and McEwen, 2018). Mice with distinct mitochondrial defects (such as mutations in mtDNA genes that cause reductions in complex I and IV activity) demonstrate deviations in their endocrine stress response to restraint (Picard et al., 2015; Picard and McEwen, 2018; Emmerzaal et al., 2020). Secondly, mitochondria seem to directly mediate certain stress-induced memory alterations. It has been shown that acute cannabinoid-induced deficits in memory formation in mice were contingent upon the activation of mitochondrial cannabinoid type 1 (mtCB1) receptors in the hippocampus. The genetic exclusion of CB1 receptors from hippocampal mitochondria effectively alleviated the cannabinoid-induced reduction in mitochondrial mobility, synaptic transmission and, importantly, impairment in memory formation (Hebert-Chatelain et al., 2016). These findings are highly interesting as it is now well known that glucocorticoid effects on memory consolidation and retrieval depend on an increased endocannabinoid signaling (Campolongo et al., 2009; Atsak et al., 2012; 2015; Morena et al., 2016). In line with this, a very recent study has provided direct evidence for the involvement of neuronal mtCB1 receptors in mediating CORT-induced impairment of memory retrieval (Skupio et al., 2023). However, to what extent stress and stress hormone effects on qualitative aspects of memory, such as changes in memory specificity and generalization, also depend on mitochondrial function has not been explored.

6. Outline of the thesis

The existing literature indicates that NE and CORT not only regulate memory consolidation and retrieval, but also exert a hormone-specific impact on memory quality. NE was found to enhance memory specificity whereas CORT was found to reduce memory specificity, leading to an increase in memory generalization. However, the precise neural mechanisms responsible for these effects remain unknown. The primary focus of my thesis was to examine the effects of the stress hormones NE and CORT on memory specificity and their neural substrate using mice.

In **Chapter 2**, I aimed to set up the dual-event inhibitory avoidance task for mice, since this allows for the assessment of memory specificity vs. strength. The dual-evet inhibitory avoidance task was originally designed for rats, yet mice have gained paramount significance in neuroscience investigations owing to the advent of genome-editing technologies. These advancements enable researchers to explore the influence of individual genes on development and behavior (Cryan et al., 2005). Secondary to this, I aimed to replicate the previously reported effects of NE and CORT on memory specificity and strength. I administered YOH and CORT immediately after training on the dual-event inhibitory avoidance task and assessed later memory retention.

In **Chapter 3**, I examined whether mitochondrial activity regulates episodic-like specificity of memory and whether acute stress exposure prior to retention testing interacts with mitochondrial activity in influencing episodic-like specificity of memory. A previous study had indicated that mitochondrial function is involved in mediating glucocorticoid effects on memory retrieval (Skupio et al., 2023), but whether stress (hormone) effects on the specificity of the recalled memories also involve changes in mitochondrial function has not been investigated. To investigate this, I used a mouse line with a genetic deficiency in the *nuclear-encoded NADH dehydrogenase [ubiquinone] iron-sulphur protein 4* (*Ndufs4*$^{GT/GT}$) (Kahlhöfer et al., 2021), which is characterized by an approximately 25% reduction in respiratory chain complex I activity (Emmerzaal et al., 2021). *Ndufs4*$^{GT/GT}$ mice as well as wildtype control mice were trained on the dual-event inhibitory avoidance task and memory specificity (and memory strength) was tested 48 h later. Thirty minutes prior to the retention test, a subgroup of both *Ndufs4*$^{GT/GT}$ and wildtype mice was exposed to a forced swim stress to examine whether this would affect memory specificity of the recalled memory. After the retention test, I examined whether the stress exposure had reduced mitochondrial activity within both the hippocampus and PFC. I

also investigated the pattern of neuronal activity in both brain regions through immunofluorescence for the neuronal activity marker c-Fos in both excitatory and inhibitory neurons.

The findings of these experiments suggested that the dual-event inhibitory avoidance task may not be the optimal choice for addressing the present research question. Consequently, in **Chapter 4**, I opted to investigate stress hormone effects on episodic-like specificity of memory in mice using an OiC task. Although the overall concepts of the two tasks share similarities, involving the training of mice in two distinct contexts and associating a specific event (shock or objects) with each of the two contexts, the OiC task is considered more suitable for my investigative purposes. I first validated that posttraining noradrenergic stimulation enhanced memory specificity on this particular task. Subsequently, I used the FosTRAP2xtdTomato transgenic mouse line which enabled me to tag the activated neurons during training/early consolidation, and compare this to later retention-induced neuronal activity. My main region of interest was the hippocampus since it has been extensively implicated in pattern separation and episodic-like specificity of memory (Yassa and Stark, 2011). Furthermore, as previous findings had indicated that NE administration into the BLA enhanced memory specificity on the dual-event inhibitory avoidance task by stimulating a miR-134-regulated consolidation process with the dDG (Atucha et al., 2024), I also used iDISCO and whole-brain neuronal activity analysis to explore whether posttraining YOH administration induces changes in neuronal activity beyond the hippocampus. Lastly, I tested whether systemic YOH administration following training on the OiC task could similarly recruit a miR-134-mediated consolidation process within the dDG. Additionally, I explored changes in mRNA expression, particularly *Creb* and *Bdnf*, which are regulated by miR-134.

Lastly, in **Chapter 5**, I have summarized the key findings of my thesis and presented overarching conclusions, as well as outlined potential avenues for future research.

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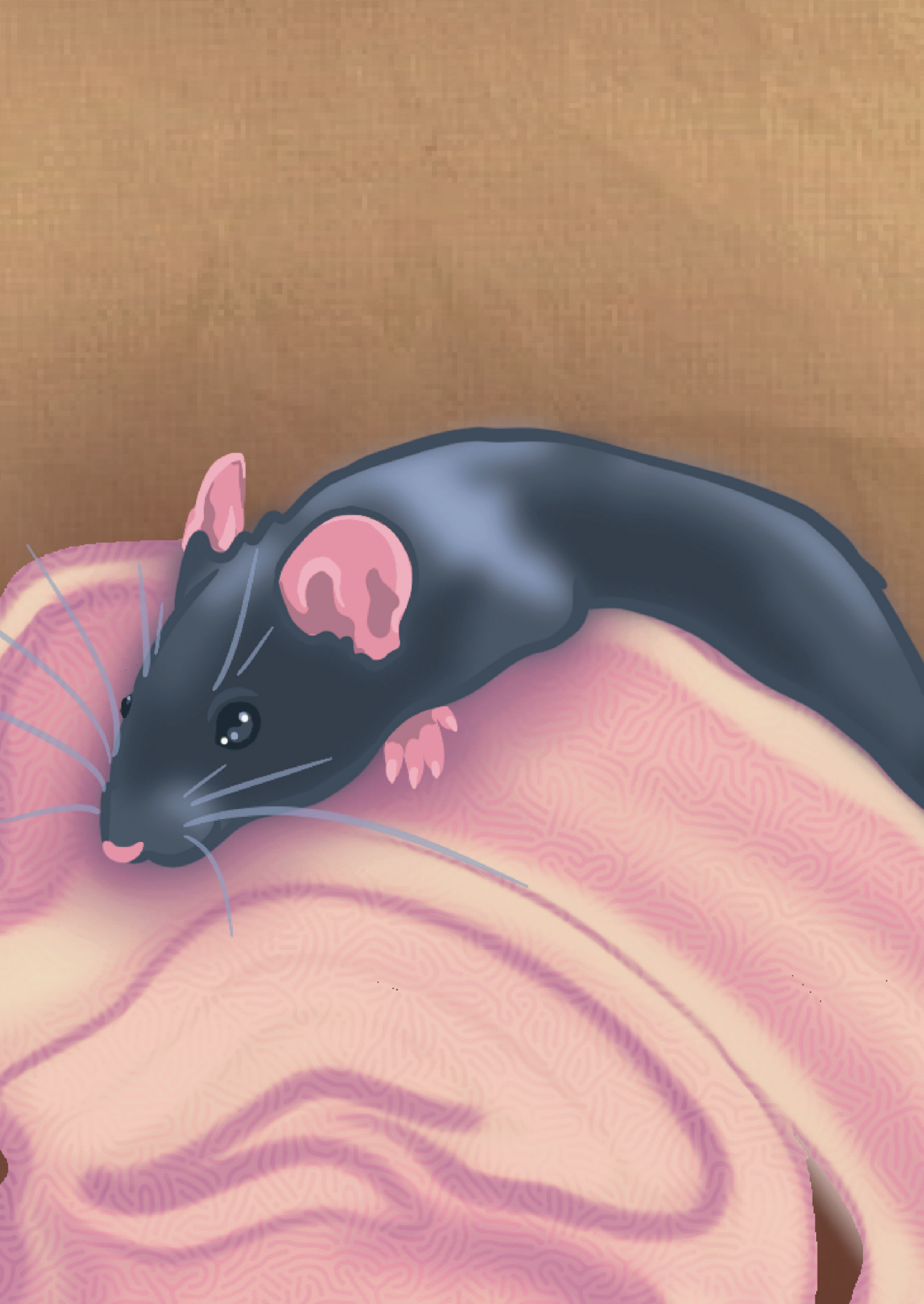

CHAPTER 2

Exploring stress hormone effects on memory specificity and strength in mice using the dual-event inhibitory avoidance task

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Abstract

Stressful and emotionally arousing experiences activate hormonal systems that not only create strong memories but also regulate several qualitative aspects of memory. Whereas both noradrenergic and glucocorticoid hormones are well known to synergistically strengthen memories, recent findings indicate that their influence on qualitative aspects of memory is more subtle and hormone specific. This highlights the need for sophisticated behavioral tasks that allow for the assessment of memory quality. The dual-event inhibitory avoidance task for rats is one such recently developed behavioral task designed to evaluate both strength and specificity of memory. In this task, rats are trained in two contextually distinct inhibitory avoidance apparatuses with a short interval, receiving a footshock only upon entering the dark compartment of the latter context. The noradrenergic stimulant YOH given systemically immediately after the training session was found to enhance both the strength and specificity of memory, as shown by longer retention latencies in the shock context but shorter latencies in the safe non-shock context. By contrast, the corticosterone (CORT) induced a generalized strengthening of memory across the two training events and increased retention latencies in both the shock and non-shock contexts. As mice are the preferred species for targeted gene and neural circuit manipulations, we here aimed to set up the dual-event inhibitory avoidance task for mice. We sought to identify the most effective training and test conditions and to replicate the effects of systemic YOH and CORT administration on memory strength and specificity in mice. Whereas non-injected control mice were capable of efficiently acquiring the task and selectively avoided the test context previously associated with footshock, the introduction of posttraining intraperitoneal injections caused problematic test context X context order interaction effects on retention latencies. Moreover, retention latencies became highly variable both within groups and across experiments, precluding a thorough investigation of YOH and CORT effects on memory specificity. Thus, whereas the dual-event inhibitory avoidance task has been proven to be highly suitable for the investigation of stress hormone effects on memory specificity in rats, our findings indicate that this task appears to be less recommendable for experiments with mice.

Keywords: *stress hormones; norepinephrine; corticosterone; memory specificity; dual-event inhibitory avoidance task*

1. Introduction

Stressful and emotionally arousing experiences are known to create strong and lasting memories (McGaugh, 2004; McGaugh, 2013). Upon emotional stimulation, norepinephrine (NE) is rapidly released in the brain and from the adrenal medulla and sympathetic nerve endings (Ramey and Goldstein, 1957; Mason, 1968; Aston-Jones et al., 1984; Galvez et al., 1999), whereas glucocorticoids (corticosterone in rodents, cortisol in humans (CORT)) are released from the hypothalamic-pituitary-adrenocortical (HPA) axis in a more delayed fashion (McEwen, 2003; McEwen, 2007; Ulrich-Lai and Herman, 2009). It is now well established that both NE and glucocorticoids, in a predominantly synergistic fashion, enhance the consolidation of stressful and emotionally arousing experiences into long-term memory (Roozendaal and McGaugh, 2011; Joëls et al., 2011; Roozendaal and Hermans, 2017; Bahtiyar et al., 2020). Importantly, more recent studies have reported that stress and emotional arousal, in addition to such memory-strengthening effects, also regulate several qualitative aspects of memory such as its specificity, accuracy, flexibility and/or context dependency (Dandolo and Schwabe, 2016; Pedraza et al. 2016; Atucha et al., 2017; dos Santos Corrêa et al., 2019; Simon-Kutscher et al., 2019; Quaedflieg et al., 2020). These novel findings suggest that stress hormones may affect multiple memory systems by which these different aspects of our experiences are encoded and/or stored (Bahtiyar et al., 2020; Schwabe et al., 2022). Yet, the majority of rodent behavioral studies investigating the neural mechanisms underlying stress hormone effects on memory have employed behavioral tasks in which only quantitative aspects of memory (i.e., memory strength) can be assessed. Given the simplicity of these tasks, the functional contribution of specific brain regions to memory performance and the identification of specific modulatory roles of different stress hormones on each memory system is limited (Bahtiyar et al., 2020). To overcome this limitation, some rodent studies have employed more refined behavioral tasks that allow for the investigation of both quantitative and qualitative aspects of memory. These tasks involve, but are not restricted to, training animals on multiple events (aimed at creating distinct memory "episodes"), testing them in several environments, or at multiple time points to assess time-dependent changes in memory (Mumby et al., 2002; Winocur et al., 2009).

The dual-event inhibitory avoidance task is one such recently developed behavioral task that allows for the assessment of both strength and episodic-like specificity of memory in rodents (Atucha and Roozendaal, 2015; Atucha et al., 2017; Colucci et al., 2019; Roozendaal and Mirone, 2020). Briefly, animals are trained in two distinct inhibitory avoidance apparatuses with a short interval, but only receive a footshock

upon entering the dark compartment of the latter context. On a later retention test, retention latencies to enter the dark compartment of these two previously encountered training contexts (i.e., the Shock box and Non-Shock box) as well as a Novel box are assessed. Longer retention latencies in the Shock box reflect a strengthening of memory for the footshock experience *per se*, whereas longer latencies in the Shock box relative to the Non-Shock box indicate episodic-like specificity of memory for the association of footshock with the correct training context. In previous studies, we showed that the noradrenergic stimulant YOH administered systemically immediately after the training session not only enhanced the strength of memory, but also facilitated its episodic-like specificity as indicated by longer retention latencies in the Shock box, but shorter retention latencies in the Non-Shock box (Atucha and Roozendaal, 2015; Roozendaal and Mirone, 2020). In contrast, posttraining CORT administration induced a generalized strengthening of memory across the two training events, increasing retention latencies in both the Shock box and Non-Shock box (Roozendaal and Mirone, 2020). These findings thus illustrate that while NE and CORT exert similar enhancing effects on memory strength, they have opposing effects on the episodic-like specificity of memory. This exemplifies the necessity of such more sophisticated behavioral paradigms to investigate the neuronal circuits that regulate stress hormone effects on also qualitative aspects of memory.

The dual-event inhibitory avoidance task was originally developed for rats. However, given the more readily available toolbox in mice for conducting gene manipulations (e.g., optogenetics, chemogenetics or generic gene knockdown or overexpression) to explore the neural circuits involved, rodent research has shifted over the last decades from using rats to mice. It is therefore necessary to also have such more sophisticated behavioral tasks available for mice. Here, we aimed at establishing the dual-event inhibitory avoidance task for mice. We adapted previously identified parameters for rats (Atucha and Roozendaal, 2015; Roozendaal and Mirone, 2020) and aimed to determine the optimal training and testing settings for mice. To replicate prior findings on the opposite effects of NE and CORT on memory specificity in rats, we additionally examined the effects of posttraining systemic YOH and CORT administration on the specificity and strength of memory in mice.

2. Material and methods

2.1. Subjects

Male adult C57BL/6J mice (8-11 weeks old at the time of the behavioral experiment) from Charles River Breeding Laboratories (France/Germany) were housed in conventional open lid cages in a temperature-controlled (22 °C) vivarium room with a regular 12:12-h light:dark cycle (07:00-19:00 h lights on). The vivarium room had a light intensity of 47 lux and humidity of 72%. Mice had *ad libitum* access to food and water. In the first experiment, mice were group housed (3-5 animals per cage), but in all subsequent experiments mice were housed individually, starting one week prior to the behavioral experiments, to prevent testing order effects (Lyte et al., 2005). Training and testing were performed during the light phase of the cycle between 10:00 and 15:00 h, at the nadir of the diurnal cycle of CORT. All experimental procedures were in compliance with European Union Directive 010/63/EU, and were approved by the Central Authority for Scientific Procedures on Animals, The Hague, The Netherlands. All efforts were made to minimize animal suffering and to reduce the number of animals needed.

2.2. Dual-event inhibitory avoidance task

2.2.1. General procedures

Mice were consecutively trained in two distinct inhibitory avoidance apparatuses within a single training session, but footshock was delivered only in the latter context. On the retention test, retention latencies were tested in the two training contexts as well as in a novel context. Each apparatus had the same geometry and consisted of a trough-shaped alley (36 cm long, 8.5 cm deep, 17 cm wide at the top and 4 cm wide at the bottom) divided into two compartments, separated by a sliding door that could be inserted or removed manually. The apparatuses also had a manually operated lid that was closed after placing the animal into the apparatus. The starting compartment (12 cm long) was made of opaque white plastic and was well lit. The shock compartment (24 cm long) was made of two dark, electrifiable metal plates and was not illuminated. The training apparatus in which footshock was given (Shock box) did not have any contextual modifications. The two other apparatuses had some contextual modifications that made them distinctly different training and/or test contexts (Figure 1). The initial modifications closely followed the design used for rats by Atucha and Roozendaal (2015), with adjustments made to the box size. The safe training context (Non-Shock box) had five white stripes (2 cm wide, 8 cm long) taped on each wall of the dark compartment together with tape placed on the floor, closing the gap between the two plates along the entire

length of the apparatus. The Novel box (used on the retention test only) had three white circles (4 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.

First, mice were handled for 3 min per day for five-seven consecutive days to habituate them to the experimenter. For training, mice were placed into the starting compartment of the first inhibitory avoidance apparatus (i.e., Non-Shock box). Once the mice reached the end of the dark compartment, they were removed from the apparatus without footshock being delivered. After a short interval (i.e., 90 or 180 s, see below), timed from the moment of crossing to the dark compartment of the Non-Shock box, mice were placed into the starting compartment of the second inhibitory avoidance apparatus (i.e., Shock box). Mice were first trained in the Non-Shock box, followed by the Shock box, based on the assumption that animals are less inclined to explore a new environment shortly after shock delivery, as well as to exclude potential stress effects on new memory encoding. During the interval, they were placed back into their home cage (or holding cage in case of group-housed animals). When the mice had stepped into the dark compartment of the Shock box, the sliding door was inserted and a single inescapable footshock (see below) was delivered for 1 s. Mice remained in the dark compartment for 30 s after footshock delivery such that they had time to form a memory of the environment. Afterwards, they were placed back into their home cage. Retention testing took place 48 or 72 h after training (see below), and retention latencies were assessed, in a randomized order and without delay, in the two training contexts (i.e., Shock box and Non-Shock box) and in the Novel box. For all three boxes, the mice were placed into the starting compartment, facing away from the door, and their latency to enter the dark compartment with all four paws was recorded (maximum latency of 600 s). Mice were removed from each box as soon as they had fully entered the dark compartment. Longer latencies in the Shock box were interpreted as a stronger memory. Memory specificity was defined as longer latencies in the Shock box relative to the Non-Shock box, indicative of successful separation of memory for the two trainings events. Longer latencies in the Novel box were interpreted as generalization of memory beyond the two training events to different environments.

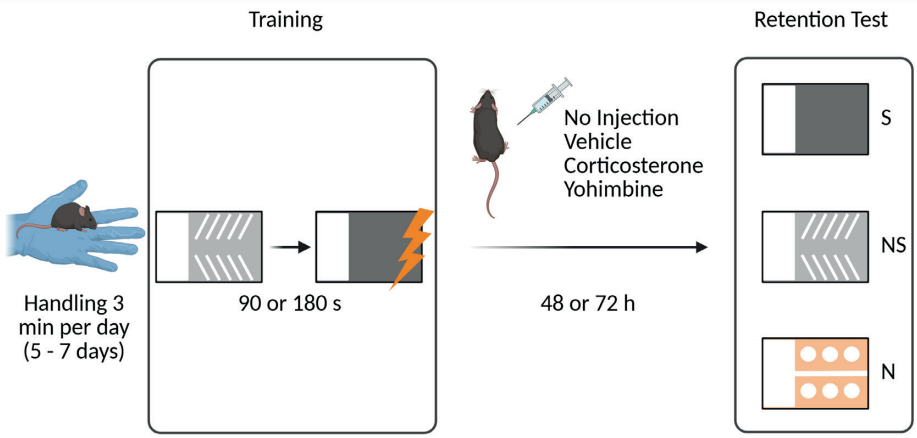


Figure 1. Schematic overview of the general experimental design. Mice were initially handled 3 min per day for 5 - 7 consecutive days. For training, mice were first trained in the Non-Shock Box (depicted in light grey) and after a short interval in the Shock Box (depicted in dark grey). A single inescapable footshock was given only after entering the dark compartment of the Shock box. Some mice received no injection and others received an intraperitoneal administration of vehicle, corticosterone or the noradrenergic stimulant yohimbine immediately after the training session. On the retention test, 48 or 72 h later, retention latencies were assessed, in a randomized order and without delay, in the two training contexts (i.e., Shock box and Non-Shock box) and in a Novel box the mice had not visited before. S, Shock box; NS, Non-Shock box; N, Novel box.

2.2.2. Specific procedures and implemented parameters

To optimize the experimental procedures of the dual-event inhibitory avoidance task for mice, we adjusted five different task parameters along the progression of experiments (Table 1). First, we changed the housing conditions from group housing to single housing to prevent test order effects (Lyte et al., 2005). Second, we used different footshock intensities (i.e., 0.20, 0.38 and 0.40 mA) to modify memory strength (Pang et al., 1992, Pittenger et al., 2006, Bentefour et al., 2014, Canto-de-Souza and Mattioli, 2016). Third, we tried to improve encoding of the starting compartment in order to facilitate box discrimination by placing mice into the starting compartment of the two training boxes with the sliding door initially inserted. Thereby, the mice were provided a specific time to explore (and encode) this compartment. Only after 20 s, the sliding door was removed, allowing the animals to cross to the dark compartment. Fourth, we used two different intervals (i.e., 90 and 180 s) between training in the Non-Shock box and Shock box to manipulate the difficulty of the task (Atucha and Roozendaal, 2015). Previous findings have indicated that rats trained with a short interval of 60 s were unable to associate the shock experience with the correct training context, whereas animals trained with a longer interval of 120 s successfully acquired this shock-context association (Atucha and Roozendaal, 2015; Atucha et al., 2017; Roozendaal

and Mirone, 2020). Lastly, we provided additional tactile cues to the Novel box (sandpaper floor) to support discrimination of this test environment.

For all experiments, we implemented a maximum training latency of 60 s to reach the end of the dark compartment for either of the two training boxes, with longer latencies indicating either inattentiveness or excessive anxiety (freezing behavior within the starting compartment). For this reason, 10 mice were excluded. Further, we excluded 6 mice that escaped from the apparatus during training.

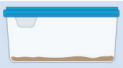
		Housing	*Shock Intensity*	*Wait in light compartment*	*Novel Box modification*	*Training box delay*
						
No Injection	Group 1	group	0.40 mA	no wait	modified	180 s
	Group 2	**single**	0.40 mA	no wait	modified	180 s
CORT	Group 1	single	**0.38 mA**	no wait	modified	180 s
	Group 2	single	0.38 mA	**20 s**	modified	180 s
	Group 3	single	0.38 mA	20 s	**distinct**	180 s
YOH	Group 1	single	0.38 mA	20 s	distinct	**90 s**
	Group 2	single	**0.20 mA**	20 s	distinct	90 s

Table 1. Task parameters per experimental group. In total five parameters were changed over the course of the experiments (shown in bold): Housing conditions (group vs single housed), shock intensity, wait time in the starting compartment, extra Novel box modifications, and the interval between the two training events. All changes were implemented after obtaining the results of the previous experimental group.

2.3. Systemic drug administration

CORT (1, 3 or 10 mg/kg, Sigma-Aldrich) was first dissolved in 100% ethanol and then diluted in saline to reach a final ethanol concentration of 5%. The vehicle contained 5% ethanol in saline only. YOH (17-hydroxyyohimban-16-carboxylic acid methyl ester hydrochloride, 0.3, 1.0 or 3 mg/kg, Sigma-Aldrich), an α_2-adrenoceptor antagonist that increases NE levels in the periphery and brain (Szemeredi et al., 1991), was dissolved in saline. Saline was used as vehicle for the YOH experiments. Drugs were administered intraperitoneally, in a volume of 0.1 mL/10 g body weight,

during short restraint immediately after the training session. Doses of YOH and CORT were selected based on their memory-enhancing effect on other behavioral tasks in mice (Cai et al., 2006; Song et al., 2020; Brosens et al., 2023). Drug solutions were freshly prepared before each experiment. Injections took place in a room adjacent to the behavioral room.

2.4. Statistical analysis

Statistical analyses were performed using IBM SPSS statistics, version 27. Retention latencies were analyzed using a general linear mixed model with test context (Shock box, Non-Shock box, Novel box) as within-subject factor and drug treatment (if applicable) as between-subject factor. Although the order of testing mice in the different contexts during the retention test was randomized, we also included context order (first, second or third box) as a within-subject factor to the analysis. Significant main effects of drug treatment, test context or context order were followed up by general linear mixed models comparing two conditions at a time, allowing direct comparison. Significant interaction effects were followed up by isolating one of the interacting factors (e.g., test context) and running a general linear model on the remaining factors (e.g., context order and drug treatment). For all comparisons, $P < 0.05$ (two-tailed) was accepted for statistical significance. The number of mice per group is indicated in the figure legends.

3. Results

3.1. Setting up the dual-event inhibitory avoidance task in mice

To examine whether mice are able to acquire the dual-event inhibitory avoidance task, we started off using training settings that would generate a strong and specific memory. Therefore, we trained mice on an easy version of the task with a long interval of 180 s between the two training events (Atucha and Roozendaal. 2015). Based on prior findings with the standard, one-trial inhibitory avoidance task in mice, we used a relatively high footshock intensity of 0.4 mA for 1 s (Pittenger et al., 2006; Bentefour et al., 2014). On the training session, mice readily entered the dark compartment of both the Non-Shock box (8.6 ± 2.6 s, mean ± s.e.m.) and Shock box (12.6 ± 1.8 s, see Table S1 for training latencies of all experiments). On the 48-h retention test, a linear mixed model for retention latencies in the three test contexts did not reveal a main effect of test context ($F(2,36.88) = 2.05$, $P = 0.14$, Figure 2A). Yet, retention latencies in both the Shock box ($t(9) = 4.68$, $P = 0.001$) and Non-Shock box ($t(9) = 7.99$, $P = 0.02$) were significantly longer than their entrance latencies during training, indicating that the mice had acquired a memory of the

shock experience *per se*. Remarkably, several mice (n = 8) did not cross to the dark compartment in at least two of the boxes, and were therefore assigned the maximum latency of 600 s. Instead, they displayed freezing behavior after placing them into the starting compartment. Interestingly, mice were group housed in this experiment, and six out of these eight animals with maximum latencies were tested as the last one from their home cage (shown in red in Figure 2A). Potentially, the behavior of these mice was affected by anticipatory stress caused by the removal and testing of their cage mates.

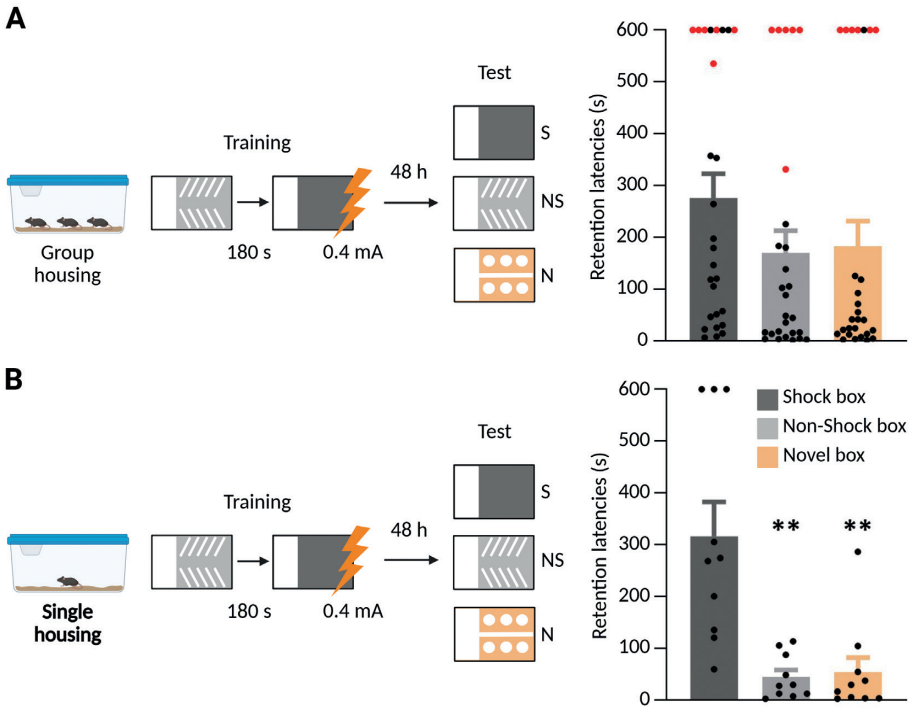


Figure 2. Setting up the dual-event inhibitory avoidance task in mice. **A**. In group-housed animals (n = 27), 48-h retention latencies did not significantly differ across the three test contexts. However, group housing was associated with marked freezing behavior of mice that were tested as the last ones of their cage (n = 6, depicted in red), making that they did not cross to the dark compartment in several of the test contexts. Therefore we switched to single-housing conditions for the next experiment. **B.** Training of single-housed mice (n = 10), using the same experimental parameters, induced memory specificity on a 48-h retention test, reflected by longer retention latencies in the Shock box compared to those in both the Non-Shock box and Novel box . Data represent mean + s.e.m. Dots indicate individual data points. $^{**}P < 0.01$ different from Shock box.

Therefore, in the next experiment, we switched to single housing conditions, while the training and test conditions remained unaltered. A linear mixed model for 48-h

retention latencies now indicated a significant main effect of test context (F(2,15.60) = 8.06, P = 0.004, Figure 2B). Retention latencies in the Shock box were significantly longer than those in both the Non-Shock box (F(1,9.73) = 16.11, P = 0.003) and Novel box (F(1,12.04) = 13.28, P = 0.003), whereas retention latencies in the Non-Shock box and Novel box did not differ from each other (F(1,21.97) = 0.10, P = 0.76). Retention latencies were not affected by context order (F(2,5.73) = 4.13, P = 0.08) or a test context X context order interaction (F(4,2.08) = 6.44, P = 0.13), indicating that the order in which animals were tested in the three test contexts did not influence retention performance.

3.2. Effect of posttraining corticosterone treatment on memory in the dual-event inhibitory avoidance task

Our previous findings in rats indicated that systemic CORT administration given immediately after training on the dual-event inhibitory avoidance task induced a strong, yet unspecific memory, increasing retention latencies in both the Shock box and Non-Shock box, without significantly increasing retention latencies in the Novel box (Roozendaal and Mirone, 2020). To examine whether we could replicate these findings in mice, we used the same settings as described above, except for a slight reduction in footshock intensity (0.38 mA) to facilitate the detection of a CORT effect on memory strength. CORT (3 or 10 mg/kg) or vehicle was administered intraperitoneally immediately after the training session. Retention of the memory was tested 48 h later (Figure 3A).

A linear mixed model for 48-h retention latencies revealed no significant main effect of CORT treatment (F(2,71.66) = 1.29, P = 0.28) or test context (F(2,50.04) = 2.82, P = 0.07), and no CORT treatment X test context interaction effect (F(4,49.62) = 0.57, P = 0.69, Figure 3A). Remarkably, even vehicle-treated mice displayed similar retention latencies in all three test contexts (F(2,19.56) = 0.84, P = 0.45), contrary to our earlier observation in non-injected mice. Moreover, unlike in the experiment with non-injected mice, we now found a statistically significant effect of context order (F(2,56.57) = 3.15, P = 0.03) and a context order X test context interaction effect (F(4,40.22) = 2.79, P = 0.04). These findings indicate that retention performance was influenced by the order in which animals were tested in the three test contexts. This effect was independent of CORT treatment (F(4,57.33) = 1.49, P = 0.22, Figure 3A). Further analyses on the main effect of context order revealed that, across all CORT treatment conditions, retention latencies in the first test context were significantly longer than those in both the second (F(1,46.96) = 4.41, P = 0.04) and third test context (F(1,50.44) = 5.69, P = 0.02), whereas retention latencies in the second context and third context did not differ (F(1,50.50) = 0.11, P = 0.74). Apparently,

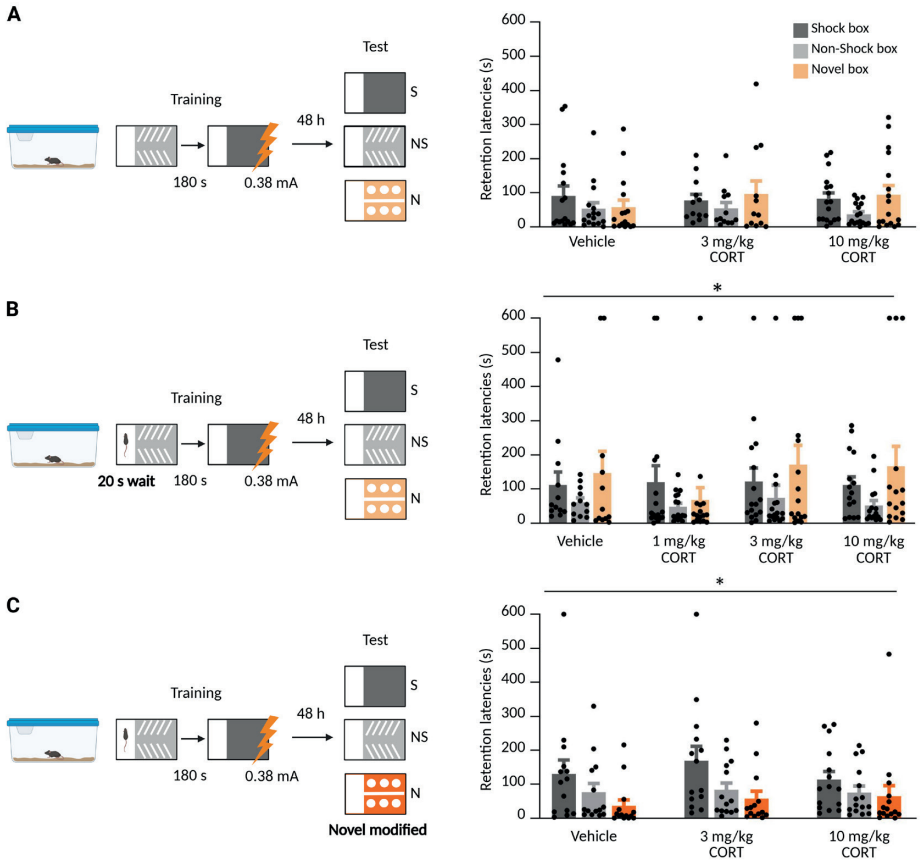


Figure 3. Effect of corticosterone treatment on strength and specificity of memory in the dual-event inhibitory avoidance task in mice. A. Shock intensity was slightly decreased (0.38 mA) compared to prior settings to facilitate the detection of a memory-enhancing effect of corticosterone (CORT), yet all other experimental settings remained the same. No effect of CORT treatment or test context was found (Vehicle: n = 16, CORT 3 mg/kg: n = 12, CORT 10 mg/kg: n = 17). **B.** To facilitate discrimination across training contexts, a 20-s waiting time in the starting compartment during the training session was included. This resulted in a main effect of test context, but no main effect of CORT treatment or CORT treatment X test context interaction. Instead, long retention latencies were found in the Novel box (Vehicle: n = 12, CORT 1 mg/kg: n = 16, CORT 3 mg/kg: n = 16, CORT 10 mg/kg: n = 16). **C.** To also facilitate discrimination of the Novel box, we added extra tactile information (sandpaper) to the Novel box. This resulted in a main effect of test context, but no main effect of CORT treatment or interaction effect (Vehicle: n = 15, CORT 3 mg/kg: n = 14, CORT 10 mg/kg: n = 15). Data represent mean + s.e.m. Dots indicate individual data points. *P < 0.05: main effect of test context.

retention latencies became progressively shorter by the repeated testing of the animals across the different test contexts. Yet, the significant context order X test context interaction effect indicated that this context order effect differed across the test contexts. Follow up tests revealed that mice that were first tested in the Shock box had significantly longer retention latencies in this context than mice that were tested last in this context ($F(1,19.00) = 6.56$, $P = 0.02$, Figure 4A). No such context order effect was observed for the Non-Shock box ($F(1,20.52) = 1.36$, $P = 0.26$) or Novel Box ($F(1,18.99) = 3.62$, $P = 0.07$). To further grasp the functional implications of this context order effect in the Shock box only, we plotted retention latencies in all three test contexts according to the order in which mice were tested in the Shock box (Figure 4B). When animals were tested first in the Shock box, then retention latencies in this box were significantly longer than those in the Non-Shock box ($F(1,16.69) = 6.63$, $P = 0.02$) but not in the Novel box ($F(1,19.96) = 0.09$, $P = 0.77$). This test context effect was however absent when mice were tested in the Shock box as second ($F(1,16.31) = 2.81$, $P = 0.11$) or third context ($F(1,21.94) = 1.47$, $P = 0.24$).

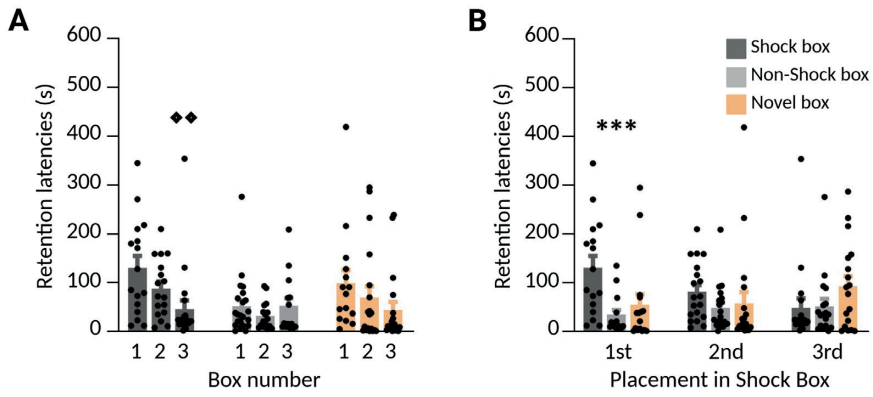


Figure 4. Effect of context order on retention latencies across test contexts. A. Retention latencies in the Shock box depended on the order in which mice were tested in this box, displaying longer retention latencies when tested there as first vs third test context. **B.** Mice that were tested first in the Shock box displayed longer retention latencies in the Shock box compared to those in the Non-Shock box, whereas this difference was not significant for mice that were tested as second or third in the Shock box. Data represent mean + s.e.m. Dots indicate individual data points. ***$P < 0.001$: different from Shock box, ◆◆$P < 0.01$: different from first box.

While the memory specificity observed in the non-injected group in the previous experiment indicated that mice are capable of acquiring memory specificity and differentiate between the safe (Non Shock box/Novel Box) and aversive (Shock box) contexts, the vehicle group of the CORT experiment did not display this memory

specificity. Possibly, the intraperitoneal injection procedure *per se* was stressful and triggered a glucocorticoid response that could be responsible for this loss of memory specificity (Drude et al., 2011). To facilitate memory specificity, in the next experiment we added a 20-s waiting time in the starting compartment of the Shock box and Non-Shock box during the training session before allowing animals to step into the dark compartment (Figure 3B). With these adapted task settings, we did observe a significant main effect of test context on 48-h retention latencies (F(2,45.08.17) = 6.31, P = 0.004), but still no effect of CORT treatment (F(3,61.65) = 1.13, P = 0.34) or CORT treatment X test context interaction (F(6,44.56) = 0.42, P = 0.86). Yet, the main effect of text context appeared to be driven by significantly longer retention latencies in the Shock box compared to those in the Non-Shock box (F(1,43.99) = 0.09, P = 0.007) but, rather unexpectedly, also significantly longer retention latencies in the Novel box compared to those in the Non-Shock box (F(1,44.88) = 7.15, P = 0.01). Furthermore, similar to the first CORT experiment, we found a significant main effect of context order (F(2,47.76) = 3.58, P = 0.03) and context order X test context interaction (F(2,47.76) = 7.95, P = 0.001, Figure 3B).

To reduce retention latencies in the Novel box, in a next experiment, we made the Novel box more easily distinguishable from the Shock box and Non-Shock box by adding more discriminatory cues (a sandpaper floor); a modification that was maintained in all following experiments. A linear mixed model for 48-h retention latencies revealed a main effect of test context (F(2,46.58) = 6.71, P = 0.03), but again no main effect of CORT treatment (F(2,46.93) = 0.73, P = 0.49) or CORT treatment X test context interaction (F(4, 46.52) = 0.25, P = 0.91, Figure 3C). Follow up tests revealed that retention latencies in the Shock Box were now significantly longer than those in both the Non-Shock box (F(1,36.95) = 8.62, P = 0.006) and Novel box (F(1,36.99) = 13.38, P = 0.001), whereas retention latencies in the Non-Shock box did not differ significantly from those in the Novel box (F(1,29.29) = 1.58, P = 0.22). We further found again a significant effect of context order (F(2,43.41) = 12.77, P < 0.001), in the absence of a significant context order X test context interaction effect (F(4,29.95) = 0.19, P = 0.94). This main effect of context order was caused by longer retention latencies in the first test context compared to those in the second (F(1,38.18) = 22.58, P < 0.001) or third test context (F(1,38.55) = 21.77, P < 0.001).

3.3. Effect of posttraining yohimbine treatment on memory in the dual-event inhibitory avoidance task

In the next series of experiments, we aimed to replicate the YOH effect in enhancing both the specificity and strength of memory (Atucha and Roozendaal, 2015; Roozendaal and Mirone, 2020). As this would require an unspecific memory (i.e.,

similar latencies in the Shock and Non-Shock box) in saline-treated control animals, we increased task difficulty by having a shorter interval of 90 s between training in the Non-Shock box and Shock box (Atucha and Roozendaal, 2015). YOH (0.3, 1, or 3 mg/kg) or saline control was administered intraperitoneally immediately after the training session, and retention was tested 72 h later. A linear mixed model for retention latencies in the three test contexts indicated a main effect of YOH treatment ($F(3,105.33 = 3.38$, $P = 0.02$) and test context ($F(2,93.52) = 13.69$, $P < 0.001$), but no significant YOH treatment X test context interaction effect ($F(6,94.29) = 0.27$, $P = 0.95$, Figure 5A). The main effect of test context was caused by longer retention latencies in the Shock box compared to the Non-Shock box ($F(1,68.09) = 23.41$, $P < 0.001$) and Novel box ($F(1,81.52) = 22.59$, $P < 0.001$), whereas retention latencies in the Non-Shock box and Novel box did not differ ($F(1,70.07) = 0.38$, $P = 0.54$). Despite the short training interval, saline-treated mice also displayed a significant test context effect ($F(2,21.84) = 7.01$, $P = 0.004$), with longer latencies in the Shock box compared to the Non-Shock box ($F(1,16.58) = 6.87$, $P = 0.02$) and the Novel Box ($F(1,16.57) = 14.02$, $P = 0.002$), without any difference in retention latencies in the Non-Shock box and Novel box ($F(1,15.15) = 2.48$, $P = 0.12$). The two higher dosages of YOH increased retention latencies across boxes compared to saline treatment (1 mg/kg: $F(1,48.65) = 4.43$, $P = 0.04$; 3 mg/kg: $F(1,32.57) = 8.90$, $P = 0.005$), whereas the lower dosage of YOH (0.3 mg/kg) was ineffective ($F(1,34.58) = 1.10$, $P = 0.31$). Furthermore, similar to the CORT experiments, we observed a main effect of context order ($F(2,82.08) = 12.60$, $P < 0.001$), without any further interactions (all P's > 0.09). Again, this was caused by shorter retention latencies in the second context compared to the first context ($F(1,67.82) = 25.28$, $P < 0.001$). Retention latencies did not further reduce from the second to third context ($F(1,63.09) = 1.71$, $P = 0.20$). Thus, we did not observe any enhancing effect of YOH administration on memory specificity, whereas its memory-strengthening effect was rather weak and unspecific across boxes.

In the last experiment, we attempted to reduce the impact of context order on retention latencies. As both memory strength and memory specificity are determined only based on retention latencies in the Shock box and Non-Shock box (and not in the Novel box), we here counterbalanced only the order of retention testing in the Shock and Non-Shock box (Figure 5B). Thus, mice were tested either first or second in the Shock box (and vice versa in the Non-Shock box) and always last in the Novel box. We also further reduced the footshock intensity (0.2 mA) to create more room for a memory enhancement effect by YOH. A general linear mixed model for 72-h retention latencies showed no effect of YOH treatment ($F(2,62.48) = 0.07$, $P = 0.94$), but did reveal a significant main effect of test context ($F(1,43.53) = 43.53$, $P < 0.001$), yet again without a significant YOH treatment X test context interaction

effect ($F(2,43.50) = 0.04$, $P = 0.96$). The main effect of test context was caused by longer retention latencies in the Shock Box compared to those in the Non-Shock box ($F(1,43.53) = 39.68$, $P < 0.001$) and Novel box ($F(1,70.50) = 63.26$, $P < 0.001$), and longer retention latencies in the Non-Shock box compared to the Novel box ($F(1,77.76) = 4.52$, $P = 0.04$). This latter difference could be related to the significant main effect of context order ($F(1,43.53) = 39.68$, $P < 0.001$) that we still observed. Retention latencies became progressively shorter upon test context exposures, with retention latencies in the second test context being shorter compared to those in the first test context ($F(1,43.53) = 43.53$, $P < 0.001$), and retention latencies in the third test context being shorter compared to those in both the second ($F(1.79.97) = 4.70$, $P = 0.03$) and first test context ($F(1,72.92) = 66.31$, $P < 0.001$). Thus, we did not replicate the rat findings where YOH enhanced both the strength and specificity of memory (Atucha and Roozendaal, 2015; Roozendaal and Mirone, 2020).

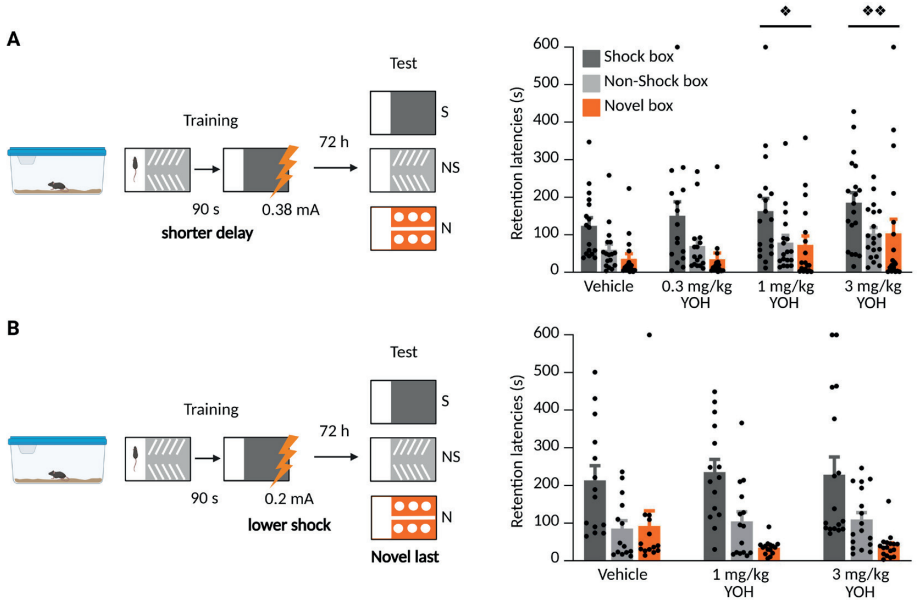


Figure 5. Effect of yohimbine treatment on strength and specificity of memory in the dual-event inhibitory avoidance task in mice. A. Main effect of yohimbine (YOH) treatment on 72-h retention latencies (following training with a 90 s interval and 0.38 mA footshock), caused by longer retention latencies across test contexts of mice administered the two higher dosages of YOH (1 and 3 mg/kg). No significant YOH treatment X test context interaction effect was found (Vehicle: $n = 17$, YOH 0.3 mg/kg: $n = 16$, YOH 1 mg/kg: $n = 19$, YOH 3 mg/kg: $n = 20$). **B.** We only counterbalanced the order of retention testing in the Shock box and Non-Shock box (Novel box always last) and lowered the footshock intensity (0.20 mA). Still, we found no effect of YOH treatment or YOH treatment X test context interaction on retention latencies (Vehicle: $n = 14$, YOH 1 mg/kg: $n = 15$, YOH 3 mg/kg: $n = 17$). Data represent mean + s.e.m. Dots indicate individual data points. ❖: $P < 0.05$, ❖❖: $P < 0.01$: different from saline.

4. Discussion

We aimed here to set up the dual-event inhibitory avoidance task for mice. This task allows for the assessment of both strength and episodic-like specificity of memory in rodents. This task was previously used in rats to demonstrate that YOH enhances both the episodic-like specificity and strength of memory while CORT induces an unspecific strengthening of memory. The present study aimed to investigate whether the dual-inhibitory avoidance is suitable to investigate stress hormone effects on memory specificity in mice. We observed that mice were capable of acquiring episodic-like memory specificity, displaying longer retention latencies in the Shock box than in the other two boxes. However, we failed to observe any modulatory effect of CORT or YOH administration on episodic-like specificity of memory as previously reported for rats. We further only found a weak memory-strengthening effect of YOH and were not able to replicate the memory-strengthening effect of CORT. As doses of CORT and YOH were selected based of their memory-enhancing effects on other behavioral tasks in mice (Cai et al., 2006; Song et al., 2020; Brosens et al., 2023) and we used a broad spectrum of dosages for both drugs, it appears unlikely that we might have overlooked the effective dose. Particularly the introduction of intraperitoneal injections in mice appeared problematic, resulting in loss of memory specificity in vehicle-treated mice, test order effects and interactions that rendered the task impracticable for investigating the effects of stress hormone manipulations on episodic-like memory specificity in mice.

We first established the training parameters that would generate a specific, episodic-like memory of moderate strength in mice. We started off by group housing our mice, but during the first experiment noticed substantial freezing behavior of mice that were tested as last from their cage. We considered that the long retention latencies, irrespective of the test context, of these mice were caused by anticipatory stress, as they witnessed the repeated removal of their cage mates, potentially similarly to what they had experienced during the training session. A switch to single housing was successful in preventing these exceptionally long retention latencies. This aligns with prior research indicating that anxiety-like behavior depends on the testing order of group-housed mice (Lyte et al., 2005). Subsequent findings indicated that mice were able to successfully associate the experience of footshock with the Shock box and distinguish the two training events.

Remarkably, whereas non-injected mice were able to display memory specificity, this effect was no longer observed when mice had received an intraperitoneal

injection after the training session using the same training conditions. Noteworthy, the brief period of restraint that is required for such injections is stressful to mice, and previously found to activate the HPA axis and induce the endogenous release of adrenocorticotropin and CORT (de Kloet et al., 2011; Baek et al., 2015). Although we did not measure CORT levels in our animals, a possible effect of injection stress would be consistent with our hypothesis that stress and glucocorticoids impair memory specificity. However, such stress-induced impairment of memory specificity might occlude possible effects of stress hormone administration on this readout. In rats, systemic injections were not found to affect memory specificity, but here less stressful subcutaneous injections were used (Roozendaal and Mirone, 2020), which do not require restraint and exposure of the vulnerable body parts (Miner et al., 1969; Levin-Arama et al., 2016). In mice, the procedure of intraperitoneal injection with restraint might therefore be even more aversive and salient than the actual training session. One could speculate that the injection event is inherently distinct from the two training events (including the shock), leading to a linking of memory the two training events following injection stress. To make the task more salient and facilitate memory specificity, we modified some of the training parameters, including an extension of the time spent in the training boxes and incorporating additional cues into the Novel box. These modifications indeed enhanced memory specificity, but this effect was not very robust.

Another issue that emerged upon introducing the intraperitoneal injections was the context order effects with mice displaying the longest retention latencies in the first box they were tested in. This shortening of retention latencies upon repeated box exposures seems to point toward a rather unspecific extinction of the fear association across testing contexts, and thus might fit the idea of proposedly unspecific memory for the contexts induced by injection stress. Noteworthy, we had attempted to avoid new safety learning during the retention test by always removing mice from each context as soon as they had entered the dark compartment. In some of our experiments, this context order effect interacted with the test context, being most pronounced when the mice were tested first in the Shock box, corroborating the idea of generalized extinction of fear. This context order X test context interaction severely confounded our results. In the first experiment without intraperitoneal injection, no such context order effect was observed. Similarly, also our previous studies in rats did not observe such a context order effect (Atucha and Roozendaal 2015). We attempted to reduce this confounding effect of context order by limiting the potential context order exposures, with either the Shock box or Non-Shock box being presented as the first box (and the Novel box always presented as the third box). We opted for this

approach as both memory strength and specificity are solely based on retention latencies in these two boxes, and prior research never revealed any effects of stress hormones on retention latencies in the Novel box. Yet, this did not reduce the order effects.

Noteworthy, in the dual-event inhibitory avoidance task animals are exposed only once to each of the two training contexts and only receive a single shock in the Shock box. Furthermore, the only outcome measure is retention latency which is a noisy readout. This makes the results highly variable, and like other inhibitory avoidance tasks, a large number of animals is required to achieve the necessary statistical power (Castellano el al., 1989; Mele et al., 1995; Blake et al., 2008). As such, other, more robust training paradigms might be preferred to assess stress hormone effects on memory specificity in mice. A potentially suitable candidate would be the object-in-context (OiC) task; a hippocampus-dependent episodic-like memory task in which two object presentation events during a training session are distinguished by the contexts in which they appear. During the subsequent retention test, the mice encounter one of each of the objects they explored during training, with one object being novel in the testing context, whereas the other object was already encountered in this test context before (Kanatsou et al., 2015; Pillai et al., 2018; Sep et al., 2021). Memory specificity is defined by a preferential exploration of the object that was not previously encountered in the testing context (Barsegyan et al., 2014). Thus, similar to the dual-event inhibitory avoidance task, the OiC task tests for the specificity of an associative memory across two training events. Whereas the OiC task still consists of a single training session, the task offers the benefit of a prolonged training duration, that can easily be modified to create a more robust memory (Barsegyan et al., 2014). Another advantage of the OiC task is a single context exposure on the retention test, thus avoiding any potential effects of repeated testing. Moreover, behavior on the retention test is less volatile, as it assesses exploratory behavior of rodents over the course of several minutes.

In conclusion, our findings indicate that mice can acquire episodic-like memory specificity in the dual-event inhibitory avoidance task. Yet, the introduction of the intraperitoneal injection to assess stress hormone effects severely impacted memory readouts; introducing context order interactions that complicated data interpretation. As such, other tasks might be better suitable to assess memory specificity in mice.

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7. Supplements

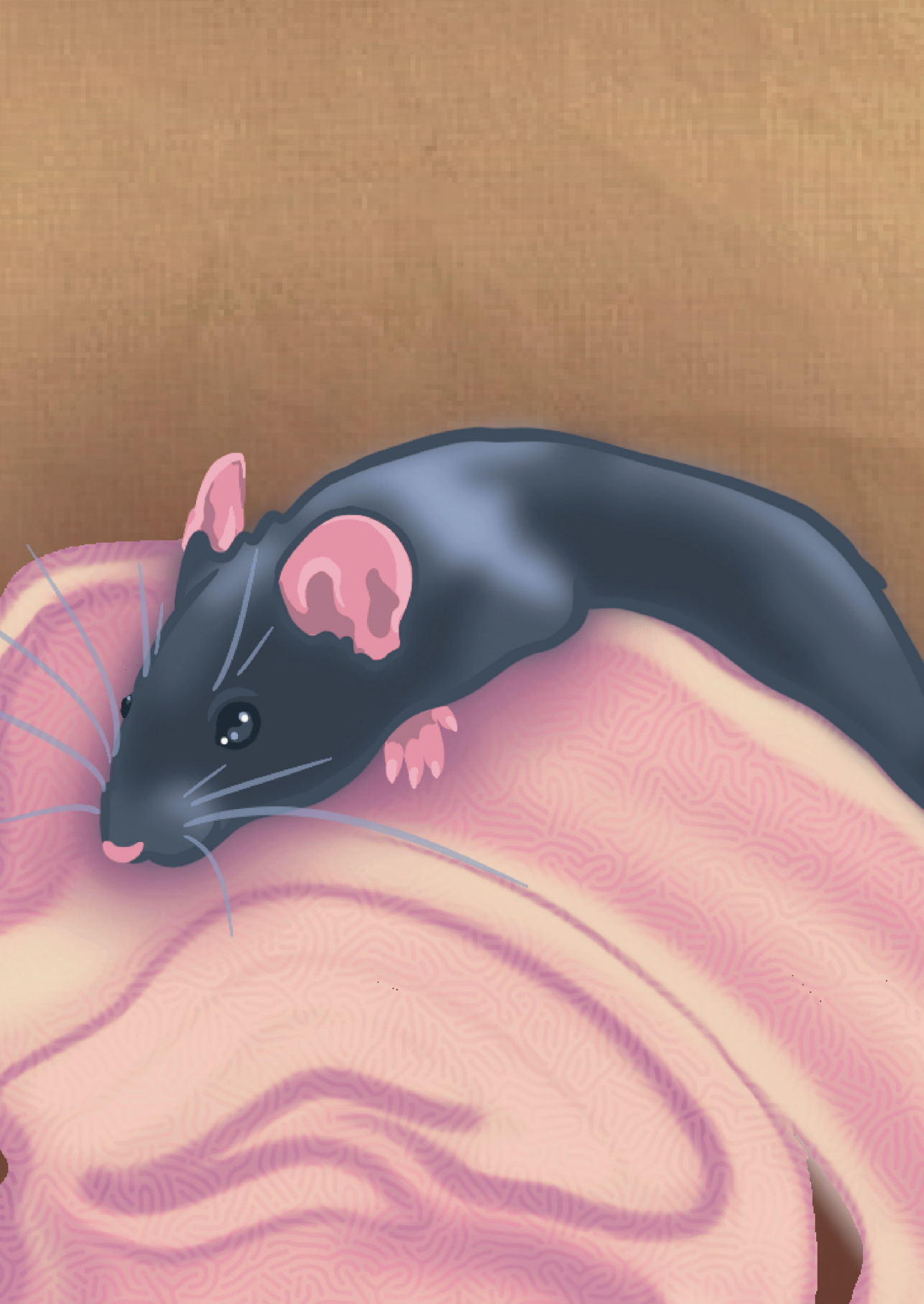
Table S1. Training and retention test latencies in seconds for animals shown in Figure 2, 3 and 5.

Figure 2	**TRAINING**		**RETENTION TEST**		
	Non-Shock mean ± s.e.m.	**Shock** mean ± s.e.m.	**Shock** mean ± s.e.m.	**Non-Shock** mean ± s.e.m.	**Novel** mean ± s.e.m.
(A) No Inj. (n = 27)	8.6 ± 2.6	12.6 ± 1.8	275.3 ± 47.5	169.5 ± 43.1	182.5 ± 48.8
(B) No Inj. (n = 10)	7.1 ± 2.2	8.8 ± 1.8	316.1 ± 66.4	44.2 ± 13.4	53.9 ± 27.7
Figure 3A	**TRAINING**		**RETENTION TEST**		
	Non-Shock mean ± s.e.m.	**Shock** mean ± s.e.m.	**Shock** mean ± s.e.m.	**Non-Shock** mean ± s.e.m.	**Novel** mean ± s.e.m.
Vehicle (n = 16)	7 ± 1.8	12.3 ± 2.2	90.3 ± 28.9	53.4 ± 17.7	57.3 ± 21.2
3 mg CORT (n = 11)	6.2 ± 1.4	14.5 ± 4.2	75.7 ± 20.0	57.6 ± 18.7	105.3 ± 40.7
10 mg CORT (n = 15)	5.1 ± 1.0	12.4 ± 2.5	80.6 ± 19.5	30.4 ± 7.4	71.2 ± 23.6
Figure 3B	**TRAINING**		**RETENTION TEST**		
	Non-Shock mean ± s.e.m.	**Shock** mean ± s.e.m.	**Shock** mean ± s.e.m.	**Non-Shock** mean ± s.e.m.	**Novel** mean ± s.e.m.
Vehicle (n = 15)	20.8 ± 2.4	23.4 ± 2.3	112.1 ± 23.9	52.2 ± 14.5	166.5 ± 58.9
1 mg CORT (n = 12)	26.4 ± 1.4	29.0 ± 1.9	111.3 ± 38.6	61.8 ± 12.7	146.6 ± 63.7
3 mg CORT (n = 16)	21.2 ± 2.5	23.6 ± 2.4	119.5 ± 49.1	48.1 ± 10.4	67.9 ± 36.5
10 mg CORT (n = 16)	19.7 ± 1.9	25.6 ± 2.4	122.6 ± 39.2	74.2 ± 36.3	171.2 ± 56.8
Figure 3C	**TRAINING**		**RETENTION TEST**		
	Non-Shock mean ± s.e.m.	**Shock** mean ± s.e.m.	**Shock** mean ± s.e.m.	**Non-Shock** mean ± s.e.m.	**Novel** mean ± s.e.m.
Vehicle (n = 14)	26.6 ± 1.0	26.6 ± 0.9	130.2 ± 41.3	77.0 ± 25.8	36.9 ± 17.4
3 mg CORT (n = 14)	25.8 ± 0.9	28.7 ± 1.2	168.9 ± 42.9	83.0 ± 20.9	57.6 ± 22.1
10 mg CORT (n = 28)	24.4 ± 0.8	29.8 ± 1.4	102.3 ± 23.0	67.8 ± 18.1	68.6 ± 33.5

Table S1. Continued

Figure 5A	**TRAINING**		**RETENTION TEST**		
	Non-Shock mean ± s.e.m.	**Shock** mean ± s.e.m.	**Shock** mean ± s.e.m.	**Non-Shock** mean ± s.e.m.	**Novel** mean ± s.e.m.
Vehicle (*n* = 17)	6.1 ± 0.4	7.3 ± 0.8	123.9 ± 21.2	59.8 ± 14.9	35.9 ± 13.5
0.3 mg YOH (*n* = 16)	6.3 ± 0.8	8.7 ± 1.3	150.3 ± 37.7	69.7 ± 19.0	34.4 ± 16.9
1 mg YOH (*n* = 18)	8.1 ± 1.2	10.1 ± 1.2	162.5 ± 34.0	78.7 ± 19.5	72.5 ± 24.0
3 mg YOH (*n* = 19)	6.6 ± 0.7	11.0 ± 1.6	185.1 ± 27.4	102.6 ± 16.2	103.1 ± 37.9
Figure 5B	**TRAINING**		**RETENTION TEST**		
	Non-Shock mean ± s.e.m.	**Shock** mean ± s.e.m.	**Shock** mean ± s.e.m.	**Non-Shock** mean ± s.e.m.	**Novel** mean ± s.e.m.
Vehicle (*n* = 14)	32.3 ± 3.3	29.6 ± 1.0	213.2 ± 39.3	85.2 ± 21.6	92.4 ± 40.3
1 mg YOH (*n* = 15)	27.1 ± 1.2	30.3 ± 1.9	235.9 ± 33.2	104.0 ± 26.2	34.9 ± 5.1
3 mg YOH (*n* = 17)	29.8 ± 2.6	29.6 ± 1.3	228.9 ± 46.7	109.0 ± 19.2	39.1 ± 8.5

CORT = corticosterone, YOH = yohimbine, No inj. = No injection.



CHAPTER 3

Effect of stress exposure prior to retention testing on episodic-like specificity of memory: Involvement of mitochondrial function

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Abstract

Stress exposure or glucocorticoid administration prior to retention testing is known to impair memory recall. However, it had not been investigated whether such stress exposure also affects qualitative aspects of the recalled memory, such as its episodic-like specificity. Here, we examined whether acute stress exposure shortly before retention testing affects episodic-like specificity of memory recall. Further, as findings have indicated that glucocorticoids affect mitochondrial function in regulating several memory processes, we also examined whether stress exposure interacts with mitochondrial activity in influencing memory recall. To investigate this, we used both wildtype (WT) mice and a mouse line with a genetic deficiency in the *nuclear-encoded NADH dehydrogenase [ubiquinone] iron-sulfur protein 4* (*Ndufs4*), characterized by a reduced mitochondrial complex I activity. Both WT and *Ndufs4*$^{GT/GT}$ mice were trained on an dual-event inhibitory avoidance task, in which they explored two different contexts, but footshock was administered only in the latter context. Forty-eight hours later, retention latencies were tested in the two training contexts as well as in a novel context. Thirty minutes prior to the retention test, a subgroup of both *Ndufs4*$^{GT/GT}$ and WT mice was exposed to a 15-min forced swim stress. One hour after the retention test, we examined mitochondrial activity as well as expression of the neuronal activity marker c-Fos within both the hippocampus and prefrontal cortex (PFC). We found that stress exposure similarly increased plasma corticosterone (CORT) levels in both genotypes. Non-stressed WT mice displayed long latencies specific to the shock context, and mitochondrial dysfunction impaired this episodic-like specificity of memory, reflected by similar retention latencies in all test contexts. Stress exposure 30 min prior to the retention test interacted with mitochondrial dysfunction in influencing retention performance. Whereas stress exposure in WT mice selectively shortened latencies in the shock context, reflecting impaired memory recall, stress exposure in *Ndufs4*$^{GT/GT}$ mice generally increased latencies in both training contexts, but not the novel context. As expected, non-stressed *Ndufs4*$^{GT/GT}$ mice had lower complex I mitochondrial activity in the hippocampus compared to non-stressed WT mice. Stress exposure in WT mice selectively reduced mitochondrial complex I activity in the hippocampus, whereas stress exposure in *Ndufs4*$^{GT/GT}$ mice did not further reduce mitochondrial complex I activity in the hippocampus. In contrast, stress exposure in *Ndufs4*$^{GT/GT}$ mice significantly increased mitochondrial complex IV activity in the PFC. Further, stress exposure was found to reduce c-Fos activity in the hippocampus but to increase c-Fos activity within the PFC. Both of these effects were more pronounced in *Ndufs4*$^{GT/GT}$ mice. These findings emphasize the critical role of mitochondrial function in the hippocampus in regulating stress effects on recall of episodic-like specificity of memory.

Keywords: *memory specificity; memory strength; stress; mitochondrial dysfunction; Ndufs4$^{GT/GT}$; dual-event inhibitory avoidance task*

1. Introduction

Extensive evidence indicates that stress and glucocorticoid hormones are potent modulators of memory, affecting both the consolidation and retrieval of memory (de Quervain et al., 1998; Roozendaal, 2000; Roozendaal, 2011; Schwabe et al., 2012; de Quervain et al., 2017; Schwabe et al., 2022). Recent findings have indicated that the effect of posttraining stress exposure or glucocorticoid administration on memory consolidation not only influences the strength of memory but also several qualitative aspects of memory, such as its specificity, detailedness, and contextualization (Schwabe et al., 2022). In our laboratory, we previously found that systemic CORT administration immediately after training on a dual-event inhibitory avoidance task enhanced memory of the footshock experience *per se*, but impaired the episodic-like specificity of memory regarding the association of shock exposure with the specific training context (Roozendaal and Mirone, 2020). These findings are in agreement with other findings indicating that posttraining stress exposure or CORT administration enhances generalization of fear memory to other contexts or innocuous stimuli (Kaouane et al., 2012; Dos Santos Corrêa et al., 2019; Lesuis et al., 2021). Most studies investigating such stress and glucocorticoid effects on episodic-like specificity of memory have used posttraining manipulations to selectively affect the consolidation phase of memory. However, stress exposure or glucocorticoid administration prior to retention testing is well known to impair the retrieval of hippocampus-dependent memory (de Quervain et al., 1998; de Quervain et al., 2003; Roozendaal et al., 2004; Yang et al., 2004). It has not been investigated whether such stress hormone manipulations prior to retention testing also influence the episodic-like quality of these recalled memories.

Previous studies have indicated that the effect of posttraining glucocorticoid administration on reducing episodic-like specificity of memory involves an enduring shift from hippocampal toward PFC engagement in the memory (Dos Santos Corrêa et al., 2019; Lesuis et al., 2021; Bahtiyar et al., 2020). Such glucocorticoid effects on memory specificity during the consolidation phase likely require coordinated changes in gene expression in both brain regions (Atucha et al., 2017; Bahtiyar et al., 2020; Gulmez-Karaca et al., 2025). In contrast, the effect of glucocorticoid administration prior to retention testing on impairing memory retrieval are rapidly induced and temporary in nature, and therefore do not seem to

depend on gene transcription (de Quervain et al., 1998; Sajadi et al., 2006). Several studies have revealed that glucocorticoid effects on memory retrieval involve fast and non-genomic interactions with the endocannabinoid system (Atsak et al., 2012; Morena et al., 2015), with glucocorticoid effects on memory retrieval at least being partially mediated by cannabinoid type 1 (CB1) receptors located on mitochondrial membranes (mtCB1) (Skupio et al., 2023). These findings are consistent with more general evidence indicating that glucocorticoids, via both genomic and non-genomic actions, can modulate mitochondrial function (Moutsatsou and Papavassiliou, 2007), and that they assist in energy release during stress (Hunter et al., 2016). Mitochondria are highly dynamic within neurons, and are trafficked to regions of high metabolic demand such as the synapse, where they play a central role in multiple biochemical pathways that regulate neural plasticity and memory (Mattson et al., 2008; Tanaka et al., 2008; Hebert-Chatelain et al., 2016). However, it has not been investigated whether changes in mitochondrial activity *per se* regulate episodic-like specificity of memory and whether acute stress exposure prior to retention testing interacts with mitochondrial activity in influencing episodic-like specificity of memory.

In the present study, we examined whether acute stress exposure shortly before retention testing on a dual-event inhibitory avoidance task affects episodic-like specificity of the recalled memory and whether this effect involves changes in mitochondrial activity. Moreover, we investigated the effect of mitochondrial activity *per se* on episodic-like memory specificity and its modulation by stress. To test this, we used a mouse line with a genetic deficiency in the *nuclear-encoded NADH dehydrogenase [ubiquinone] iron-sulfur protein 4* (*Ndufs4*) (Kahlhöfer et al., 2021), which is characterized by a reduced mitochondrial complex I (CI) activity compared to wildtype (WT) mice (Emmerzaal et al., 2020). Both *Ndufs4*$^{GT/GT}$ and WT mice were trained on the dual-event inhibitory avoidance task where they could explore two similar, but contextually distinct, inhibitory avoidance apparatuses with a short interval. They received a single inescapable footshock only after entering the dark compartment of the second of these two contexts (Atucha and Roozendaal, 2015). Forty-eight hours later, retention latencies to enter the dark compartment of these two previously encountered contexts (Shock box and Non-Shock box) as well as a Novel box were assessed in a random order, allowing us to determine both the specificity and strength of memory (Atucha and Roozendaal, 2015). Thirty minutes prior to the retention test, a subgroup of both *Ndufs4*$^{GT/GT}$ and WT mice was exposed to a 15-min forced swim stress. One hour after the retention test, we examined mitochondrial activity as well as expression of the neuronal activity marker c-Fos in both excitatory and inhibitory neurons within both the hippocampus and PFC.

2. Material and Methods

2.1. Animals

Male adult *Ndufs4*$^{GT/GT}$ (n = 58) and WT (n = 58) mice, 7-14 weeks old at the start of the experiment, were used in the study. Mice were generated in-house by heterozygous breeding (strain from Jackson: B6.129S4-Ndufs4 tm1.1Rpa/J // Jax (stock 027058)). As previously described (Emmerzaal et al., 2020), *Ndufs4*$^{GT/GT}$ mice were generated through a gene trap insertion within the first intron of the Ndufs4 gene. This insertion was previously shown to cause reduced NDUFS4 protein production, resulting in an approximately 25% reduction in mitochondrial CI activity relative to WT mice (Emmerzaal et al., 2021; Emmerzaal, 2022). This genetic modification will be referred to as mitochondrial dysfunction. Mice were maintained on a 12:12-h light:dark cycle (07:00-19:00 h lights on) in a temperature-controlled (22 °C) vivarium room with a light intensity of 47 lux and humidity of 72%. They had *ad libitum* access to food and water. Ten days prior to the start of the behavioral experiment, mice were housed individually to prevent fighting between cage mates as well as testing order effects (Chapter 2). Training and testing were performed during the light phase of the cycle between 10:00 and 15:00 h, at the nadir of the circadian cycle of CORT. All experimental procedures were in compliance with European Union Directive 2010/63/EU and were approved by the Central Authority for Scientific Procedures on Animals, The Hague, The Netherlands. All efforts were made to minimize animal suffering and to reduce the number of animals needed.

2.2. Dual-event inhibitory avoidance task

Mice 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 48 h later, mice were tested in the two training contexts as well as in a novel context (Figure 1). Each apparatus had the same geometry and consisted of a trough-shaped alley (36 cm long, 17 cm wide at the top and 4 cm wide at the bottom) divided into two compartments, separated by a sliding door that could be manually operated. The starting compartment (12 cm) was made of opaque white plastic and was well lit. The shock compartment (24 cm long) consisted 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 five white stripes (2 cm wide, 8 cm long) taped on each wall of the dark compartment together with tape placed on the floor, closing the gap

between the two plates along the entire length of the apparatus. The Novel box (used on the retention test only) had three white circles (4 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 another in a sound- and light-attenuated room. For a detailed description of the task see Atucha and Roozendaal (2015) and Chapter 2 of this thesis.

Prior to the training session, mice were handled for 2 min each on five consecutive days to habituate them to the experimenter. For training, mice were initially placed into the starting compartment of the Non-Shock box and could explore the apparatus, including the dark compartment, for 20 s, without footshock being delivered. Afterwards, the mice were removed from the apparatus and, after a 2-min interval in their home cage, placed into the starting compartment of the Shock box. When the mice had stepped into the dark compartment of the Shock box, the sliding door was closed and a single inescapable footshock of 0.5 mA was delivered for 1 s. Mice were removed from the apparatus 20 s after termination of footshock and returned to their home cages.

The retention test took place 48 h after the training. Retention latencies were assessed, in a randomized order and without delay, in the two training contexts (i.e., Shock box and Non-Shock box) and in the Novel box they had not visited before. For all three boxes, the mice were placed into the starting compartment, and their latency to enter the dark compartment with all four paws was recorded (maximum latency of 600 s). Longer latencies in the Shock box were interpreted as indicative of stronger memory of the shock experience, and longer latencies in the Shock box relative to the Non-Shock box as episodic-like specificity of memory for the association of shock with the specific training context. Latencies in the Novel box were used to determine whether changes in memory performance were restricted to the two training contexts or also generalized to another, not previously seen context. After the retention test (which took a maximum of 30 min), animals were placed back in their home cage until tissue collection which took place 1 h after the start of the retention test.

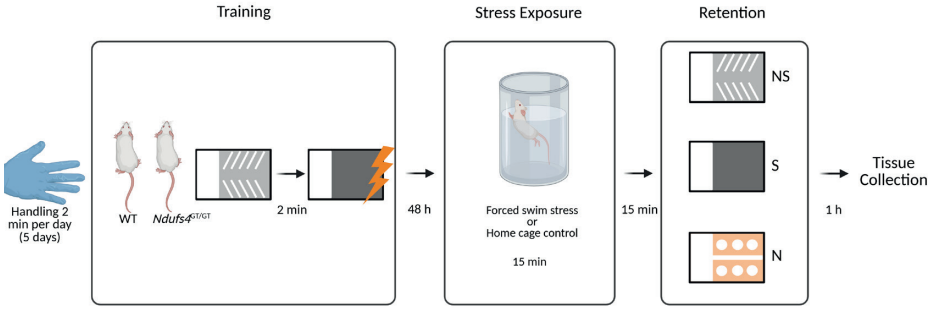

Figure 1. Experimental design. Both *Ndufs4*$^{GT/GT}$ and wildtype (WT) mice were trained first in the Non-Shock box and 2 min later in the Shock box. Footshock was only delivered in the Shock box. On the 48-h retention test, retention latencies were assessed in the two training contexts, i.e., Non-Shock box and Shock box , and in a Novel box. Stressed groups (*Ndufs4*$^{GT/GT}$ stress, n = 19, WT stress, n = 15) were exposed to a 15-min forced swim stress procedure starting 30 min prior to the retention test. Non-stressed control groups (*Ndufs4*$^{GT/GT}$ control, n = 21, WT control, n = 18) were left undisturbed in their home cage until the retention test. Blood and brain tissue was collected 1 h after the start of the retention test.

2.3. Stress manipulation

Mice that were assigned to the stress condition were subjected to a 15-min forced swim stress 30 min prior to the start of the retention test. Mice were individually placed in a glass cylinder (1000 mL capacity, 6 cm diameter, 45 cm high) filled with water up till 35 cm height at room temperature (RT) for 15 min. Afterwards, they were dried with a towel and placed back in their home cage until the start of the retention test. Non-stressed control mice were not subjected to this stress procedure and left undisturbed in their home cage until the retention test. Other groups of trained mice were killed 60 min after the stress manipulation without retention testing to determine the effect of the stress manipulation on plasma CORT levels in the two genotypes *per se*.

2.4. Blood and brain tissue collection

One hour after the start of the retention test (when animals were back in their home cage), mice were killed by rapid decapitation without the use of anesthetics. Trunk blood, for plasma CORT measurement, was collected in 0.5-mL EDTA K2 tubes and placed on ice until centrifugation (1,000xg for 15 min at 4 ^{0}C). The supernatant was stored at −20 ^{0}C. Brains were rapidly removed and the two hemispheres separated. The left hemisphere was used for mitochondrial complex activity measurements. For this, the hippocampus and PFC were rapidly dissected and directly frozen on dry ice. Frozen hemispheres were stored at -80 °C. The right hemisphere was used for immunohistochemical analysis of neuronal activity, and placed in a beaker filled with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) (pH 7.4)

that was placed inside a larger beaker filled with water. Then, the samples were microwave-perfused at 650 W for 5 s, shaken and again microwaved at 650 W for 5 s. They were post-fixed in 4% PFA in 0.1 M PBS (pH 7.4) for 24 h, cryoprotected in 30% sucrose in 0.1 M PBS for at least 2 days, and then stored at -80 ^{0}C. Fixed hemispheres were used for the two different analyses based on prior reports on lateralization of stress effects, typically increasing engagement of the right hemisphere (Carlson et al., 1993; Sullivan, 2004).

2.5. Corticosterone measurement

Plasma CORT levels were measured in duplicate by using a CORT double antibody radioimmunoassay (RIA) kit (sensitivity: 12.5 ng/mL, SKU:07120103, MP Biomedicals, Huissen, The Netherlands). RIA CORT response assessment was performed by using linear fitting and logarithmic plotting software for standard curve and data extrapolation, developed at the Dept. Chemical Endocrinology of Radboudumc. The coefficient of variance among the duplicates equaled 15.8%. Samples were excluded if the variance between duplicates was >20%.

2.6. Mitochondrial electron transport chain complex activity measurements

Mitochondrial electron transport chain (MtETC) complex activity within the hippocampus and PFC was assessed spectrophotometrically using a KoneLab 20XT analyzer (Thermo Scientific) following an established protocol (Janssen et al., 2007; Rodenburg, 2011). These analyses were performed in the laboratory of Prof. Tamas Kozicz at the Mayo Clinic, Rochester, USA. For the assessment of mitochondrial CI activity, 2,6-dichlorophenolindophenol (DCIP) served as the terminal electron acceptor. CI oxidizes nicotinamide adenine dinucleotide + hydrogen (NADH), and the generated electrons reduce the substrate coenzyme Q1, subsequently reducing DCIP. The reduction of DCIP was monitored spectrophotometrically at 600 nm. Rotenone, a specific inhibitor of CI, was included as a blank measurement. For the determination of complex II (CII) activity, DCIP was also employed as the terminal electron acceptor (Fischer et al., 1985). CII converts succinate + flavin adenine dinucleotide (FAD) to fumarate + flavin adenine dinucleotide + hydrogen (FADH2), and electrons from FADH2 are transferred to deubiquitinate (DUB), forming deubiquitinate + hydrogen (DUBH) and reducing DCIP. The reduction of DCIP was tracked at 600 nm. Malonate, a specific inhibitor of CII, was added as a blank measurement. Complex III (CIII) activity was measured with the artificial substrate DUBH and (reduced) cytochrome c, which is converted by CIII into DUB and oxidized cytochrome c (Mourmans et al., 1997). This results in an increased extinction at 550 nm. Complex IV (CIV) activity was measured by determining cytochrome c oxidase

(COX) activity (Cooperstein and Lazarow, 1951). COX oxidizes reduced cytochrome c, in which oxygen is reduced to water. The decrease of reduced cytochrome c can be measured at 550 nm. mtETC complex activities were normalized to total protein content, a commonly used method for normalization of mitochondrial activity (Bender et al., 2010; Schmitt et al., 2014; Musyaju et al., 2023).

2.7. Immunohistochemistry

To determine neuronal activity, 30 µm-thick coronal slices of the hippocampus and PFC were cut on a cryostat, collected in 0.1 M PBS with 0.1% sodium azide and stored at 4 °C. Five sections of both the hippocampus (anteroposterior (AP), -1.46 to -1.94 mm from Bregma) and PFC (AP, +1.98 to +1.18 mm from Bregma) of each animal were selected according to the Franklin and Paxinos mouse brain atlas (2007). Approximately 15–16 slices were collected from the hippocampus and 25–26 slices from the PFC. For analysis, every 4th, 6th, 8th, 10th, and 12th slice was selected from the hippocampus, and every 4th, 8th, 12th, 18th, and 22nd slice from the PFC. If a designated slice was deemed suboptimal, an adjacent slice was selected instead. General tissue quality was checked under a light microscope prior to immunostaining. Eight animals with extensive tissue damage, likely caused by the perfusion method, were excluded from further analyses. Selected sections were rinsed in 0.5% Triton X-100 in PBS for 30 min at RT, washed three times in PBS for 10 min per wash and blocked in 5% normal donkey serum (NDS, Jackson ImmunoResearch Laboratories) in PBS for 1 h at RT. Sections were then incubated with primary antibodies (guinea pig anti-c-Fos; 1:500, Synaptic Systems #226004; mouse anti-glutamic acid decarboxylase 67 (GAD67); 1:500, Sigma-Aldrich #MAB5406) in PBS with 5% NDS and 0.1% acetylated bovine serum albumin (BSA-c; Aurion) overnight at RT. Afterwards, the sections were washed three times in PBS for 10 min per wash, followed by incubation with fluorophore-conjugated secondary antibodies (donkey anti-guinea pig Alexa Fluor 647, 1:500, Jackson ImmunoResearch #706-605-148; donkey anti-mouse Alexa Fluor 488, 1:500, Invitrogen #A21202) for 3 h at RT. All procedures starting from the secondary antibody incubation onwards were performed in the dark. During the last 15 min of incubation, 4,6-diamidine-2-phenylindole dihydrochloride (DAPI, 1:5,000) was added. Then, sections were washed three times in PBS for 10 min per wash, mounted on gelatin-coated slides, air-dried and coverslipped with FluoroSave mounting medium (Sigma-Aldrich) and stored at -20 °C.

2.8. Imaging and quantification

Fluorescent images of the hippocampus and PFC were taken at 20x magnification using an automated high-content fluorescence microscope (Leica DMI 6000B,

Germany), and image processing was performed in FIJI (NIH, version 1.0) (Schindelin et al., 2012). First, image tiles were corrected for differences in background signal due to signal bleaching by the BaSiC plugin (Peng et al., 2017), and stitched to a single image by the Grid/StitchCollection plugin in FIJI. Then, a set of regions of interest (ROIs) for each brain region was drawn manually based on the Allen Mouse Brain Atlas (https://portal.brain-map.org/). For the analysis of c-Fos$^+$ neurons, the hippocampus was divided into its three main subregions: dentate gyrus (DG), cornu ammonis 3 (CA3) and cornu ammonis 1 (CA1) (Figure 2A). As GABAergic interneurons are mostly found within dendritic areas of the hippocampus (Klausberger, 2009), we analyzed double labeling for c-Fos and GAD67, as proxy for GABAergic activity, selectively within the *stratum molecularis* (*mo*) and *hilus* of the DG and the *stratum oriens* (*so*) and *stratum radiatum* (*sr*) of the CA3 and CA1 (Figure 2B). Subregional cell counts were combined to get a single value for each main region (DG, CA3 and CA1). Selected PFC regions were the anterior cingulate (AC), prelimbic (PL) and infralimbic (IL) cortices (Figure 2C). ROIs were drawn with the help of the DAPI staining. Counts of all sections of an animal were averaged per ROI to generate a mean value per animal. All analyses were performed by a researcher blind to experimental condition.

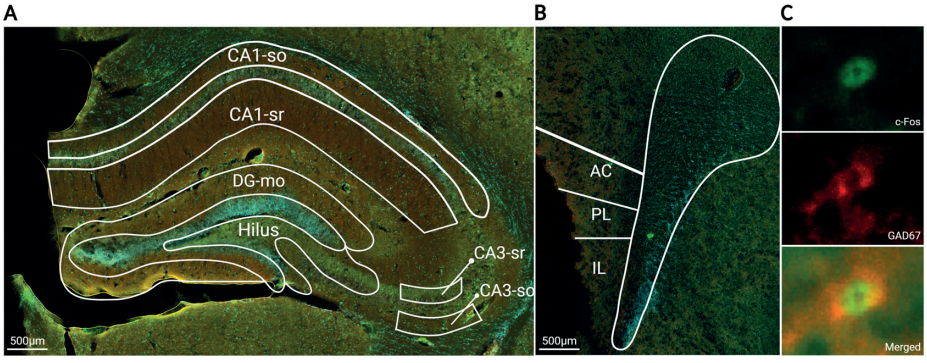

Figure 2. Depiction of ROIs in hippocampus and PFC. A. For the analyses of c-Fos$^+$ neurons, the hippocampus was divided into its three main subregions: dentate gyrus (DG), cornu ammonis 3 (CA3) and cornu ammonis 1 (CA1). For the analyses of the double labeling for c-Fos and GAD67, as proxy for GABAergic activity, ROIs comprised the *stratum molecularis* (mo) and *hilus* of the DG and the *stratum oriens (so)* and *stratum radiatum (sr)* of the CA3 and CA1. **B.** The PFC was divided into the anterior cingulate (AC), prelimbic (PL) and infralimbic (IL) cortices. **C.** Example of c-Fos and GAD67 staining and overlap in an activated GABAergic neuron (50 µm).

2.9. Statistics

Data were analyzed in SPSS (IBM SPSS Statistics, Version 27). Training latencies on the dual-event inhibitory avoidance task were analyzed with a linear mixed model with mitochondrial dysfunction (WT vs. *Ndufs4*$^{GT/GT}$) as between-subject

factor and training context (Shock box, Non-Shock box) as within-subject factor. Retention latencies where analyzed with a linear mixed model with stress exposure (control vs. stress) and mitochondrial dysfunction (WT vs. *Ndufs4*$^{GT/GT}$) as between-subject factors and test context (Shock box, Non-Shock box, Novel box) as within-subject factor. Subsequent analyses examined stress exposure effects on retention performance in WT and *Ndufs4*$^{GT/GT}$ mice separately. *Post hoc* comparisons used independent or paired samples *t*-tests (all two-tailed), when appropriate. Deviations from normal distribution were checked for by Shapiro-Wilk tests. In case data significantly deviated from normal distribution, between-subject effects of stress exposure and mitochondrial dysfunction were confirmed by Mann Whitney U tests, whereas within-subject effects of test context were verified by Friedman's test and/or Wilcoxon's test. For sake of consistency, we report *F*- and *t*-test results throughout, as the inclusion of non-parametric tests did not alter overall conclusions.

Plasma CORT levels and MtETC complex activity levels were analyzed by two-way ANOVAs with stress exposure and mitochondrial dysfunction as between-subject factors, followed by independent samples *t*-tests (two-tailed), when appropriate. Immunohistochemistry data were analyzed with linear mixed models with stress exposure and mitochondrial dysfunction as between-subject factors and hippocampal/PFC subregion as within-subject factor. Subsequently, data were analyzed for each ROI separately with two-way ANOVAs with stress exposure and mitochondrial dysfunction as factors, followed by independent samples *t*-tests (two-tailed), when appropriate.

Lastly, Pearson correlations were used to test for correlations of c-Fos counts between hippocampal and PFC subregions for each of the four experimental groups. Pearson correlations were also used to examine whether changes in mitochondrial activity were associated with changes in neuronal activity in the hippocampus and PFC. For all statistical tests, $P < 0.05$ was accepted for statistical significance, except for the Pearson correlations where we kept a more stringent threshold of $P < 0.01$.

3. Results

3.1. Effect of stress exposure on corticosterone levels in *Ndufs4*$^{GT/GT}$ and WT mice

Previous studies have indicated that mitochondrial activity can alter the stress response (Zhao et al., 2002). Therefore, we initially determined the effect of acute

exposure to forced swim stress on plasma CORT levels in WT and *Ndufs4*$^{GT/GT}$ mice. For this, separate groups of trained WT and *Ndufs4*$^{GT/GT}$ mice were exposed to a 15-min forced swim stress, and blood was collected 60 min later without retention testing on the dual-event inhibitory avoidance task. Control groups were not exposed to the stress procedure but remained undisturbed in their home cage. A two-way ANOVA for plasma CORT levels indicated a main effect of stress exposure ($F_{1,41}$ = 19.78, P < 0.001), but no main effect of mitochondrial dysfunction ($F_{1,41}$ = 1.17, P = 0.28) or stress exposure x mitochondrial dysfunction interaction effect ($F_{1,41}$ = 0.90, P = 0.84). *Post hoc* analyses revealed that plasma CORT levels of both stressed WT ($t_{14.21}$ = 2.86, P = 0.01) and stressed *Ndufs4*$^{GT/GT}$ mice ($t_{17.01}$ = 4.03, P = 0.001) were significantly higher than those of their respective non-stressed control group (Figure 3A). Plasma CORT levels of stressed WT and *Ndufs4*$^{GT/GT}$ mice did not differ significantly from each other (t_{21} = 1.27, P = 0.22). Also, plasma CORT levels of control WT and *Ndufs4*$^{GT/GT}$ mice did not differ significantly (t_{20} = 0.25, P = 0.80). Thus, these findings indicate that both basal and stress-induced CORT levels were comparable across genotypes.

Additionally, we assessed plasma CORT levels in both stressed and control WT and *Ndufs4*$^{GT/GT}$ mice 60 min after the retention test (i.e., roughly 90 min after the start of the stress exposure). At that timepoint, a two-way ANOVA revealed no significant effect of stress exposure ($F_{1,52}$ = 0.29, P = 0.59), mitochondrial dysfunction ($F_{1,52}$ = 0.27, P = 0.60) or stress exposure x mitochondrial dysfunction interaction effect ($F_{1,52}$ = 1.18, P = 0.19, Figure 3B). These findings thus indicate that plasma CORT levels in both WT and *Ndufs4*$^{GT/GT}$ mice had returned to baseline at the time of tissue collection.

3.2. Effect of stress exposure on retention performance of WT and *Ndufs4*$^{GT/GT}$ mice

We then assessed the impact of stress exposure, 30 min before the retention test, on both the strength and episodic-like specificity of the recalled memory. Entrance latencies into the dark compartments of the two contexts during the training session did not differ significantly between WT and *Ndufs4*$^{GT/GT}$ mice (mitochondrial dysfunction: $F_{1,70}$ = 1.83, P = 0.55; context: $F_{1,70}$ = 4.30, P = 0.052; mitochondrial dysfunction x context: $F_{1,70}$ = 0.03, P = 0.86). Further, entrance latencies during the training session did not differ between groups assigned later to the stress exposure or control condition ($F_{1,70}$ = 1.70, P = 0.57). A linear mixed model for 48-h retention latencies in the three test contexts indicated significant main effects of stress exposure ($F_{1,194.55}$ = 13.76, P < 0.001), mitochondrial dysfunction ($F_{1,194.55}$ = 17.85, P < 0.001) and test context ($F_{2,144.29}$ = 22.81, P < 0.001, Figure 4). In addition, we

found significant stress exposure x mitochondrial dysfunction ($F_{1,194.55}$ = 48.98, $P < 0.001$) and stress exposure x mitochondrial dysfunction x test context interaction effects ($F_{2,144.29}$ = 8.79, $P < 0.001$).

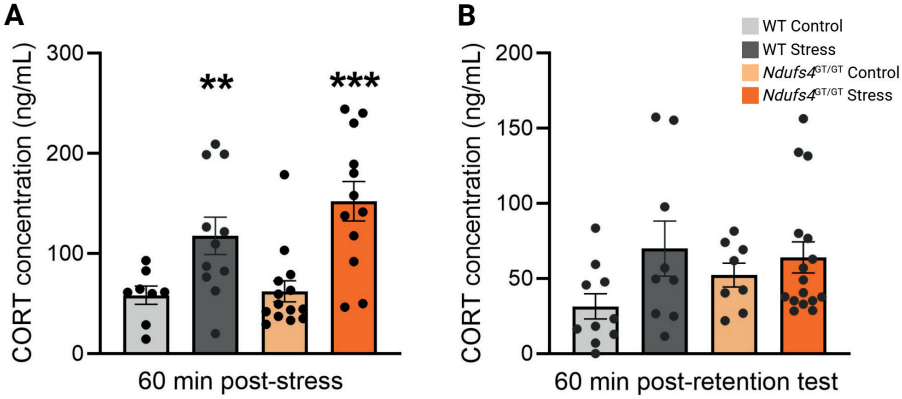

Figure 3. Plasma corticosterone levels in stressed and control mice. A. Stress exposure (15-min forced swim stress) induced elevated plasma CORT levels in both WT and *Ndufs4*$^{GT/GT}$ mice 60 min later. Animals of these groups were not subjected to a retention test. WT control: n = 8, WT stress: n = 14, *Ndufs4*$^{GT/GT}$ control: n = 11, *Ndufs4*$^{GT/GT}$ stress: n = 12. **B.** Plasma CORT levels 60 min after the retention test of stressed WT and *Ndufs4*$^{GT/GT}$ mice did not differ from those of non-stressed mice. WT control: n = 14, WT stress: n = 16, *Ndufs4*$^{GT/GT}$ control: n = 12, *Ndufs4*$^{GT/GT}$ stress: n = 16. $^{**}P < 0.01$, $^{***}P < 0.001$ vs respective control group. Graphs depict mean ± SEM and individual data points.

In WT mice, we observed significant main effects of stress exposure ($F_{1,51.10}$ = 7.62, P = 0.008) and test context ($F_{2,49.63}$ = 9.60, $P < 0.001$), as well as a significant stress exposure x test context interaction effect ($F_{2,49.63}$ = 3.91, P = 0.03). Non-stressed WT mice accurately discriminated the shock context from the two safe contexts (main effect of test context: $F_{2,27.56}$ = 8.95, P = 0.001), as indicated by longer retention latencies in the Shock box compared to those in both the Non-Shock box (paired samples t-test: t_{16} = 4.22, $P < 0.001$) and Novel box (t_{16} = 3.74, P = 0.002). Retention latencies in the Non-Shock box and Novel box did not significantly differ from each other (t_{16} = 0.18, P = 0.86). Stress exposure in WT mice reduced retention latencies in the Shock box (independent samples t-test: t_{30} = 2.80, P = 0.009), whereas it did not significantly affect retention latencies in either the Non-Shock box (t_{30} = 0.47, P = 0.65) or Novel box ($t_{27.44}$ = -1.72, P = 0.10). As a result, retention latencies of stressed WT mice in the three test contexts did not differ significantly from each other ($F_{2,21.42}$ = 3.13, P = 0.06).

In contrast to WT mice, non-stressed *Ndufs4*$^{GT/GT}$ control mice displayed similar retention latencies in all three test contexts ($F_{2,47.15}$ = 2.10, P = 0.13). Retention

latencies of non-stressed *Ndufs4*$^{GT/GT}$ mice in the Shock box were significantly shorter than those of non-stressed WT mice (independent samples *t*-test: $t_{36} = 2.96$, $P = 0.005$), whereas retention latencies in the Non-Shock box ($t_{36} = 0.37$, $P = 0.72$) and Novel box ($t_{36} = 0.57$, $P = 0.57$) did not differ significantly from those of WT control mice. Thus, these findings indicate that non-stressed *Ndufs4*$^{GT/GT}$ mice were unable to discriminate the shock context. Yet, retention test latencies of *Ndufs4*$^{GT/GT}$ mice were significantly longer than their training latencies (Shock box: $t20 = 3.64$, $P = 0.002$, Non-Shock box: $t20 = 4.54$, $P < 0.001$,Table S1), indicating that the mice had acquired a memory of the shock experience *per se*.

In *Ndufs4*$^{GT/GT}$ mice, we also found significant main effects of stress exposure ($F_{1,112.32} = 50.21$, $P < 0.001$) and test context ($F_{2,71.92} = 14.70$, $P < 0.001$), together with a significant stress exposure x test context interaction effect ($F_{2,71.92} = 6.42$, $P = 0.003$). However, the impact of stress exposure on retention latencies in *Ndufs4*$^{GT/GT}$ mice was entirely different from that in WT mice. Stress exposure in *Ndufs4*$^{GT/GT}$ mice significantly increased retention latencies in both the Shock box (independent samples *t*-test: $t_{38} = 7.16$, $P < 0.001$) and Non-Shock box ($t_{20.45} = 3.39$, $P = 0.003$), and non-significantly increased retention latencies in the Novel box ($t_{21.03} = 1.72$, $P = 0.09$). Retention latencies in the Shock box were significantly longer than those in the Non-Shock box ($t_{18} = 3.56$, $P = 0.002$) and Novel box ($t_{18} = 3.75$, $P = 0.001$). Retention latencies in the Non-Shock box and Novel box did not differ from each other ($t_{18} = 1.13$, $P = 0.27$).

Hence, these findings indicate that stress exposure prior to the retention test interacted with mitochondrial dysfunction in influencing retention performance. Whereas stress exposure in WT mice selectively shortened retention latencies in the Shock box, impairing memory recall, stress exposure in *Ndufs4*$^{GT/GT}$ mice increased retention latencies in both the Shock box and Non-Shock box (and non-significantly in the Novel box).

3.3. Effect of stress exposure on mitochondrial activity in the hippocampus and PFC following the retention test in WT and *Ndufs4*$^{GT/GT}$ mice

Next, we determined the effect of stress exposure on mtETC activity in both the hippocampus and PFC of WT and *Ndufs4*$^{GT/GT}$. For this, the hippocampus and PFC were dissected 60 min after the retention test, and mitochondrial CI, CII, CIII and CIV activity, normalized to total protein content, was analyzed.

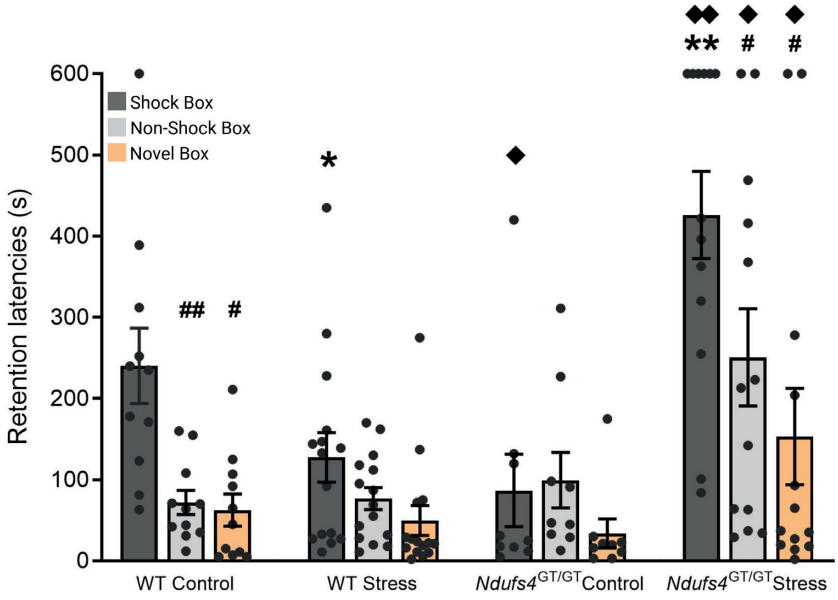

Figure 4. Stress exposure effects on retention test performance in the dual-event inhibitory avoidance task in WT and *Ndufs4*$^{GT/GT}$ mice. Data presented are step-through latencies in seconds on the 48-h retention test. WT control mice had significantly longer retention latencies in the Shock box as compared to both the Non-Shock box and Novel box, indicating that they accurately discriminated the shock context. Stressed WT mice had similar retention latencies in all three context, and retention latencies in the Shock box significantly shortened as a consequence of stress. *Ndufs4*$^{GT/GT}$ control mice had similar retention latencies in all three contexts, and retention latencies in the Shock box were significantly shorter than those of WT control mice. Stress exposure in *Ndufs4*$^{GT/GT}$ mice induced significantly longer retention latencies in the Shock box and Non-Shock box. Graphs depict mean ± SEM and individual data points. WT control: n = 17, WT stress: n = 21, *Ndufs4*$^{GT/GT}$ control: n = 15, *Ndufs4*$^{GT/GT}$ stress: n = 19. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, different from Shock box, $^{*}P < 0.05$, $^{**}P < 0.01$, different from its respective control condition, $^{\blacklozenge}P < 0.05$, $^{\blacklozenge\blacklozenge}P < 0.01$ different from corresponding WT group.

3.3.1. Hippocampus

A two-way ANOVA for CI activity indicated significant main effects of stress exposure ($F_{1,53}$ = 8.42, P = 0.005) and mitochondrial dysfunction ($F_{1,53}$ = 11.88, P = 0.001), as well as a significant stress exposure x mitochondrial dysfunction interaction effect ($F_{1,53}$ = 6.27, P = 0.02). *Post hoc* analyses indicated that CI activity of *Ndufs4*$^{GT/GT}$ control mice was significantly lower than that of WT control mice (t_{28} = 3.68, P = 0.001). Stress exposure significantly reduced CI activity of WT mice (t_{24} = 3.28, P = 0.003), but did not further reduce CI activity of *Ndufs4*$^{GT/GT}$ mice (t_{29} = 0.33, P = 0.74, Figure 5A). For CII activity, we found no stress exposure ($F_{1,53}$ = 1.56, P = 0.22), mitochondrial dysfunction ($F_{1,53}$ = 0.14, P = 0.71) or stress exposure x mitochondrial dysfunction interaction effect ($F_{1,53}$ = 1.13, P = 0.29).

For CIII activity, we also found no effects of stress exposure ($F_{1,54}$ = 0.33, P = 0.57), mitochondrial dysfunction ($F_{1,54}$ = 0.63, P = 0.43) or stress exposure x mitochondrial dysfunction interaction ($F_{1,54}$ = 2.25, P = 0.14). For CIV activity, we found no main effect of stress exposure ($F_{1,52}$ = 0.04, P = 0.84), but we did observed a significant main effect of mitochondrial dysfunction ($F_{1,52}$ = 22.43, P < 0.001), and a significant stress exposure x mitochondrial dysfunction interaction effect ($F_{1,52}$ = 4.17, P = 0.046). *Post hoc* analyses did not reveal a significant stress exposure effect in either WT (t_{23} = -1.28, P = 0.22) or *Ndufs4*$^{GT/GT}$ mice (t_{29} = 1.66, P = 0.11). However, we found that CIV activity of stressed *Ndufs4*$^{GT/GT}$ mice was significantly lower than that of stressed WT mice (t_{25} = 4.43, P = 0.001), whereas CIV activity of *Ndufs4*$^{GT/GT}$ control and WT control mice did not differ from each other (t_{27} = 1.82, P = 0.08).

3.3.2. Prefrontal cortex

A two-way ANOVA for CI activity in the PFC indicated no main effect of stress exposure ($F_{1,55}$ = 0.22, P = 0.64), mitochondrial dysfunction ($F_{1,55}$ = 0.13, P = 0.72), or stress exposure x mitochondrial dysfunction interaction ($F_{1,55}$ = 2.15, P = 0.15, Figure 5B). For CII activity, we found no main effect of stress exposure ($F_{1,55}$ = 3.33, P = 0.07), but we did observe a significant main effect of mitochondrial dysfunction ($F_{1,55}$ = 6.04, P = 0.02), without a further stress exposure x mitochondrial dysfunction interaction effect ($F_{1,55}$ = 0.01, P = 0.91). The main effect of mitochondrial dysfunction was related to significantly higher CII activity in stressed *Ndufs4*$^{GT/GT}$ compared to stressed WT mice (t_{26} = 2.38, P = 0.03), whereas differences in the control groups did not reach significance ($t_{21.91}$ = 1.42, P = 0.17). For CIII activity, we found no effect of stress exposure ($F_{1,52}$ = 0.25, P = 0.62), mitochondrial dysfunction ($F_{1,52}$= 0.09, P = 0.77) or stress exposure x mitochondrial dysfunction interaction ($F_{1,52}$ = 0.08, P = 0.77). For CIV activity, we found significant main effects of both stress exposure ($F_{1,54}$ = 8.46, P = 0.005) and mitochondrial dysfunction ($F_{1,54}$ = 6.16, P = 0.02), without a significant stress exposure x mitochondrial dysfunction interaction effect ($F_{1,54}$ = 1.70, P = 0.20). *Post hoc* analyses indicated that whereas stress exposure did not significantly affect CIV activity of WT mice (t_{27} = 1.29, P = 0.27), stress exposure significantly increased CIV activity of *Ndufs4*$^{GT/GT}$ mice (t_{27} = 3.00, P = 0.006). Further, we found that CIV activity of stressed *Ndufs4*$^{GT/GT}$ mice was significantly higher than that of stressed WT mice (t_{26} = 2.58, P = 0.02), whereas that of control WT and control *Ndufs4*$^{GT/GT}$ mice did not differ (t_{27} = -0.86, P = 0.39).

Thus, these findings indicate that stress exposure selectively reduced CI activity in the hippocampus of WT mice whereas it increased CIV activity in the PFC of *Ndufs4*$^{GT/GT}$ mice.

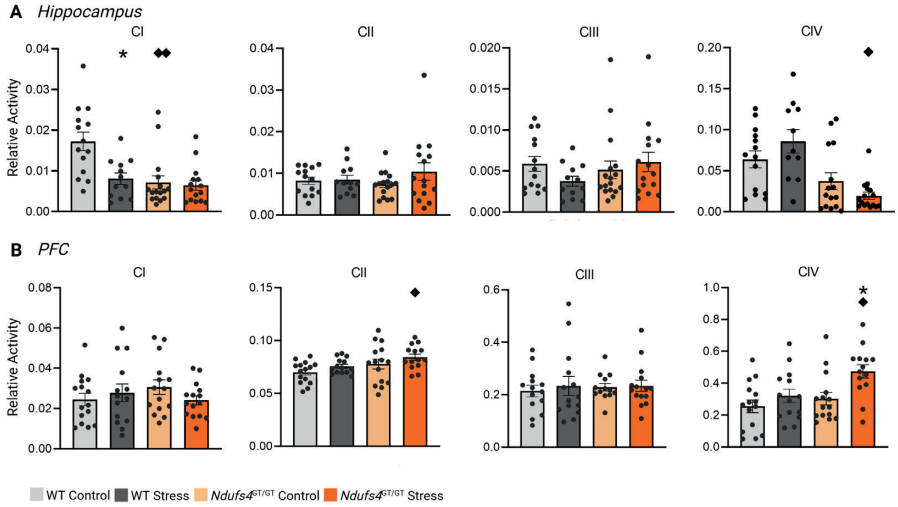

Figure 5. Effect of stress exposure on mitochondrial activity in the hippocampus and PFC following the retention test in WT and *Ndufs4*$^{GT/GT}$ mice. A. Stress exposure significantly reduced CI activity in the hippocampus of WT mice, whereas it did not further reduce CI activity of *Ndufs4*$^{GT/GT}$ mice. Stress exposure had no effect on the activity of other complexes in the hippocampus. CI activity: WT control: n = 14, WT stress: n = 12, *Ndufs4*$^{GT/GT}$ control: n = 16, *Ndufs4*$^{GT/GT}$ stress: n = 15; CII activity: WT control: n = 14, WT stress: n = 12, *Ndufs4*$^{GT/GT}$ control: n = 16, *Ndufs4*$^{GT/GT}$ stress: n = 15; CIII activity: WT control: n = 14, WT stress: n = 12, *Ndufs4*$^{GT/GT}$ control: n = 17, *Ndufs4*$^{GT/GT}$ stress: n = 15; CIV activity; WT control: n = 14, WT stress: n = 11, *Ndufs4*$^{GT/GT}$ control: n = 15, *Ndufs4*$^{GT/GT}$ stress: n = 16. **B.** Stress exposure significantly increased CIV activity in the PFC of *Ndufs4*$^{GT/GT}$ mice. Stress exposure had no effect on the activity of other complexes in the PFC. WT control: mice. CI activity: WT control: n = 14, WT stress: n = 13, *Ndufs4*$^{GT/GT}$ control: n = 14, *Ndufs4*$^{GT/GT}$ stress: n = 14; CII activity: WT control: n = 14, WT stress: n = 14, *Ndufs4*$^{GT/GT}$ control: n = 13, *Ndufs4*$^{GT/GT}$ stress: n = 14; CIII activity: WT control: n = 14, WT stress: n = 14, *Ndufs4*$^{GT/GT}$ control: n = 13, *Ndufs4*$^{GT/GT}$ stress: n = 14; CIV activity: WT control: n = 15, WT stress: n = 14, *Ndufs4*$^{GT/GT}$ control: n = 15, *Ndufs4*$^{GT/GT}$ stress: n = 13. *P < 0.05, different from its respective non-stress control condition, ◆P < 0.05, ◆◆P < 0.01 different from WT. Graphs depict means ± SEM and individual data points.

3.4. Effect of stress exposure on neuronal activity in the hippocampus and PFC following the retention test in WT and *Ndufs4*$^{GT/GT}$ mice

Then, we determined the effect of stress exposure and mitochondrial dysfunction on neuronal activity in both the hippocampus and PFC of WT and *Ndufs4*$^{GT/GT}$ 60 min after the retention test.

3.4.1. Hippocampus

A linear mixed model for the number of c-Fos^{+} cells in the hippocampus, with stress exposure and mitochondrial dysfunction as between-subjects factors and hippocampal subregion as within-subject factor, revealed significant main effects of stress exposure ($F_{1,105.54}$ = 13.74, P < 0.001) and mitochondrial dysfunction

($F_{1,105.54}$ = 6.74, P = 0.01), but no main effect of subregion ($F_{2,67.96}$ = 1.29, P = 0.28, Figure 6A) or interaction effects (all P's > 0.34). Further exploratory analyses for each genotype separately revealed a significant main effect of stress exposure on the number of c-Fos$^+$ cells in *Ndufs4*$^{GT/GT}$ mice ($F_{1,55.01}$ = 12.36, P = 0.01), but that this stress exposure effect failed to reach significance in WT mice ($F_{1,50.13}$ = 3.35, P = 0.07). *Post hoc* tests indicated that stress exposure significantly reduced the number of c-Fos$^+$ cells of *Ndufs4*$^{GT/GT}$ mice in the DG ($t_{16.25}$ = 2.22, P = 0.04) and CA3 ($t_{15.07}$ = 2.45, P = 0.03), whereas this effect failed to reach significance in the CA1 ($t_{18.28}$ = 2.05, P = 0.06). Further, we found that stressed *Ndufs4*$^{GT/GT}$ mice had a lower number of c-Fos$^+$ cells compared to stressed WT mice in the DG (t_{16} = 2.15, P = 0.047) and CA3 region (t_{16} = 2.39, P = 0.03). This effect failed to reach significance in the CA1 (t_{17} = 1.53, P = 0.14).

To determine whether stress effects on neuronal activity might involve inhibitory neurons, we also investigated c-Fos expression within GABAergic neurons. For this, we counted the number of neurons showing double labeling for c-Fos and GAD67, a marker for GABAergic neurons (Ito et al., 2015). Also here, we observed significant main effects of stress exposure ($F_{1,96.16}$ = 20.10, P < 0.001) and mitochondrial dysfunction ($F_{1,96.16}$ = 6.19, P = 0.02), but no main effect of subregion ($F_{2,71.9}$ = 3.62, P = 0.44, Figure 6B). No significant interaction effects were observed (all P's > 0.55). Further exploratory analyses revealed a significant stress exposure effect on the number of c-Fos$^+$+GAD67$^+$ cells in both WT mice ($F_{1,44.72}$ = 7.53, P = 0.009) and *Ndufs4*$^{GT/GT}$ mice ($F_{1,50.84}$ = 13.64, P = 0.001). Whereas *post hoc* analyses for each subregion separately did not reveal a significant stress exposure effect in WT mice, stress exposure in *Ndufs4*$^{GT/GT}$ mice significantly reduced the number of c-Fos$^+$+GAD67$^+$ cells in the DG ($t_{13.66}$ = 2.24, P = 0.04), CA3 ($t_{12.95}$ = 2.61, P = 0.02) and CA1 ($t_{13.15}$ = 2.49, P = 0.03). *Post hoc* tests to follow up on the mitochondrial dysfunction effect indicated no significant difference in non-stressed control mice, but that stressed *Ndufs4*$^{GT/GT}$ mice had fewer c-Fos$^+$+GAD67$^+$ cells in the CA3 (t_{16} = 2.71, P = 0.02) compared to stressed WT mice. This effect failed to reach significance in the DG ($t_{12.39}$ = 2.14, P = 0.053) and CA1 (t_{14} = 1.35, P = 0.20).

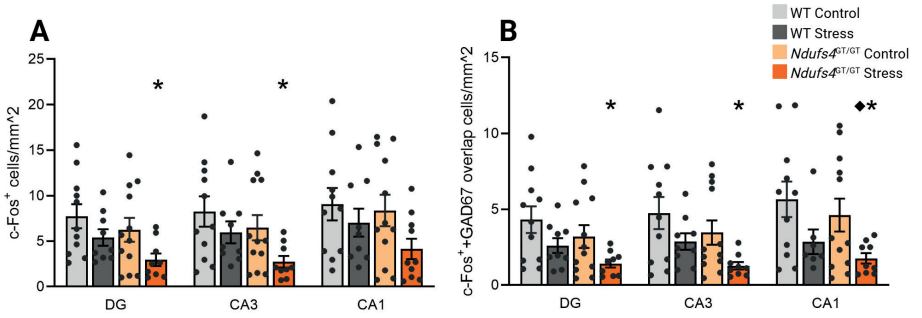

Figure 6. Effect of stress exposure on neuronal activity in the hippocampus following the retention test in WT and *Ndufs4*$^{GT/GT}$ mice. A. Stress exposure significantly reduced the number of c-Fos$^+$ cells in the hippocampus 60 min after the retention test. Exploratory analysis per genotype indicated that stress exposure in WT mice did not significantly affect the number of c-Fos$^+$ cells in the hippocampus, but stress exposure in *Ndufs4*$^{GT/GT}$ mice significantly reduced the number of c-Fos$^+$ cells in both the dentate gyrus (DG) and cornu ammonis 3 (CA3). Mitochondrial dysfunction alone did not significantly affect the number of c-Fos$^+$ cells in the hippocampus, but stressed *Ndufs4*$^{GT/GT}$ mice had a lower number of c-Fos$^+$ cells compared to stressed WT mice in the DG and CA3 region. WT control: n = 11, WT stress: n = 9, *Ndufs4*$^{GT/GT}$ control: n = 12, *Ndufs4*$^{GT/GT}$ stress: n = 9. **B.** Stress exposure significantly reduced the number of c-Fos$^+$+GAD67$^+$ cells in the hippocampus 60 min after the retention test. Exploratory analysis per genotype indicated that stress exposure in WT mice did not significantly affect the number of c-Fos$^+$+GAD67$^+$ cells in the hippocampus, but stress exposure in *Ndufs4*$^{GT/GT}$ mice significantly reduced the number of c-Fos$^+$+GAD67$^+$ cells in all hippocampal subregions. Mitochondrial dysfunction alone did not significantly affect the number of c-Fos$^+$+GAD67$^+$ cells in the hippocampus, but stressed *Ndufs4*$^{GT/GT}$ mice had a lower number of c-Fos$^+$+GAD67$^+$ cells compared to stressed WT mice in the CA3. *P < 0.05, different from its respective control. ◆P < 0.05, different from WT. Graphs depict means ± SEM and individual data points.

3.4.2. Prefrontal cortex

A linear mixed model for the number of c-Fos$^+$ cells in the PFC, with stress exposure and mitochondrial dysfunction as between-subjects factors and PFC subregion as within-subject factor, revealed a significant main effect of stress exposure ($F_{1,111.84}$ = 19.07, P < 0.001; Figure 7A). No other main or interaction effects were observed (all P's > 0.93). Exploratory analyses per genotype revealed that stress exposure increased the number of c-Fos$^+$ cells of both WT mice (D = 0.66, $F_{1,54.93}$ = 6.50, P = 0.01) and *Ndufs4*$^{GT/GT}$ mice (D = 0.91, $F_{1,56.33}$ = 12.63, P = 0.001). *Post hoc* comparisons for each subregion separately failed to reach significance in WT mice (IL: t_{19} = -1.65, P = 0.12, PL: t_{21} = 0.83, P = 0.42, AC: t_{21} = 0.28, P = 0.78), but indicated greater numbers of c-Fos$^+$ cells in stressed compared to control *Ndufs4*$^{GT/GT}$ mice in the IL ($t_{14.32}$ = -2.69, P = 0.02). This effect failed to reach significance in the PL (t_{19} = 1.70, P = 0.11) and AC (t_{19} = 1.93, P = 0.07).

To assess also changes in c-Fos$^+$ expression selectively in GABAergic neurons, we determined the number of c-Fos$^+$+GAD67$^+$ neurons. A linear mixed model revealed no main or interaction effects of stress exposure, mitochondrial dysfunction, or subregion (all *P*'s > 0.2, Figure 7B).

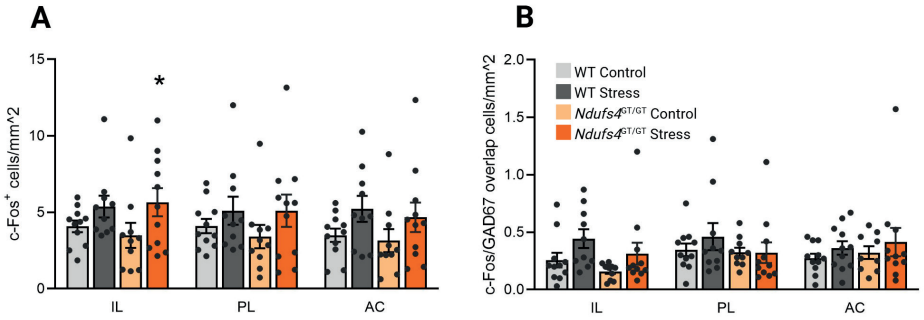

Figure 7. Effect of stress exposure on neuronal activity in the PFC following the retention test in WT and *Ndufs4*$^{GT/GT}$ mice. A. Stress exposure in both WT and *Ndufs4*$^{GT/GT}$ significantly increased the number of c-Fos$^+$ cells within the prefrontal cortex (PFC). Specifically, stress exposure in *Ndufs4*$^{GT/GT}$ mice significantly increased the number of c-Fos$^+$ cells in the infralimbic (IL) subregion. Mitochondrial dysfunction did not significantly affect the number of c-Fos$^+$ cells in the PFC, prelimbic or anterior cingulate. WT control: *n* = 11, WT stress: *n* = 10, *Ndufs4*$^{GT/GT}$ control: *n* = 10, *Ndufs4*$^{GT/GT}$ stress: *n* = 11. **B.** Stress exposure or mitochondrial dysfunction did not significantly affect the number of c-Fos$^+$+GAD67$^+$ cells activity in the PFC 60 min after the retention test. *P < 0.05 different from its respective control. Graphs depict means ± SEM and individual data points.

3.4.2. Correlations in neuronal activity between the hippocampus and PFC

Pearson correlations were performed to examine the relationship between c-Fos expression in hippocampal and PFC subregions. We examined both correlations in activity between the different subregions of either hippocampus or PFC as well as correlations in activity between hippocampal and PFC subregions. We were particularly interested in determining whether the observed decrease in hippocampal activity and increase in PFC activity after stress exposure were related. These correlations were examined separately for each experimental group.

Across all experimental groups, we consistently found strong positive correlations in c-Fos$^+$ cell counts between different subregions of the hippocampus and PFC, respectively. However, we did not find any significant correlation in the number of c-Fos$^+$ cells between hippocampal and PFC subregions (all *P*'s > 0.47). (Figure 8).

Lastly, we also wanted to determine whether changes in mitochondrial activity in hippocampus and PFC were associated with neuronal activity within these regions however we did not find any significant correlations (all *P*'s > 0.84. Additionally, our

correlational analyses revealed no significant association between c-Fos expression or MtETC complex activity in the hippocampus and/or PFC and stress-induced alterations in memory performance (all *P*'s > 0.09).

Overall, three of the experimental conditions except WT control, stress exposure found to have strong positive correlations in c-Fos activity between the different hippocampal or PFC subregions. This highlights that in mice with pre-existing mitochondrial dysfunction, neuronal activation in both regions is elevated to levels comparable to those observed in stressed WT mice .

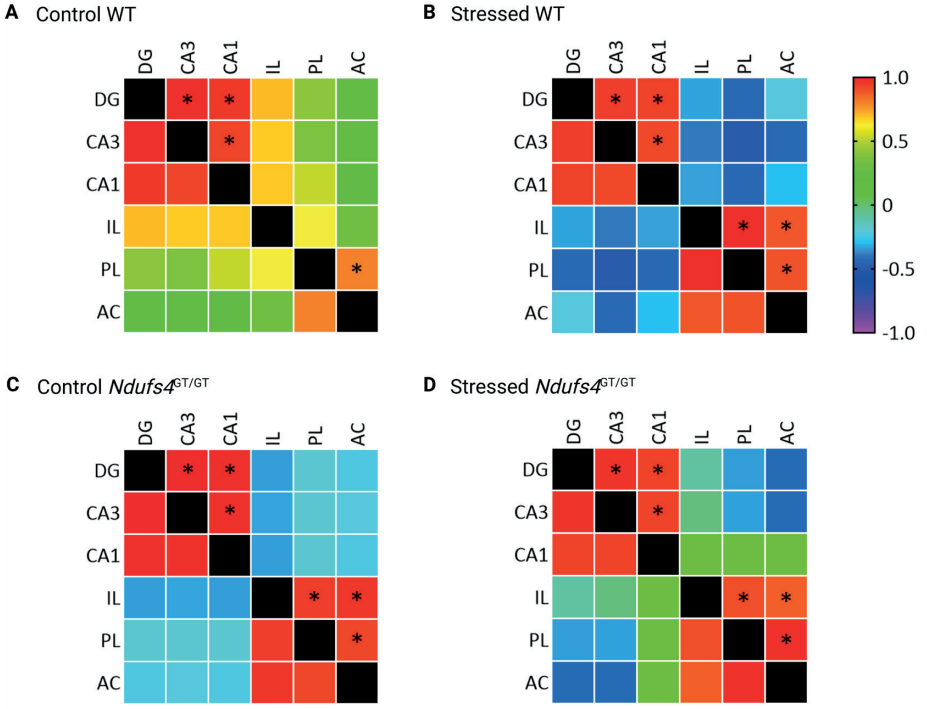

Figure 8. Correlations in neuronal activity between the hippocampus and PFC after the retention test in WT and *Ndufs4*$^{GT/GT}$ mice. In all experimental groups, we found strong positive correlations in c-Fos^{+} cell counts between individual hippocampal and PFC subregions, respectively. c-Fos^{+} cell counts did not show any significant correlations between hippocampus and PFC. *P < 0.01

4. Discussion

In this study, we examined whether acute stress exposure prior to retention testing affects the episodic-like specificity of the recalled memory. The interest in this question stems from the findings of several recent studies indicating that acute stress exposure or glucocorticoid administration after a learning experience induces a generalized strengthening of memory during memory consolidation. To examine whether stress exposure prior to the retention test also affects the specificity of the recalled memory, we trained mice on the dual-event inhibitory avoidance task and exposed them to a stressful forced swim experience shortly before the retention test. We found that while non-stressed WT control mice accurately discriminated the training context in which they had received footshock, stressed mice were unable to discriminate this shock context and showed similar retention latencies across all three test contexts. However, the stress exposure selectively reduced retention latencies in the former shock context, but did not increase retention latencies in the safe training and novel retention contexts, which would be indicative of memory generalization. As such, our findings do not provide evidence as of whether the stress exposure had a specific influence on the episodic-like specificity of the recalled memory or only impaired memory recall. A second aim of this study was to examine whether stress exposure interacts with mitochondrial activity in influencing retention performance. We found that stress exposure in WT mice reduced CI activity within the hippocampus. To explore whether such reduced CI activity could influence retention performance, we used *Ndufs4*$^{GT/GT}$ mice which have a genetically induced 25% reduction in CI activity. We found that non-stressed *Ndufs4*$^{GT/GT}$ mice, similar to stressed WT mice, displayed short retention latencies in all three test contexts and failed to distinguish the Shock box during the retention test. In response to stress, *Ndufs4*$^{GT/GT}$ mice demonstrated longer retention latencies across all boxes.

Our finding that stress exposure prior to the retention test impaired memory retrieval aligns with prior studies in both animals and humans demonstrating that stress exposure or glucocorticoid administration before a retention test impairs memory retrieval. This includes impairments of hippocampus-dependent declarative memory in humans and of spatial or contextual memory in rodents (de Quervain et al., 1998; Kuhlmann et al., 2005; Park et al., 2008; Wolf, 2009). A few studies had examined whether such stress and/or glucocorticoid administration might also reduce the specificity of the recalled memory. In one study, rats were trained on an inhibitory avoidance task and retention testing took place in the training context as well as a novel context. Similar to the present findings, CORT

administration 30 min prior to the retention test induced shorter retention latencies when tested in the former shock context but did not affect retention latencies in a novel inhibitory avoidance context (Roozendaal et al., 2004). In another study, rats were administered CORT prior to retention testing on a contextual fear conditioning task (Atsak et al., 2012). Also similar to the present findings, the CORT treatment selectively reduced freezing behavior in the former shock context and not in a safe novel context. These findings would thus suggest that stress exposure (or glucocorticoid administration) prior to retention testing primarily reduces the strength of the recalled memory without directly influencing the episodic-like specificity of the recalled memory.

We further found that stress exposure in WT mice reduced CI mitochondrial activity in the hippocampus after the retention test. We became interested in this research question because of prior findings that glucocorticoid effects on memory retrieval are at least partially mediated by cannabinoid type 1 (CB1) receptors located on mitochondrial membranes (mtCB1) in GABAergic interneurons of the hippocampus (Skupio et al., 2023). These findings fit with previous findings from our laboratory indicating that glucocorticoids effects on memory retrieval involve a rapid increase in endocannabinoid signaling within the hippocampus (Atsak et al., 2012). Our finding that stress exposure selectively affected CI activity in the hippocampus, and not that of other mitochondrial complexes is consistent with other findings indicating that chronic stress exposure selectively reduced hippocampal CI activity (Clement et al., 2022). Additionally, prolonged stress in adult male rats has been reported to significantly reduce mitochondrial respiratory chain activity, with a 69% inhibition of CI activity (Madrigal et al., 2001). While we found that the stress exposure selectively reduced CI activity in the hippocampus, and not PFC after the retention test, other studies have shown that intense stress (a PTSD induction paradigm) showed reduced CI activity in cerebellum (Emmerzaal, 2022). Since Skupio et al. (2023) reported that the effect of glucocorticoid administration on memory retrieval was mediated by an activation of CB1 receptors on mitochondrial membranes in GABAergic interneurons, we also examined whether our stress manipulation affected the activity of GABAergic neurons within the hippocampus. In support of this view we found that stress exposure to WT mice significantly reduced the activity of GABAergic neurons within the hippocampus after the retention test, but it should be stated that analysis of stress exposure effects on each hippocampal subregion separately failed to reach significance. In light of the present findings, future studies are warranted to examine whether the stress-induced reduction in CI activity is mediated by endocannabinoid signaling or related pathways.

To further explore the role of mitochondrial activity in regulating episodic-like specificity of memory and how this might interact with stress exposure, we used *Ndufs4*$^{GT/GT}$ mice which display a genetically induced reduction of CI activity (Emmerzaal, 2022). At the behavioral level, we found that non-stressed *Ndufs4*$^{GT/GT}$ mice showed short retention latencies in all three test contexts, but significantly longer latencies than during the training session, indicating a generalization of a relatively weak memory. However, since this is a permanent dysfunction, it remains unclear whether the observed effect is due to an impairment in memory consolidation or retrieval. Our findings are consistent with that of several prior studies indicating that the impact of mitochondrial dysfunction on memory performance is profound. A decline in mitochondrial function within the hippocampus has previously been linked to hippocampus-dependent memory impairments (Olesen et al., 2020). This decline may occur naturally with aging or, as observed in our study, through genetic modifications resulting in disrupted CI functioning (Mishra and Thakur, 2022; Javani et al., 2023). Moreover, research has shown that enhancing mitochondrial CI activity by improving the coupling efficiency of the respiratory chain can prevent cognitive decline (Zhang et al., 2015) and effectively reverse memory impairments associated with CI dysfunction. We also found that stressed *Ndufs4*$^{GT/GT}$ mice had lower CIV levels in the hippocampus than stressed WT mice. Although a role of CIV activity in memory performance is not yet fully understood, evidence suggests that CIV is essential for maintaining the stability of CI (Li et al., 2007). This finding highlights a clear connection between CI dysfunction and CIV stability, suggesting a potential indirect effect of CIV on memory performance.

Stress exposure in *Ndufs4*$^{GT/GT}$ mice prior to the retention test severely impacted retention performance. Interestingly, stress exposure did not, as was found in WT mice, further reduce retention latencies but resulted in a rather generalized increase in retention latencies across test contexts. Although we found that stress exposure in *Ndufs4*$^{GT/GT}$ mice did not further reduce mitochondrial CI activity in the hippocampus, we did find that stress exposure reduced both total c-Fos^{+} cell counts as well as c-Fos^{+} cell counts of GABAergic neurons within the hippocampus and modestly increased PFC activity. Further, stress exposure in *Ndufs4*$^{GT/GT}$ mice was found to increase CIV mitochondrial activity in the PFC. This shift from hippocampal to PFC engagement has been associated with memory generalization (Bahtiyar et al., 2020). However, disrupted mitochondrial function has also been implicated in general deficits in behavioral coping (Picard et al., 2015). A particularly intriguing observation is the strong association between mitochondrial diseases and behavioral abnormalities, including a notable prevalence of psychiatric disorders

such as depression (Carrozzo et al., 2007; Morava et al., 2006a; Morava et al., 2006b; Zeviani and Di, 2004). Research has identified suboptimal mitochondrial function as a critical factor contributing to the etiology of stress-related psychopathologies. Individuals with suboptimal mitochondrial function are characterized by sufficient mitochondrial capacity to support daily activities but insufficient reserve capacity to cope with severe challenges and mount an adaptive stress response. This distinction excludes individuals with overt mitochondrial diseases, as their mitochondrial function is markedly impaired (Morava and Kozicz, 2013). Therefore, impaired mitochondrial function might increase vulnerability to the effect of stress exposure. Based on these findings, we could speculate that the stress exposure in *Ndufs4*$^{GT/GT}$ might enhance the effect of mitochondrial dysfunction, causing the mice to be exhausted and to have even less energy which could explain the long retention latencies in every context.

Taken together, we found that stress exposure before retention testing impaired memory retrieval in WT mice. However, it remains unclear as of whether the stress exposure might have had an influence on the episodic-like quality of memory as well. Stress exposure in WT mice was associated with reduced CI activity within the hippocampus, potentially serving as a mechanism through which stress impairs memory retrieval via a non-genomic pathway. We further found that stress exposure intensified the shortcoming associated with mitochondrial dysfunction in *Ndufs4*$^{GT/GT}$ mice. We not only found that hippocampal activity of stressed *Ndufs4*$^{GT/GT}$ mice was significantly lower than that of stressed WT mice, but also that this effect was associated with longer retention latencies in all test contexts.

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6. Supplements

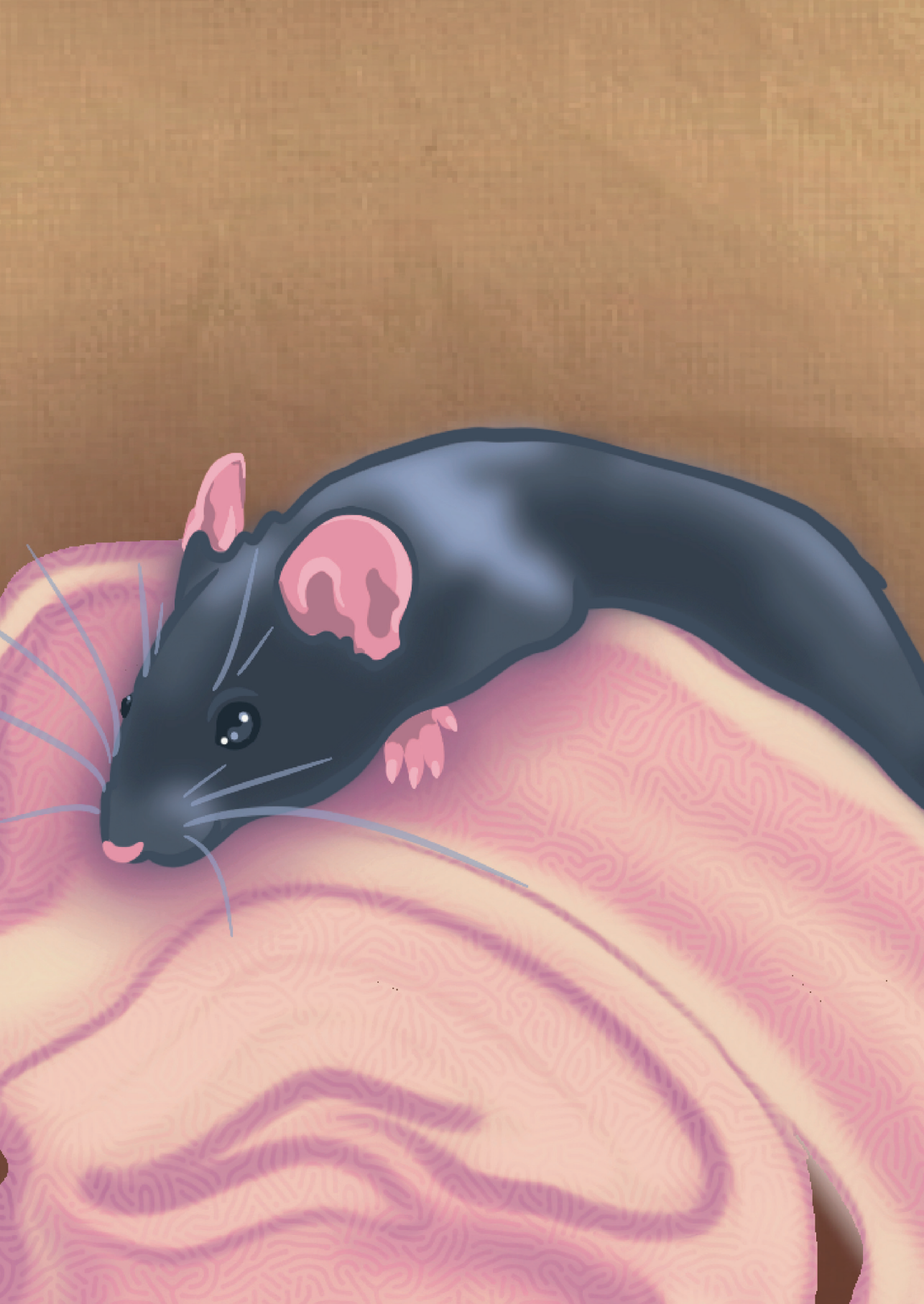
Table S1. Training and retention test latencies of animals

	Training[1] (s)		Retention test[2] (s)		
	Non-Shock mean ± s.e.m. (mean ± s.e.m.)	**Shock** mean ± s.e.m. (mean ± s.e.m.)	**Shock** mean ± s.e.m. (mean ± s.e.m.)	**Non-Shock** mean ± s.e.m. (mean ± s.e.m.)	**Novel** mean ± s.e.m. (mean ± s.e.m.)
WT control ($n = 17$)	14.7 ± 3.4[3]	20.5 ± 2.7	243.7 ± 43.4	54.8 ± 11.6	58.1 ± 16.3
WT stress ($n = 21$)	14.2 ± 3.4	16.5 ± 3.2	103.5 ± 24.2	60.8 ± 11.5	45.6 ± 14.5
Ndufs4$^{GT/GT}$ control ($n = 15$)	16.5 ± 3.9	18.4 ± 3.7	92.1 ± 30.2	66.3 ± 22.8	24.4 ± 11.0
Ndufs4$^{GT/GT}$ stress ($n = 19$)	15.0 ± 2.5	19.5 ± 3.8	422.5 ± 38.5	215.6 ± 44.1	137.7 ± 50.0

[1] Mice were trained first in the Non-Shock box and subsequently in the Shock box

[2] Retention testing in the Shock box, Non-Shock box and Novel box occurred in a randomized order and without delays

[3] Training and retention test latencies in seconds



CHAPTER 4

Exploring the effects of posttraining noradrenergic activation on episodic-like memory specificity using the object-in-context task in mice

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**Equal Contribution*

Abstract

Emotionally arousing events are remembered better than mundane, every day events. Noradrenergic activation is known to critically contribute to this memory-strengthening effect by enhancing the consolidation of memory after a learning experience. Less is known about the effects of noradrenergic activity on the quality of episodic memories and the neural mechanisms supporting these effects. Here, we investigated the effect of posttraining administration of the noradrenergic stimulant yohimbine (YOH) on episodic-like memory in an object-in-context (OiC) task. To study the associated neural mechanisms, we used male transgenic FosTRAP2xtdTomato mice to assess the training/early consolidation and retention test-induced neuronal activity in the hippocampus, as well as engram reactivation rates. In the same mice, we additionally assessed neuronal activity during the training/early consolidation at the whole-brain level, combining iDISCO+ with light-sheet microscopy. In both cases, the effects of YOH were contrasted to conditions of more extensive training to determine whether memory effects in both conditions were supported by similar neural mechanisms. Lastly, in male C57Bl/6 mice, we studied the molecular substrate of the altered hippocampal activity by assessing subregional changes in microRNa (miR)-134 levels and its downstream effector genes. As expected, both YOH administration and extensive training enhanced OiC memory. We found no evidence that YOH or extensive training affected the number of activated neurons during the training/early consolidation period. However, both YOH and extensive training reduced retention test-induced neuronal activity in the cornu ammonis 3 (CA3) region. Whole brain analysis further revealed reductions in activity in the ventral hippocampal CA3 and cornu ammonis 1 (CA1), ventral entorhinal cortex, parasubiculum, substantia nigra, and thalamic regions after both YOH administration and extensive training. Finally, YOH administration elevated cAMP response element-binding (*Creb*) messenger RNA (mRNA) levels in the dorsal blade of the dentate gyrus and CA1 following OiC training, and increased miR-134 levels in the CA3. Overall, these findings highlight distinct neural activity patterns during encoding/early consolidation versus retention, particularly in the hippocampus, as a consequence of posttraining noradrenergic activation; effects that were remarkably similar to those of extensive training.

Keywords: *norepinephrine; yohimbine; memory specificity; Object-in-Context task; hippocampus; qPCR; iDISCO+*

1. Introduction

Emotionally arousing events are well remembered. A large body of evidence indicates that arousal-associated noradrenergic activation contributes crucially to this memory-strengthening effect by enhancing the consolidation process (McGaugh et al., 1988; Okuda et al. 2004; Roozendaal et al., 2006). However, less is known about how noradrenergic activation might influence qualitative aspects of these memories. Recent research in our lab has indicated that posttraining noradrenergic activation enhances the episodic-like specificity of memory in a dual-event inhibitory avoidance task. In this task, rats are trained sequentially in two different inhibitory avoidance apparatuses with a brief delay, but receive an electric footshock only in the latter context. After 48 hours, retention latencies are tested in both training contexts as well as in a novel context, to test whether the rats have associated the shock experience with the correct training context (Atucha and Roozendaal, 2015). Both systemic administration of the noradrenergic stimulant yohimbine (YOH) and direct administration of norepinephrine (NE) into the basolateral amygdala (BLA) were found to improve episodic-like specificity of memory on this task by facilitating the consolidation of pattern-separated memories of the two training events (Atucha and Roozendaal, 2015; Atucha et al., 2017; Roozendaal and Mirone, 2020; Atucha et al., 2025). Further investigation revealed that NE administration into the BLA mediated this effect by inducing a down-regulation of microRNA (miR)-134 within the dorsal blade of the dentate gyrus (dDG) (Atucha et al., 2025), leading to an up-regulation of both *Creb* and brain-derived neurotrophic factor (*Bdnf*) messenger RNA (mRNA) levels; that both play a critical role in memory consolidation (Silva et al., 1998; Bramham and Messaoudi, 2005).

In Chapter 2 of my thesis, I aimed at setting up the dual-event inhibitory avoidance task in mice to replicate these observations in rats. However, the posttraining systemic injections disrupted task performance in mice, suggesting that mice might require a more robust behavioral paradigm for the assessment of NE effects on episodic-like specificity of memory. In the meantime, our lab set up the object-in-context (OiC) task for mice, where mice – similarly to the dual-event inhibitory avoidance task – are consecutively trained on two events. First, mice are exposed to a set of two identical objects in a first context and then to a different set of two identical objects in a distinct, second context. Episodic-like specificity of memory can subsequently be tested by re-exposing the mice to one of these training contexts, containing one exemplar of each of the two training objects. Increased exploration of the object previously encountered in the other training

context indicates episodic-like specificity of memory. Posttraining administration of YOH was found to improve OiC memory in a dose-dependent manner, which was associated with an increased correlated activity in the dDG - CA3 pathway during the posttraining consolidation period (Guo, 2024), suggestive of facilitated pattern separation of memory (Yassa and Stark, 2011; Kesner, 2013).

Several critical questions, however, remain unanswered. Firstly, memories are thought to be stored in the neurons activated during memory encoding, i.e., the so-called engram cells (Shapira and Eichenbaum, 1999; Guskjolen and Cembrowski, 2023), which increase their interconnectivity to facilitate their synchronized reactivation during memory recall (Ryan et al., 1979; Choi et al., 2018). The degree of reactivation of these neurons was previously found to correlate with memory performance (Josselyn et al., 2015; Choi et al., 2018; Gulmez Karaca et al., 2021). However, it has not been investigated whether posttraining noradrenergic activation induces an increase in the reactivation of hippocampal engram cells, potentially related to the enhanced episodic-like quality of memory. Here, we set out to investigate the reactivation rate (RR) of training-activated neurons during the retention test. Moreover, we were interested in analyzing hippocampal activity during memory recall, another yet unexplored avenue. Therefore, in a first experiment, we used the FosTRAP2xtdTomato transgenic mouse line allowing us to tag the activated neurons during training/early consolidation by the systemic injection of 4-hydroxytamoxifen (4-OHT), and compare this to later retention-induced neuronal activity as assessed by immunohistochemistry for the immediate early gene c-Fos. To determine whether the neurobiological mechanism underlying the effect of exogenous noradrenergic stimulation on memory is similar to that induced by more extensive training, resulting in a similarly enhancement of episodic-like memory, we included an additional group of mice that was exposed for a longer time to the two training events of the OiC task and compared these to YOH-treated mice.

Secondly, whereas previous work has clearly implicated the hippocampus in mediating changes in pattern separation and episodic-like specificity of memory (Lee et al., 2004; Leutgeb et al., 2007; Kesner and Hunsaker, 2010), many other brain regions might be involved as well. The locus coeruleus sends out noradrenergic terminals to an extensive network of forebrain structures involved in learning and memory (Sara, 2009), modulating local activity. We previously reported on increased neuronal activity in the anterior insular cortex (aIC) and perirhinal cortex (PRh) as a consequence of noradrenergic activation after training on an object recognition task (Qi, 2021). Therefore, we also investigated in these FosTRAP2xtdTomato mice the effects of systemic noradrenergic activation following training on the OiC task

on neuronal activity across the brain, and compared this effect again to that of extensive training.

Lastly, whereas previous work indicated that noradrenergic activation of the BLA after training on the dual-event inhibitory avoidance task facilitated episodic-like memory for the two training events by down-regulating miR-134 within the dDG, it is yet unclear whether a systemic administration of YOH after training on the OiC task recruits a similar molecular mechanism. Hence, we here investigated this by analyzing miR-134 and *Creb* and *Bdnf* mRNA levels within the dDG as well as the other hippocampal subregions after training (and YOH administration) on the OiC task using C57Bl/6 mice. Together, these experiments are expected to generate a more comprehensive picture of the effects of increased noradrenergic activity on hippocampal and brain plasticity in regulating episodic-like specificity of memory.

2. Materials and methods

2.1. Subjects

Eighty male FosTRAP2xtdTomato mice were used for investigating the effect of YOH on neuronal activity associated with both training/early consolidation and recall. FosTRAP2xtdTomato mice were bred in house by crossing female homozygous Fos2A-iCreER (Fos$^{tm2.1(icre/ERT2)Luo}$/J, #030323) with male homozygous conditional tdTomato (B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J, #007909) founder lines, originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA). In these mice, the injection of 4-OHT induces the fluorescent labeling, i.e., tdTomato expression, of neurons that express the immediate early gene c-Fos during a ~12-h labeling window (DeNardo et al., 2019). Fifty-four male C57Bl/6 mice (Charles River Breeding Laboratories, France/Germany) were used for investigating the effect of YOH on hippocampal gene expression after the training session. Both FosTRAP2xtdTomato and C57Bl/6 mice were 8 - 10 weeks old at the time of behavioral testing, and solitary housed in a temperature-controlled (22 °C) vivarium room with a regular 12-h/12-h light/dark cycle (lights on at 7:00 h), starting one week prior to the behavioral experiment. The vivarium room had a light intensity of 47 Lux and humidity of 72%. Mice had *ad libitum* access to food and water. Behavioral training and testing was performed during the light phase of the diurnal cycle, between 10:00 and 15:00 h. All experimental 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. All efforts were made to minimize animal suffering and to reduce the number of animals.

2.2. Object-in-Context Task

Prior to training, mice were first handled for 3 min on 5 consecutive days to become accustomed to the experimenter. Subsequently, the animals received three days of habituation to reduce novelty stress, which is required to guarantee sufficient exploration of the objects during the training session (Stefanko et al., 2009). As we previously found that prior habituation to the training contexts reduces hippocampal involvement in the task (Guo, 2024), mice were habituated to two contexts that were different from the actual training contexts, i.e., two square boxes (40 cm width, 40 cm length, 40 cm height) with different modifications (contexts X and Y). One box was gray with sawdust bedding, and the other box had white stripes on the walls and contained corncob bedding. During the habituation sessions, the animals could explore each context without any objects. The duration of habituation to each context was always identical to the duration of the training session in each context (i.e., either 5 or 10 min).

Training and testing on the OiC task was performed in two gray round plastic boxes (40 cm diameter, 40 cm height) placed next to one another in a dimly illuminated experimental room. One box had gray inner walls and the floor was covered with sawdust (context A). The other box had white dots modifying the walls and contained corncob bedding to make this a distinctly different contextual environment from the other box (context B). On the training session, the mice were initially placed in one of these two boxes (context A or B), and were able to explore a set of two identical objects (either two glass jars (5.5 cm diameter, 5 cm height) or two light bulbs (6 cm diameter, 11 cm length), placed 5 cm away from the edge of the box. Immediately after the first context exposure, mice were placed in the second box (context B or A, respectively), containing the other set of two identical objects. All potential orders and object-context combinations were counterbalanced across animals. To assess YOH-induced memory enhancement, animals were trained in each context for 5 min. In the extensive training condition mice were trained for 10 min in each context to generate memory performance similar to that of the YOH -treated mice. To avoid the presence of olfactory trails, feces were removed, bedding was stirred, and the objects were thoroughly cleaned with 70% ethanol in between animals. Immediately after the training session, FosTRAP2xtdTomato mice received an intraperitoneal injection of either YOH or saline followed immediately by an intraperitoneal injection of 4-OHT (Figure 1A). C57Bl/6 mice only received YOH or saline after the training session (Figure 1B). All animals were placed back in their home cage after the injection. C57Bl/6 mice were killed 40 min after the end of the training session to assess YOH-induced changes in hippocampal gene expression. FosTRAP2xtdTomato mice were left undisturbed

until the retention test 72 h later. This time frame was previously established to allow for optimal tdTomato expression in FosTRAP2xTdTomato mice. For retention testing, the animals were placed in one of the two training contexts with one exemplar of each of the two training objects for 5 min. The context used during the retention test, the object identity and the position of the novel object association within that context, i.e., left or right, were counterbalanced across animals.

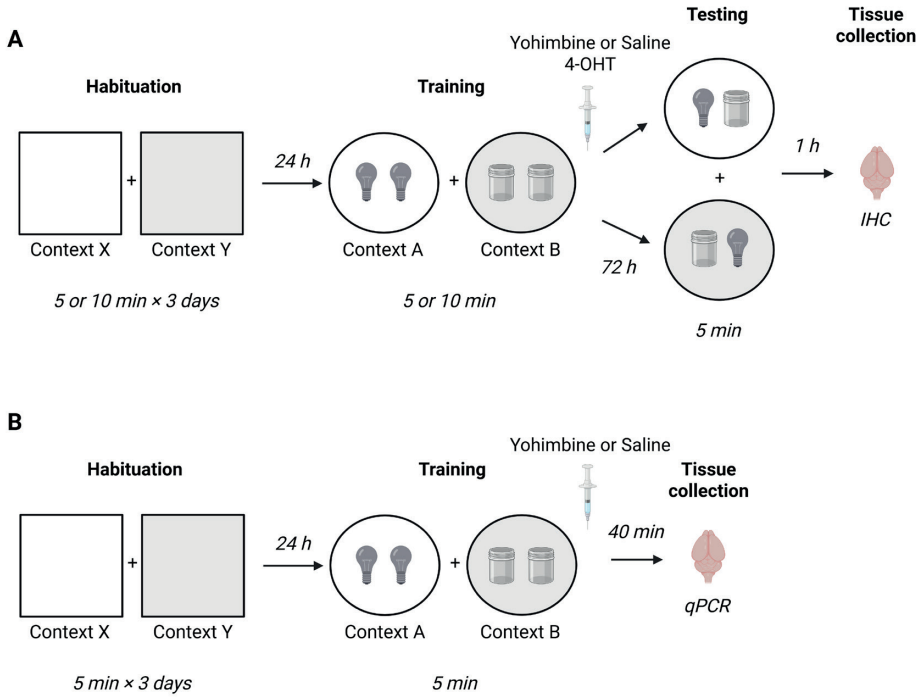

Figure 1. Experimental design of the object-in-context task. Mice were habituated to two square boxes (context X and Y) for three consecutive days. The duration of context habituation was always identical to the duration of the training trial and therefore depended on the experimental group. For OiC training, mice could first explore a set of two identical objects in one context for 5 min (or 10 min for the extensive training group) and then a different set of two identical objects in a second training context. **A.** FosTRAP2xtdTomato mice received either yohimbine (YOH 0.3 or 1 mg/kg) or saline control intraperitoneally immediately after the training session, as well as an intraperitoneal injection of 4-OHT (50 mg/kg). Retention was tested 72 h later during which the mice could explore one exemplar of each of the two training objects in one of the two training contexts for 5 min. Mice were killed 1 h after the retention test and tissue was collected for immunohistochemistry and iDISCO+ protocols. **B.** C57Bl/6 mice received either yohimbine (YOH 1 mg/kg) or saline immediately posttraining, and were killed 40 min after the training session for tissue collection for qPCR.

Mice' behavior during training and retention test was recorded with a video camera mounted above the experimental apparatus. Recordings were analyzed offline by a trained observer blind to treatment condition, and the time spent exploring each object was scored. Object exploration was defined as active interaction with an object, i.e., pointing the nose to the object at a distance of <1 cm and/or touching it with the nose (Okuda et al., 2004; Leger et al., 2013; Song et al., 2021). Turning around, climbing or sitting on an object *per se* was not included as the animals then often do not actively engage in exploring the object, but rather exhibit grooming behavior or are using the object as platform to scan the environment (Bianchi et al., 2006; Roozendaal et al., 2006; Li et al., 2011; Wimmer et al., 2012; Leger et al., 2013; Vogel-Ciernia and Wood, 2014; Pezze et al., 2017). To analyze memory performance, a discrimination index (DI%) was calculated as the difference in time exploring the novel and familiar OiC combination, expressed as the ratio of the total time spent exploring both objects (i.e., [Time Novel - Time Familiar] / [Time Novel + Time Familiar] x 100%).

2.3. Systemic drug administration

The noradrenergic stimulant YOH (17-hydroxyyohimban-16-carboxylic acid methyl ester hydrochloride; Sigma-Aldrich), an α2-adrenoceptor antagonist that increases NE levels in the periphery and brain (Szemeredi et al., 1991), was dissolved in saline. YOH or saline control was administered intraperitoneally in a volume of 0.01 mL/g of body weight, immediately after the training session. For the behavioral experiments in FosTRAP2xtdTomato mice, two different doses of YOH (0.3 and 1 mg/kg) were used (Song et al., 2021, Guo, 2024). The extensive training group was injected only with saline control. To investigate the effect of YOH administration on hippocampal gene expression (in C57Bl/6 mice), only the behaviorally most effective dosage of YOH (i.e., 1 mg/kg) was used.

4-OHT (50 mg/kg; Sigma-Aldrich), in a volume of 0.005 mL/g of body weight, was administered intraperitoneally to FosTRAP2xtdTomato mice immediately after the training session. 4-OHT was first diluted in 0.5 mL absolute ethanol, sonicated for 1 h at 55-60 °C, and then further diluted in 4.5 mL corn oil (Sigma Aldrich) and sonicated again for 1 h at 45-55 °C. The final solution contained 10% ethanol and 90% corn oil. All drug solutions were prepared freshly before each experiment.

2.4. Brain tissue collection for immunohistochemistry and iDISCO+

One hour after behavioral testing, FosTRAP2xtdTomato mice were anesthetized with an overdose of sodium pentobarbital, perfused transcardially with ice-cold 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by ice-cold 4%

paraformaldehyde (PFA). Brains were extracted and the two hemispheres separated. The left hemispheres, used for slice immunohistochemistry, were post-fixed in 4% PFA in 0.1 M PBS (pH 7.4) for 24 h, and then transferred to a 30% sucrose solution in 0.1 M PBS at 4 °C for 72 h. They were then wrapped in aluminum foil and stored at -80 °C. The right hemispheres, reserved for iDISCO+ experiments, were post-fixed in 4% PFA in 0.1 M PBS at 4 °C overnight, then washed three times in 0.1 M PBS for 30 min at room temperature (RT), and stored in 0.1 M PBS with 0.02% sodium azide at 4 °C. Fixed brain hemispheres (right vs left) were chosen for both experiments to minimize intra-experiment variance, based on reports of laterality in whole brain circuits and stress responding (Wittling and Pflüger, 1990; Sullivant, 2004). Brain analyses were restricted to the comparison of the behaviorally most effective dosage of YOH (i.e., 1 mg/kg) to the saline and extensive training groups.

2.5. Immunohistochemistry

Thirty-micrometer-thick coronal slices of the hippocampus were cut on a cryostat (Leica, CM 30502), collected in PBS with 0.03% sodium azide, and stored at 4 °C. Three to six sections containing the dorsal hippocampus (anteroposterior (AP), -1.43 to -2.27 mm from Bregma) from each animal were selected according to the Franklin and Paxinos mouse brain atlas (Franklin and Paxinos, 3rd edition, 2007). Sections were first washed three times in 0.1 M PBS for 10 min per wash, rinsed in 0.5% Triton in 0.1 M PBS for 30 min at RT, washed again 3 times for 10 min in 0.1 M PBS, and then blocked in 5% Normal Donkey Serum (NDS, Jackson ImmunoResearch Laboratories) in 0.1 M PBS for 1 h at RT. Next, sections were incubated with primary antibodies for c-Fos (guinea pig anti-c-Fos, 1:500, Synaptic Systems, #226 004) and glutamic acid decarboxylase 67 (GAD67) (mouse anti-GAD67, 1:500, Sigma-Aldrich, MAB5406) in 0.1 M PBS containing 5% NDS and 0.1% acetylated BSA (BSA-c, Aurion) overnight at RT. Afterwards, sections were washed 3 times in 0.1 M PBS with 0.1% BSA-c for 10 min, followed by incubation with fluophore-conjugated secondary antibodies donkey anti-guinea pig Alexa Fluor 647 (1:500, Jackson ImmunoResearch) and donkey anti-mouse Alexa Fluor 488 (1:500, Invitrogen) in 0.1 M PBS with 5% NDS and 0.1% BSA-c for 3 h at RT. Subsequently, sections were incubated with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, 1:5,000) in 0.1 M PBS with 0.1% BSA-c for 15 min, then washed three times in 0.1 M PBS for 10 min per wash, mounted on gelatin-coated slides, left to dry, and coverslipped with Fluorsave mounting medium (Sigma-Aldrich). The slides were stored in the dark at 4 °C.

2.6. Imaging and quantification for slice immunohistochemistry

Images were acquired on an automated high-content fluorescence microscope (Leica DMI 6000B, Germany) at 20x magnification, and image processing was performed in FIJI (version 1.53g) (Schindelin et al., 2012). At least three hippocampal slices were imaged per animal. The acquired images were corrected for photobleaching with the BaSic plugin in FIJI (Peng et al., 2017). The images were stitched together using the Grid-Stitch-Collection plugin. Ten regions of interest (ROIs) within the hippocampus were defined based on the Allen Mouse Brain Atlas (https://portal.brain-map.org/): the granule cell layer of the dentate gyrus dorsal blade (dDG), granule cell layer of the dentate gyrus ventral blade (vDG), molecular layer of the dentate gyrus dorsal blade (dDG-mo), molecular layer of the dentate gyrus ventral blade (vDG-mo), pyramidal cell layer of the cornu ammonis 3 (CA3-pc), stratum radiatum of the cornu ammonis 3 (CA3-sr), stratum oriens of the cornu ammonis 3 (CA3-so), pyramidal cell layer of the cornu ammonis 1 (CA1-pc), stratum radiatum of the cornu ammonis 1 (CA1-sr), stratum oriens of the cornu ammonis 1 (CA1-so) (Figure 2). The ROIs were drawn manually using the DAPI signal, area sizes measured (Rueden et al., 2017), and the number of neurons expressing c-Fos, tdTomato, GAD67 and their overlap were counted using the Cell Counter plugin in FIJI, and then converted to number of cells per mm^2. A RR was calculated as the percentage of $tdTomato^+$ cells that was also $c\text{-}Fos^+$ [RR: (number of $tdTomato^+$+$c\text{-}Fos^+$ cells) / (number of $tdTomato^+$ cells) * 100%] (Josselyn and Tonegawa, 2020; Gulmez-Karaca et al., 2025). The observed RR was compared to the expected RR based on chance [overlap by chance = (number of $c\text{-}Fos^+$ cells / number of $DAPI^+$ cells)* (number of $tdTomato^+$ cells / number of $DAPI^+$ cells) * 100%]. Relative activation of GABAergic neurons during the retention test was defined as the fraction of $GAD67^+$ neurons that was also $c\text{-}Fos^+$ [relative GABAergic activity: (number of $c\text{-}Fos^+$+$GAD67^+$ cells) / (number of $GAD67^+$ cells) * 100%].

2.7. iDISCO+ and Light-Sheet Microscopy

The right hemispheres of the FosTRAP2xtdTomato mouse brains were used for iDISCO+. The standard protocol used for clearing the brains can be found at https://idisco.info/ (Renier et al. 2016). This standard protocol was followed in every step. The primary antibodies used were rabbit anti-Red Fluorescent Protein (1:1,000, RFP, Rockland, #600-401-379) and guinea pig anti-c-Fos (1:1,000, Synaptic Systems, #226 004), and the secondary antibodies used were donkey anti-rabbit Alexa fluor 647 (1:400, ThermoFisher, 706-605-148) and goat anti-rabbit Alexa fluor 555 (1:200, ThermoFisher, #A-21429). Additionally, we performed an agarose (1.5% agarose in 0.1 M PBS) embedding step prior to the final dehydration and clearing stages in order to stabilize the sample shape and prevent imaging artifacts associated with gluing the hemispheres to the microscope's sample holder.

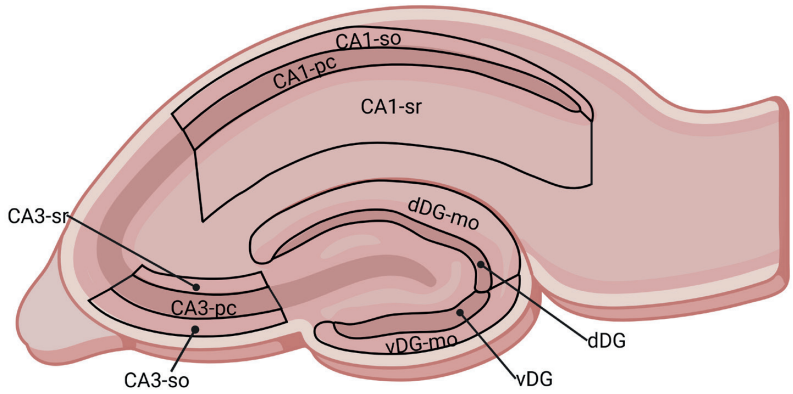

Figure 2. Region of interests (ROIs) within the hippocampus. Ten ROIs were defined based on the Allen Mouse Brain Atlas: the granule cell layer of the dentate gyrus dorsal blade (dDG), granule cell layer of the dentate gyrus ventral blade (vDG), molecular layer of the dentate gyrus dorsal blade (dDG-mo), molecular layer of the dentate gyrus ventral blade (vDG-mo), pyramidal cell layer of the cornu ammonis 3 (CA3-pc), stratum radiatum of the cornu ammonis 3 (CA3-sr), stratum oriens of the cornu ammonis 3 (CA3-so), pyramidal cell layer of the cornu ammonis 1 (CA1-pc), stratum radiatum of the cornu ammonis 1 (CA1-sr), stratum oriens of the cornu ammonis 1 (CA1-so). For immunohistochemistry, c-Fos$^+$ and tdTomato$^+$ cells were analyzed in cell layers (dDG, vDG, CA3-pc, CA1-pc) and c-Fos$^+$-GAD67$^+$ co-expression in the dendritic layers (dDG-mo, vDG-mo, CA3-sr, CA3-so, CA1-sr, CA1-so) of the hippocampus.

2.8. Imaging and data analyses for iDISCO+

2.8.1. Light-Sheet Microscopy

Agarose blocks containing a brain hemisphere were glued to the sample holder in a sagittal orientation (midline down) and placed in a reservoir filled with 100% dibenzyl ether. Images were acquired using Inspector Pro (version 7) at 2x magnification on a light-sheet microscope (Ultramicroscope II from LaVision Biotec). Samples were scanned at 560/40 nm (tdTomato) at 75% laser power, and 630/30 nm (c-Fos) at 85% laser power with a step size of 3 µm. Dynamic focus, sheet motor calibration and horizontal focus were set separately for each brain. The exposure time was set between 200 and 300 ms and the number of steps was set according to Inspector Pro's recommendations.

2.8.2. Image Analysis

The obtained images were analyzed with Arivis (https://imaging.arivis.com/), ImageJ and ClearMap software. For cell segmentation, an experimenter, blinded to the experimental conditions, first manually selected approximately 250-350 cells and background regions to train the Arivis software, after which the neurons were mapped to the anatomical reference atlas. The identified center coordinates were

subsequently transferred to ClearMap software (https://idisco.info/) (1 GUI, beta) for neuron mapping onto a standardized brain atlas. The files were down-sampled, compressing z-stacks, and mapped onto the standardized brain atlas using ImageJ. Landmarks defined in ImageJ were utilized by ClearMap and transformed into coordinates. Finally, the output was a heatmap aligned with the standardized brain atlas along with original cell coordinates (Cai et al., 2019; Kirst, 2020). All these analysis steps were applied to tdTomato$^+$ and c-Fos$^+$ cells, however, in the results section only tdTomato$^+$ cells were reported since our analysis showed that c-Fos labeling did not succeed in deeper brain structures because of poor antibody penetration (Figure 3).

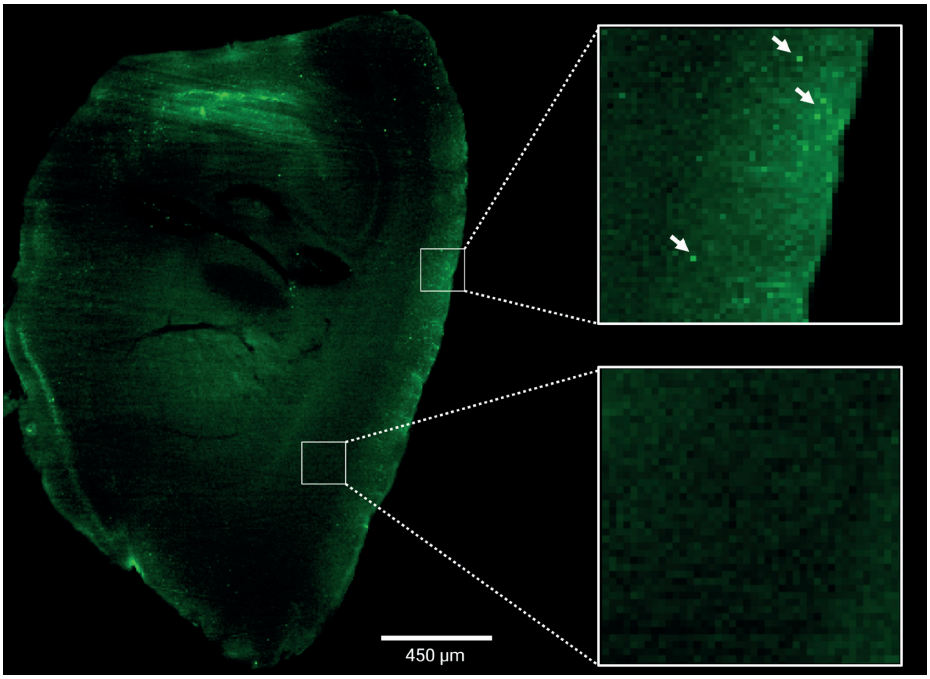

Figure 3. Light-sheet microscopy image of c-Fos$^+$ signal using the iDISCO+ method. As illustrated in the image, c-Fos$^+$ labeling was pronounced at the outside edges of the brain (zoomed image right top), but diminished in the deeper areas of the brain (zoomed image right bottom), likely due to poor antibody penetration. Despite troubleshooting efforts, optimal conditions for obtaining viable data were not achieved. White arrows pointing to labeled c-Fos$^+$ cell clusters.

2.8.3. Data Analysis

Cell counts per atlas region were extracted, and clustered into 92 clusters corresponding to the parent regions as defined in the Allen Brain Atlas (Dirven et al., 2024). Given that the variability in brain transparency resulted in substantial variation in the total cell counts between brains within experimental groups, all cell

counts were normalized to the total cell counts per animal, and normalized counts analyzed. Counts were Z-transformed and data points deviating >3 SDs from the mean were excluded as outliers (Dirven et al., 2024), which led to the exclusion of approximately 0.6% of all data points.

2.9. RNA Isolation and Quantitative PCR

For miR/mRNA measurements (Atucha et al., 2025), C57Bl/6 mice were killed by decapitation 40 min after training and drug treatment. Brains were rapidly extracted and flash frozen by submersion in a beaker filled with pre-cooled isopentane at -40 °C, placed on dry ice. Flash-frozen brains were stored at -80 °C until tissue processing. Brain tissue containing the dorsal hippocampus was cut into 200-µm-thick coronal slices on a cryostat (Leica, Model number: CM3050 S) and mounted on RNAse free slides, and further dissected using a 0.5-mm punch needle (Stoelting Co). Bilateral punches from different subfields of the hippocampus (dDG, vDG, CA1 and CA3) (AP, -1.34 to -2.37 mm from Bregma) were collected from five consecutive slices. Punches were preserved in RNALater-ICE (Ambion®Life Technologies, USA) in autoclaved tubes at -80 °C.

For further analyses, RNALater-ICE was removed and total RNA was isolated using a miRNAeasy® kit (Qiagen). The concentration and purity of total mRNA was assessed using Nanodrop™. For optimized detection and quantification of miR-134 and mRNA (*Bdnf* and *Creb*), DNAse treatment (Thermo Scientific) was carried out as indicated in the manufacturer's protocol. Then, cDNA was synthesized from total RNA using miScript II kit (Qiagen) according to the manufacturer's protocol. Using first strand cDNA as a template, real-time quantitative reverse transcription PCR (qRT-PCR) reactions were performed for miR-134 using miR-specific forward primers of the entire mature miR sequence and the universal qPCR reverse primer. For mRNA qRT-PCR reactions, mRNA-specific forward and reverse primers were used (Table 1).

Table 1. Primers used for qRT-PCR

	Forward Primer	Reverse Primer
Creb	5'- TCAGCCGGGTACTACCATTC -3'	5'- TTCAGCAGGCTGTGTAGGAA -3'
Bdnf	5'- GGTCACAGCGGCAGATAAAAAGA -3'	5'- TTCGGCATTGCGAGTTCCAG -3'
miR-134	5'- TGTGACTGGTTGACCAGAGGGG-3'	Universal primer
U6	5'- CTCGCTTCGGCAGCACA -3'	5'- AACGCTTCACGAATTTGCGT -3'
Hprt	5'- CAGTCCCAGCGTCGTGATTA-3'	5'- AGCAAGTCTTTCAGTCCTGTC-3'

qRT-PCR analysis was performed with miScript SYBR® Green PCR kit (Qiagen) in a Rotorgene qRT-PCR thermo-cycler. PCR cycles (CTs) similar to water or an empty control were excluded, as those indicated negligible input. Additionally, specific triplicate read outs were excluded if they deviated >0.5 SD from the other triplicates, making that the average of the two remaining (duplicate values) was used for further analyses. Relative expression was calculated using the comparative C_T method and normalized to the expression of *U6* for miR-134 and to the averages of *U6* and *Hprt* for *Creb* and *Bdnf*, with the ΔΔCt method (Schmittgen et al., 2008). All take off CT values used for analysis were averaged except for those values deviating more than 2CTs from the mean, which were considered outliers. Three data points from the saline group and two data points from the YOH group were excluded.

2.10. Statistics and Data Analysis

Statistical analyses were performed using IBM SPSS statistic 23. All data was first checked for deviations from normal distribution. Total object exploration time during training was analyzed using a repeated measures ANOVA with drug treatment group (saline, YOH (0.3, 1 mg/kg) or extensive training) as between-subject factor and episode (first vs. second training context) as within-subject factor. Moreover, as between-subject factors the object explored first (bulb vs. jar) and training context explored first (Context A vs. B) were included. Noteworthy, these latter factors were counterbalanced across animals. The DI% and total object exploration time during the retention test were analyzed using one-way ANOVAs to examine differences between the experimental groups (saline, YOH (0.3, 1 mg/kg), extensive training). Significant effects were followed up by independent samples *t*-tests.

Immunohistochemistry data were analyzed per hippocampal subregion by one-way ANOVAs with experimental group (saline, YOH (1 mg/kg), extensive training) as between-subject factor. Significant effects were followed up by independent samples *t*-tests. If the data deviated from normal distribution, Kruskal-Wallis were used to examine significant differences between the experimental groups. Pearson correlations were calculated to determine correlations between tdTomato$^+$ and c-Fos$^+$ cell count data between hippocampal subregions.

qRT-PCR data were also analyzed per hippocampal subregion, and contained only two experimental groups (saline and YOH (1 mg/kg)). When data were conform normal distribution, they were analyzed by independent samples *t*-tests. If data deviated from normal distribution, Mann-Whitney U tests were used to examine statistical differences between the experimental groups.

For iDISCO+ data analysis, Hedges' g's were calculated to determine effect size by subtracting the group means and dividing by the pooled standard deviation of the compared experimental groups (Hedges, 1981). This approach is driven by two factors: a) the mass univariate nature of the tests, which increases the risk of false positives when relying on *p*-value-based inferences, and b) Hedges' g is a more reliable effect size indicator than other standardized metrics for groups with $n < 20$ (Hedges and Tipton, 2010). As per convention, effect sizes of 0.2, 0.5 and 0.8 corresponded to small, medium and large effects, respectively (Sawilowsky, 2009). We here selected a 0.5 cutoff for further discussion of interesting effects, as is commonly established in the literature (Brydges, 2019).

For all data, in normally distributed groups, data points exceeding more than 2 SD from the mean were excluded, while for non-normally distributed data, exclusions were based if data points deviated more than 2 interquartile ranges (IQR). No behavioral data were excluded based on these criteria. For the immunohistochemistry data, a total of 8 data points (saline ($n = 3$ (CA3 = 2, CA1 = 1)), YOH 0.3 ($n = 2$), YOH 1 mg/kg ($n = 2$(dDG = 1, vDG = 1), or extensive training ($n = 1$ (CA3 = 1))) were excluded. For all comparisons, a $p < 0.05$ was accepted as statistical significance.

Data are expressed as mean ± SEM, and all individual data points included in the analyses are depicted. Furthermore, the number of mice per group is indicated in the figure legends.

3. Results

3.1. Effect of posttraining yohimbine administration and extensive training on object-in-context memory

First, we examined whether posttraining systemic administration of the noradrenergic stimulant YOH (0.3 and 1 mg/kg) enhances OiC memory to a similar extent as more extensive training. Object exploration times during training did not differ between the three experimental groups exposed to two training episodes for 5 min (main effect of group: $F(2,54) = 0.12$, $p = 0.66$). Object exploration times were stable across the two episodes (main effect of episode: $F(1,54) = 1.78$, $p = 0.19$), similarly across these experimental groups ($F(2,54) = 0.12$, $p = 0.94$). Moreover, object exploration times were not dependent on the object type or training context mice were exposed to first (all *p*'s > 0.58). As intended, the extensive training group exhibited significantly greater object exploration times during the training than

the other three experimental groups (all *p*'s < 0.02; Table S1). Further, we found that the extensive training group spent more time exploring the objects during the first training episode than during the second episode ($t(175) = 8.47$, $p < 0.001$), but again without any preference for either of the objects ($F(1,54) = 1.78$, $p = 0.19$).

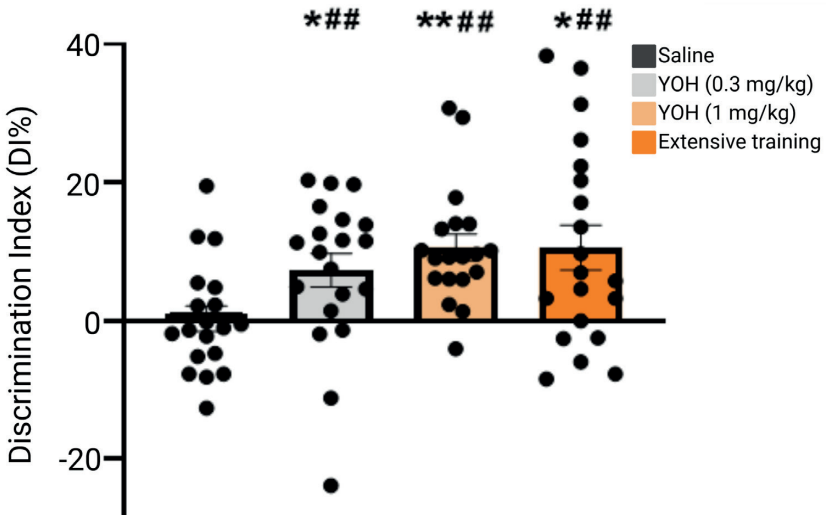

Figure 4. Effect of posttraining yohimbine administration and extensive training on object-in-context memory. The discrimination index (DI%) of saline control mice on the 72-h retention test did not differ significantly from zero (i.e.., chance level), whereas that of both yohimbine (YOH)-treated groups and the extensive training group was significantly larger than zero, indicating that only these latter groups expressed memory of the object-context association. The DI% of these three groups was significantly larger than that of the saline control group, whereas they did not differ from each other (Saline: $n = 19$, YOH (YOH, 0.3 mg/kg): $n = 20$, YOH (1 mg/kg): $n = 19$, Extensive training: $n = 20$). Data represent mean ± SEM. Dots indicate individual data points. *$p < 0.05$, **$p < 0.01$ different from saline control. ##$p < 0.01$ different from zero (i.e.., chance level).

At the 72-h retention test, a one-way ANOVA for the DI%s indicated a significant effect of experimental group ($F(3,77) = 3.86$, $p = 0.01$). *Post hoc* tests revealed that the DI% of both YOH groups and the extensive training group was significantly larger than that of the saline control group (0.3 mg/kg YOH: $t(37) = 2.28$, $p = 0.03$; 1 mg/kg YOH: $t(37) = 3.88$, $p < 0.001$; extensive training: $t(29.74) = 2.78$, $p = 0.009$) (Figure 4), without them differing significantly from each other (all *p*'s > 0.20). One-sample *t*-tests showed that the DI% of saline control mice did not differ significantly from zero, i.e., they performed at chance level ($t(18) = 0.14$, $p = 0.89$), whereas that of all other groups was significantly larger than zero (0.3 mg/kg YOH: $t(19) = 2.98$, $p = 0.008$; 1 mg/kg YOH: $t(18) = 5.45$, $p < 0.001$; extensive training: $t(19) = 3.26$, $p = 0.004$), indicating that these groups expressed memory of the object-context association. Total object exploration times during the retention test did not differ

across the four experimental groups ($F(3,77) = 1.80$, $p = 0.16$) (Table S1). Thus, these findings indicate that posttraining YOH administration and extensive training induced a comparable enhancement of OiC memory.

3.2. Effect of posttraining yohimbine administration and extensive training on neuronal activity within the hippocampus

Next, we examined the effects of posttraining YOH administration on neuronal activity within the hippocampus and whether these differed from those induced by extensive training. We examined neuronal activity both during the training/early consolidation period by assessing tdTomato expression, and following the retention test by assessing c-Fos expression. Since we observed the strongest memory-enhancing effect (and most similar memory performance compared to extensive training) for the 1 mg/kg dosage of YOH, this dosage was used for examination of neuronal effects.

3.2.1. Effect of posttraining yohimbine administration and extensive training on neuronal activity during the training/early consolidation period

In line with previous reports (Park et al., 2018), tdTomato$^+$ cells were only observed in the hippocampal cell layers (dDG, vDG, CA3-pc and CA1-pc), and not in the dendritic layers. One-way ANOVAs for the number of tdTomato$^+$ cell indicated no significant group effects in any of the hippocampal subregions (dDG: $F(2,27) = 0.24$, $p = 0.79$; vDG: $F(2,28) = 0.06$, $p = 0.94$; CA3-pc: $F(2,28) = 0.08$, $p = 0.93$; CA1-pc: $F(2,28) = 0.46$, $p = 0.64$) (Figure 5A). Posttraining administration of YOH and extensive training on the OiC were thus not associated with a significant change in the number of captured activated numbers during the training/early consolidation phase.

3.2.2. Effect of posttraining yohimbine administration and extensive training on neuronal activity following retention test

One-way ANOVAs for the number of c-Fos$^+$ cells indicated no significant group effects in the dDG ($F(2,27) = 0.21$, $p = 0.81$), vDG ($F(2,27) = 0.33$, $p = 0.72$), or CA1-pc ($F(2,28) = 0.71$, $p = 0.51$). However, we did observe a significant group effect for the number of c-Fos$^+$ cells in the CA3-pc ($F(2,26) = 4.97$, $p = 0.02$). *Post hoc* tests revealed that the number of c-Fos$^+$ cells in the CA3-pc of both the YOH ($t(14) = 1.09$, $p = 0.009$) and extensive training ($t(18) = 4.04$, $p = 0.03$) groups were significantly lower than that of the saline control group (Figure 5B), whereas the number of c-Fos$^+$ cells of the YOH and extensive training groups did not differ significantly from each other ($t(16) = 1.65$, $p = 0.55$). Thus, these findings indicate that both posttraining YOH administration and extensive training selectively reduced retention test-induced neuronal activity in the CA3-pc.

3.2.3. Effect of posttraining yohimbine administration and extensive training on retention-induced neuronal reactivation rates within the hippocampus

Next, we assessed the RR, which represents the percentage of neurons activated during both the initial training/consolidation and later retention (i.e., the number of tdTomato$^+$+c-Fos$^+$ cells), expressed as the ratio of the total number of neurons activated during the training/consolidation period (i.e., the number of tdTomato$^+$ cells). Comparison of the RRs to chance levels revealed that the RRs were consistently higher than chance (found to be <0.01%), indicating that training-activated neurons were more likely to be reactivated during recall than chance, signifying a neural substrate of memory (Figure 5C). However, a one-way ANOVA for the RRs revealed no significant difference between the three experimental groups in the dDG ($F(2,30) = 1.050$, $p = 0.36$), whereas non-parametric tests conducted in the other subregions also revealed no significant differences between experimental groups (vDG: $p = 1.00$, CA3-pc: $p = 0.09$, CA1-pc: $p = 0.34$).

3.2.4. Effect of posttraining yohimbine administration and extensive training on the activity of GABAergic neurons in the hippocampus

As potential explanation for the reduced activity of glutamatergic neurons in the CA3-pc, we next examined whether YOH and extensive training increased the activity of GABAergic neurons in the hippocampus following the retention test. GABAergic (GAD67$^+$) cells are primarily observed in the dendritic layers of the hippocampus (Brooker and Vida, 2018; Tzilivaki, 2023), which made us restrict our examination of GABAergic activity to the dDG-mo, vDG-mo, CA3-sr, CA3-so, CA1-so and CA1-sr. In general, co-localization rates were very low (<8.3%, Table S1). Kruskal-Wallis non-parametric tests revealed no significant differences in the relative fraction of GABAergic neurons activated upon the retention test in any of the subregions (all p's > 0.26). Thus, experimental groups did not differ in their relative activation of GABAergic neurons following the retention test (Figure S1).

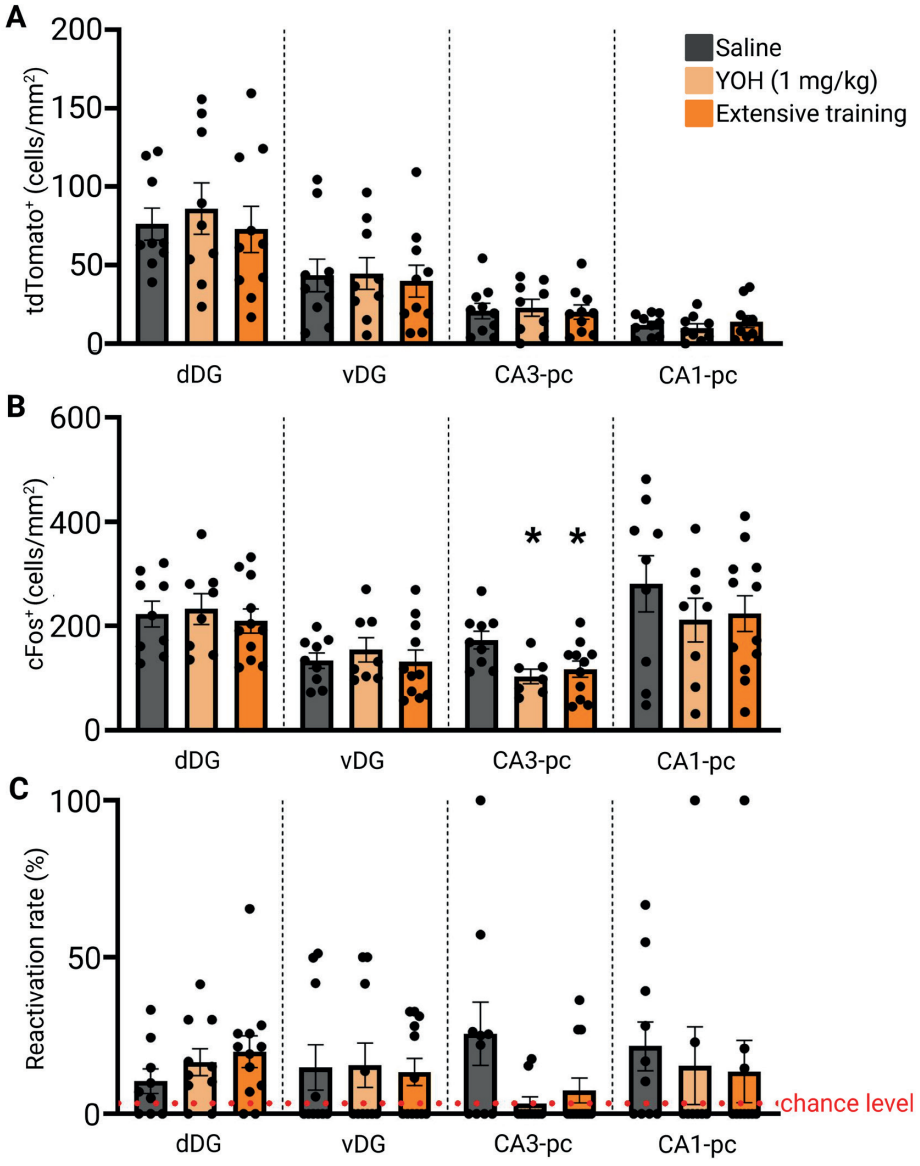

Figure 5. Effect of posttraining yohimbine administration and extensive training on neuronal activity within the hippocampus. A. Effect of posttraining yohimbine (YOH) administration and extensive training on neuronal activity during the training/early consolidation period (n = 9-10). **B.** Effect of posttraining YOH administration and extensive training on neuronal activity following the retention test (n = 8-11). **C.** Effect of posttraining YOH administration and extensive training on retention-induced neuronal RRs within the hippocampus (n = 8-12). Data represent mean ± SEM. Dots indicate individual data points. *P < 0.05: different from saline control.

3.2.5. Effect of posttraining yohimbine administration and extensive training on correlations in neuronal activity patterns between hippocampal subregions

To further investigate the effect of posttraining YOH on the hippocampus and how this compares to extensive training, we explored functional connectivity between hippocampal subregions, as approximated by calculating the correlations in the number of tdTomato$^+$ and c-Fos$^+$ cells for each of the three experimental groups (Figure 6). In saline-treated control mice, a positive correlation was observed between the number of tdTomato$^+$ cells in the dDG and vDG (r = 0.79; p = 0.01), dDG and CA3-pc (r = 0.70; p = 0.04) and vDG and CA3-pc (r = 0.64; p = 0.045). In mice treated with YOH, similar positive correlations were observed between the number of tdTomato$^+$ cells in the dDG and vDG (r = 0.79; p = 0.01), dDG and CA3-pc (r = 0.83; p = 0.005) and vDG and CA3-pc (r = 0.82; p = 0.007). Overall, the strength of these correlations appeared to be more robust in the YOH group (larger r-values), but differences were not significant (all p's > 0.11). The extensive training group also displayed a positive correlation between the number of tdTomato$^+$ cells in the dDG and vDG (r = 0.83; p = 0.003) as well as vDG and CA3-pc (r = 0.73; p = 0.02), whereas the correlation between the dDG and CA3-pc failed to reach significance (r = 0.49; p = 0.15). However, again, these correlations did not significantly differ from the saline control (all p's > 0.28) or YOH group (all p's > 0.11).

In terms of c-Fos$^+$ cells, saline-treated mice displayed a significant positive correlation between the CA3-pc and CA1-pc (r = 0.77; p = 0.02). No such positive correlation was observed in the YOH-treated group (r = 0.52, p = 0.23), yet the direct comparison of these correlations between groups revealed no significant difference (z = 0.68, p = 0.25). The extensive training group, displayed a similar positive correlations between c-Fos$^+$ cell counts in the CA3-pc and CA1-pc (r = 0.82; p = 0.002) compared to controls. Moreover, the extensive training group displayed a strong positive correlation between the number of c-Fos$^+$ cells in the dDG and vDG (r = 0.81; p = 0.003) and the dDG and CA1-pc (r = 0.65; p = 0.03), but these correlations again did not significantly differ from those observed in the saline control (both p's > 0.14) or YOH group (both p's > 0.83).

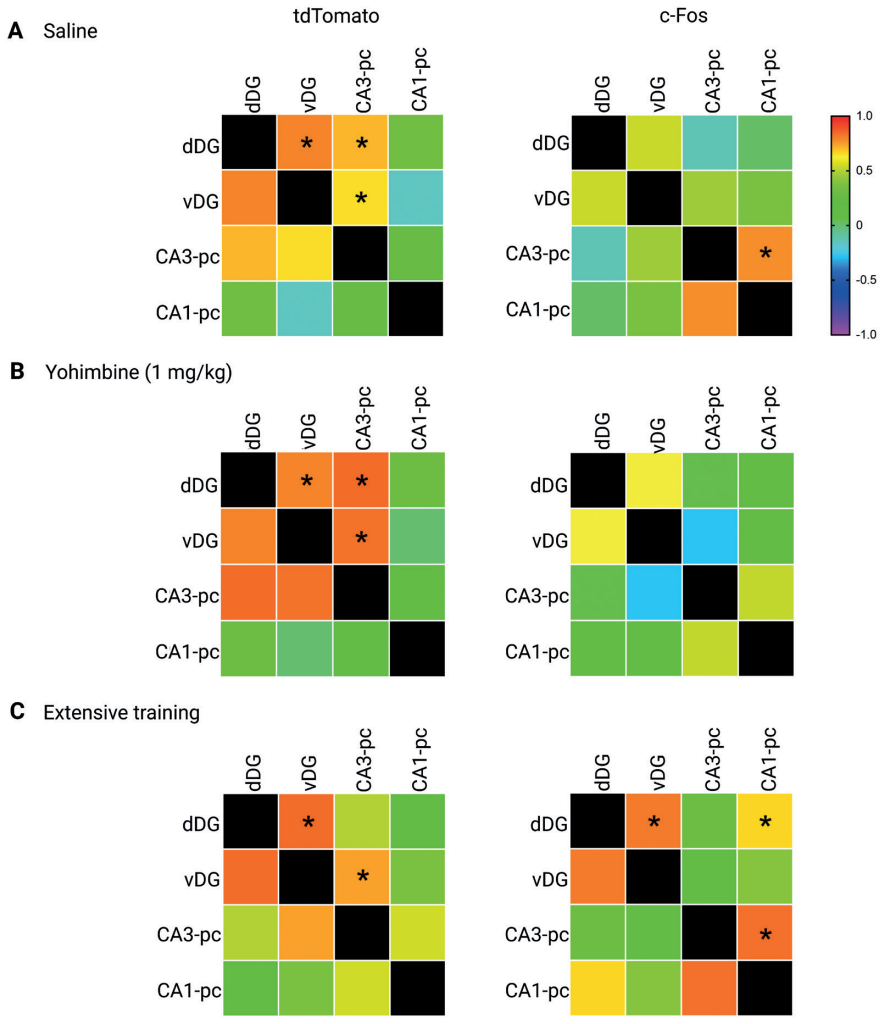

Figure 6. Effect of posttraining yohimbine administration and extensive training on correlations in neuronal activity within the hippocampus following training/early consolidation and the retention test. The saline, yohimbine (YOH 1 mg/kg) and extensive training groups showed similar patterns of correlated activity during the posttraining period (labeled by tdTomato), with significant correlations observed between the ventral dentate gyrus (vDG)- dorsal dentate gyrus (dDG) and vDG-CA3-pc in all groups, and between the dDG-CA3-pc in both the saline and YOH groups. Neuronal activity following the retention test (assessed by c-Fos) correlated between the CA3-pc and CA1-pc in the saline and extensive training group, but not in YOH-treated mice. Moreover, the extensive training group displayed correlations between the dDG and vDG and between the dDG and CA1-pc. All $n = 8\text{-}12$.*$p < 0.05$.

3.3. Effect of posttraining yohimbine administration and extensive training on brain-wide changes in neuronal activity

We next explored the effect of posttraining YOH administration and extensive training on neuronal activity during the training/early consolidation period beyond the hippocampus. For this, brain hemispheres were subjected to iDISCO+, an immunolabeling and clearing technique, in combination with light-sheet microscopy to enable visualization of the intact brain hemisphere.

In our analysis, we first compared neuronal activity in the YOH (1 mg/kg) to saline control group (Figure 7A). Findings revealed moderate-to-large reductions in the relative number of tdTomato$^+$ cells in YOH-treated mice in the hippocampus (vCA1: g = -0.58, vCA3: g = -0.86), the hippocampal formation (ventral entorhinal cortex (vEnt): g = -0.58), parasubiculum (paraSub): g = -0.55, postsubiculum (postSub): g = -0.50), the thalamus (geniculate group of the dorsal thalamus (ggd thamalus): g = -0.57) and in the substantia nigra (g = -0.51). Conversely, posttraining YOH did not show any medium or large-sized increases in relative tdTomato$^+$ cell counts.

We next analyzed the effects of extensive training by comparing this group to the saline control group (Figure 7B). Similar to what was observed after YOH treatment, extensive training was associated with a moderate-to-large reduction in relative tdTomato$^+$ cell numbers in the hippocampus (vCA1: g = -0.94, vCA3: g = -0.66), hippocampal formation (vEnt: g = -0.52, paraSub: g = -0.51), thalamus (lateral thalamus: g = -0.63, ggd thalamus: g = -0.56), ventral thalamus: g = -0.59, medial thalamus g = -0.53) and substantia nigra (g = -0.71).

Lastly, we compared posttraining neuronal activity in the YOH and extensive training groups. Both groups displayed similar memory performance, but this could potentially be supported by distinct neural mechanisms. Overall, neuronal activity was similar between groups, but the YOH group displayed a moderate increase in relative tdTomato$^+$ cell counts in the lateral thalamus (g = -0.56) and inferior colliculus (g = -0.49), compared to the extensive training group (Figure 7C).

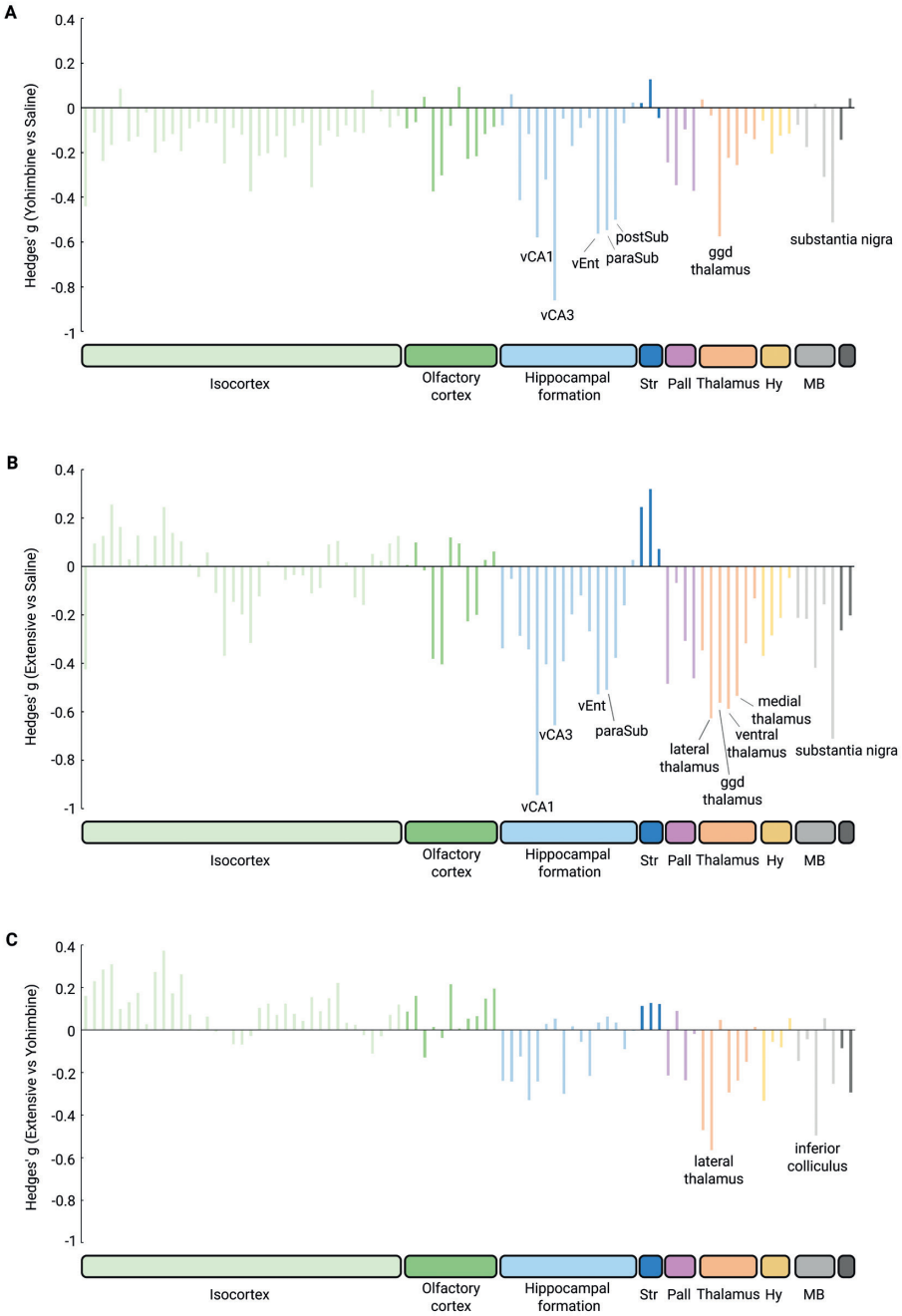

Figure 7. Effect of posttraining yohimbine administration and extensive training on brain-wide changes in patterns of neuronal activity following OiC training. Regional tdTomato$^+$ cell counts were normalized to the total cell count and compared across groups (n = 12). Only regions showing medium to strong effect sizes are marked in the graph. A complete list of all brain regions included and their Hedges' g values can be found in Table S2. Str: striatum, Pall: palladium, Hy: hypothalamus, MB: midbrain, vCA1: ventral cornu ammonis area 1, vCA3: ventral cornu ammonis area 3, vEnt: ventral entorhinal cortex, paraSub: parasubiculum, postSub: postsubiculum, ggd thalamus: geniculate group of the dorsal thalamus.

3.4. Effect of posttraining yohimbine administration on hippocampal miR-134, *Creb* and *Bdnf* expression

Previous work showed that NE administration into the BLA after training on the dual-event inhibitory avoidance task led to a down-regulation of miR-134, and an increase in mRNA levels of its target genes *Creb* and *Bdnf* in the dDG (Atucha et al., 2025). Therefore, we aimed to examine how these levels were affected by the systemic administration of a memory-enhancing dose of YOH (1 mg/kg) following OiC training. For these experiments, mice were trained for 5 min on the OiC task followed by YOH or saline administration, and killed 40 min later (Figure 1B). We then investigated miR-134, *Creb* and *Bdnf* mRNA levels in the dDG as well as in the other hippocampal subregions.

During the training session, object exploration times did not differ between the groups ($F(1,55) = 1.21$, $p = 0.74$) and were stable across the two training episodes (main effect of episode: $F(1,55) = 0.94$, $p = 0.44$), similarly for both groups ($F(1,55) = 0.84$, $p = 0.38$). Moreover, object exploration times were not dependent on the object type (jars vs bulbs: $F(1,55) = 0.80$, $p = 0.19$) or the training context in which mice were first exposed to (first vs. second: $F(1,55) = 1.01$, $p = 0.09$) (Table S3).

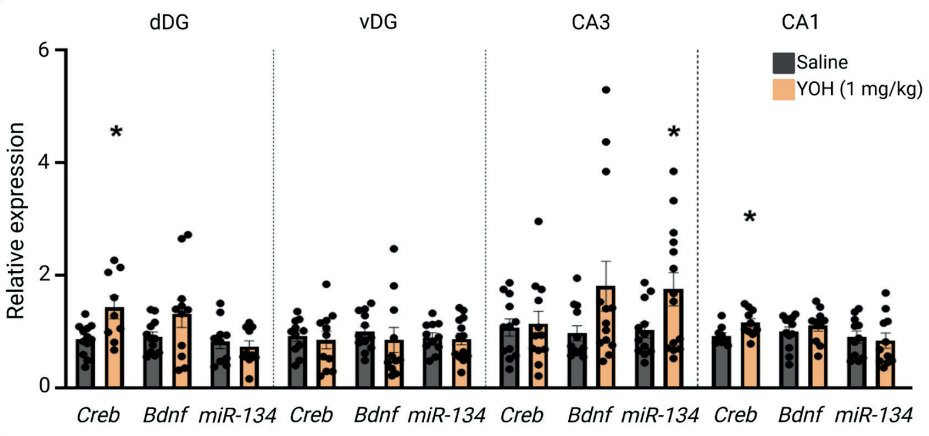

Figure 8. Effect of posttraining yohimbine administration on *Creb* and *Bdnf* mRNA and miR-134 levels in the hippocampus following OiC training. Yohimbine treatment (YOH, 1 mg/kg) after OiC training was associated with elevated levels of *Creb* mRNA in the dDG and CA1. In the CA3, YOH treatment induced elevated levels of miR-134. Data represent mean ± SEM. Dots indicate individual data points. n = 9-12, *$p < 0.05$: different from saline control.

Similar to the effects of NE administration into the BLA (Atucha et al., 2015), we observed increased *Creb* mRNA in the dDG ($t(10.50) = 2.59$, $p = 0.03$) following YOH administration. However, *Bdnf* mRNA levels ($t(12.39) = 1.57$, $p = 0.14$) and

miR-134 levels ($t(18) = 0.83$, $p = 0.58$) in the dDG were not significantly affected. YOH administration was additionally associated with a significant increase in miR-134 levels in the CA3 ($t(18.19) = 2.24$, $p = 0.006$) and elevated *Creb* mRNA levels in the CA1 region ($U = 72$, $p = 0.03$) (Figure 8).

4. Discussion

In this study, we examined the neural underpinnings of the enhancing effects of posttraining noradrenergic activation on the episodic-like specificity of memory using the OiC task and compared these to conditions of extensive training on the task. Specifically, we sought to investigate changes in a) recall-induced neuronal activity, b) the reactivation of training-activated hippocampal cells during later recall, and c) neuronal activity during the training/early consolidation phase beyond the hippocampus. Use of transgenic FosTRAP2xtdTomato mice enabled us to label active neurons during OiC training and the early posttraining consolidation phase by tdTomato expression, whereas immunohistochemistry for endogenous c-Fos expression following the retention test was used to assess retention test-induced neuronal activity. Additionally, d) we explored the molecular mechanisms underlying the effects of posttraining administration of YOH on episodic-like memory specificity focusing on miR-134 and its downstream targets *Creb* and *Bdnf* in the hippocampus (Atucha et al, 2025). We found that posttraining YOH administration and extensive training induced a similar enhancement of the episodic-like specificity of memory. Neither YOH administration nor extensive training significantly impacted the number of tdTomato$^+$ cells or their reactivation during the retention test. However, both posttraining YOH treatment and extensive training reduced neuronal activation in the hippocampal CA3 region following retention testing. Whole-brain analyses revealed that posttraining YOH treatment and extensive training affected the relative distribution of tdTomato+ cells in largely similar ways, reducing activity within several regions of the ventral hippocampus (i.e., the vCA3 and vCA1), as well as the vEnt, paraSub, ddg thalamus and substantia nigra. At the molecular level, YOH treatment posttraining elevated miR-134 levels in the CA3 region and increased levels of *Creb* mRNA in the dDG and CA1.

Whereas traditionally most memory research on the effects of NE has focused on its general memory-strengthening effects (McGaugh, 2000; Stern and Alberini, 2013; Lalumiere et al., 2017; Alberini and Stern, 2020), recent research in our lab has started investigating its effects on the episodic-like specificity of memory for multiple training events. Posttraining administration of the noradrenergic stimulant YOH was

previously found to enhance both memory specificity in the dual-event inhibitory avoidance task (Mirone and Roozendaal, 2020) and the OiC task (Guo, 2024). More recent findings indicated that noradrenergic activation enhances the episodic-like specificity by facilitating the separation of the two memory traces into two distinct memories (Atucha et al., 2025. Here, we replicated these behavioral findings, and additionally showed that extensive training on the OiC task is equally effective in improving episodic-like memory specificity. The length of the training session is a well-established factor in titrating performance in memory tasks (Stefanko et al., 2009; Song, 2021). A single 10-min training session has been shown effective in inducing strong short- and long-term memory for objects (either object location memory or object recognition memory), making it a suitable method for studying memory formation deficits (Stefanko et al., 2009; Barrett et al., 2011; Haettig et al., 2011; McNulty et al., 2012; Vogel-Ciernia et al., 2013). The OiC task shares several similarities with these tasks, as it similarly relies on object recognition and context pairing, although the exact memory process assessed (i.e., episodic-like memory specificity) differs. Similar to our findings here, increasing the training duration was previously found to improve memory detailedness in an object discrimination task to a comparable extent as posttraining administration of YOH (Song, 2021). Moreover, previous work showed that posttraining administration of epinephrine reduced the number of trials needed to reach criterion compared to vehicle on a two-way active avoidance task, but that more extensive training eventually resulted in similar performance (Nordby et al., 2006). As such, incorporating extensive training in the OiC task offers a valuable opportunity to investigate episodic-like memory specificity and compare it to the effects of posttraining noradrenergic manipulation.

Here, we assessed neuronal activity during the encoding/early consolidation phase of the OiC task, and how this was affected by the posttraining administration of YOH or extensive training, by labeling them with tdTomato using FosTRAP2xtdTomato mice. Prior work in our lab similarly investigated the effects of posttraining administration of YOH in the OiC task on hippocampal neuronal activity during the posttraining memory consolidation phase by analyzing endogenous c-Fos expression (Guo, 2024). In contrast to our null findings in the (dorsal) hippocampus, this earlier work indicated increased neuronal activity in the hippocampal CA1 as a consequence of YOH administration, as well as an increased correlation between neuronal activity between the dDG and CA3 (without any main effects on activity), suggesting improved pattern separation (Guo, 2024). We did not replicate these effects of YOH, which is in line with findings of another study from our laboratory (conducted at around the same time as this work) in which neuronal activity during the encoding/early consolidation phase of the OiC task was assessed using the

same FosTRAP2xtdTomato mice (Gulmez-Karaca et al., 2024). Whereas that study showed that the FosTRAP2-labeling system was effective in detecting increased neuronal activity in the hippocampus as a consequence of training, it also showed that overall hippocampal tdTomato expression was notably low. We similarly observed very low numbers of tdTomato$^+$ cells in the hippocampus, particularly in the CA3 and CA1, with numbers of tdTomato$^+$ cells in the CA1 region only reaching about 1/10th of the number of neurons endogenously expressing c-Fos following the retention test. As such, the low sensitivity of the FosTRAP2 system might explain why we did not replicate prior findings of enhanced neuronal activity in the CA1 following YOH administration posttraining on the OiC task. Another factor that might have influenced our outcome is the extended time window during which neuronal activity is labeled in these transgenic mice (up to 5–6 hours following 4-OHT injection (Guenthner et al., 2013). This prevents one from capturing any transient changes in hippocampal activity, as are expected following training, being first upregulated followed by a down-regulation (DeNardo et al., 2019). This might also well explain why we did not observe an increased correlation between dDG and CA3 activity posttraining as a consequence of YOH administration, as reported before (Guo, 2024). Moreover, these reasons could explain why we did not observe any differences in the reactivation rate of encoding/early consolidation activated cells across groups during the retention test. This is in line with the other work from our lab testing the effects of posttraining administration of YOH on the maintenance of episodic-like memory specificity over time on the OiC task in FosTRAP2xtdTomato mice. There, as well as in our study, consistently higher hippocampal RRs were found than could be expected based on chance, but no direct correlation with behavioral performance (Gulmez-Karaca et al., 2024). However, in that study the maintenance of OiC memory specificity over time as induced by YOH was associated with higher RRs in the hippocampus, which typically dropped because of processes of systems consolidation. Future work targeting encoding/early consolidation activated neurons in the hippocampus specifically using a viral FosTRAP system, which allows for a precise titration of viral transfection rates to optimize labeling sensitivity, should resolve the role of the hippocampus engram cells in mediating YOH-induced improved OiC memory specificity (Visser, 2022; Barykina et al., 2022).

Both posttraining YOH administration and extensive training reduced neuronal activity in the CA3 following the retention test. The hallmark role of the CA3 is its ability to retrieve patterns from partial or noisy inputs, a process referred to as pattern completion (e.g., Kesner et al., 2000; Gilbert et al., 2001; Nakazawa et al., 2002; Lee et al., 2004; Rolls and Kesner, 2006; McHugh et al., 2007; Yassa and

Stark, 2011). Pattern completion increases representational overlap at the output level compared to the input (O'Reilly and McClelland, 1994) and thereby serves an opposing process to pattern separation as mediated by the DG, which facilitates discrimination between events with overlapping features. Inefficiencies in pattern separation, or a dominance of pattern completion can lead to false recognition of similar events (Sahay et al., 2011; Stark et al., 2013; Wilson et al., 2006). Therefore, diminished retention-induced activity in the CA3 as a consequence of posttraining YOH administration as well as extensive training may reflect the suppression of pattern completion processes that might be required to express memory specificity.

Since noradrenergic activation and emotional arousal are well documented to exert widespread effects on neural activity (Morilak et al., 2005; Mather et al., 2016; Zerbi et al., 2019; Ross and Bockstaele, 2021), we continued investigating neuronal activity during the training/early consolidation phase (labeled with tdTomato) and retention test (labeled with c-Fos). Prior research in our lab demonstrated for example that enhanced memory detailedness (as induced by posttraining administration of YOH) for object memory was associated with increased retention-induced neuronal activity in the aIC and PRh (Song, 2021), regions known to be important for object recognition memory (Kafkas and Montaldi, 2014; Miranda et al., 2017; Molas et al., 2017; Chen et al., 2018; Chen et al., 2022). Other work from our lab observed increased activity in these structures posttraining on the OiC task followed by YOH administration (Guo, 2024). Because c-Fos labeling using the iDISCO+ approach was unsuccessful in staining deeper brain structures due to inadequate antibody penetration, resulting in inconsistent labeling outcomes across brains, we refrained from analyzing that signal. The tdTomato signal was superior and yielded more consistent results. We observed that both posttraining YOH administration and extensive training induced a moderately strong reduction in the number of tdTomato^{+} cells in several subregions of the hippocampal formation, specifically the ventral CA1 and CA3, as well as the vEnt and paraSub. This outcome aligns with human studies where YOH administration was observed to initially reduce hippocampal activity relative to placebo (Krenz et al., 2021); an effect proposed to be necessary to reorganize the systems consolidation process, allowing prolonged hippocampal involvement (Krenz et al., 2021). Previous research has indicated that noradrenergic activation can alter systems consolidation in rodents as well, extending hippocampal involvement in episodic-like memory (Atucha et al., 2017; Bahtiyar et al., 2020; Krenz et al., 2021; Gulmez-Karaca et al., 2024), supporting this notion. The subiculum serves as the primary output region of the hippocampus proper, specifically from area CA1, and projects predominantly to the entorhinal cortex (Swanson, 1987; Amara et

al., 1991; O'Mara et al., 2001; O'Mara, 2005), connecting these findings. Little is known about the modulatory actions of NE in the subiculum (de Melo et al., 2023), although a recent study (Lipski and Grace, 2013). Iontophoretic NE inhibited all vSub neurons tested iontophoretic NE was found to inhibit all neurons in the ventral subiculum, in line with our findings. The entorhinal cortex is a key gateway between the hippocampus and the neocortex, and involved in encoding and retrieving spatial memories, and crucial for integrating sensory information with spatial context. The vEnt in particular is thought to play a role in representing and processing temporal information as well. Studies have shown that neurons in the vEnt exhibit activity related to time, potentially contributing to the brain's ability to track elapsed time and encode temporal context, and obviously critical in OiC memory (Gerlei et al., 2021; Alexander et al., 2020; Tsanov and Manahan-Vaughan, 2008). Previous work has shown primarily activating effects of NE on the entorhinal cortex, affecting both synaptic transmission and intrinsic excitability, contrasting our findings. Noradrenaline released from the locus coeruleus modulates neuronal activity in the vEnt, with prominent effects in the superficial layers. In layers II and III, noradrenaline decreases neuronal excitability, primarily through activation of α2-adrenergic receptors that enhance potassium conductance and, in some cases, alter calcium channel activity (Sara, 2009). In parallel, noradrenaline suppresses excitatory synaptic transmission by reducing postsynaptic responses mediated by NMDA and AMPA receptors (Atzori et al., 2016; Yam et al., 2018). These effects are largely restricted to the superficial layers, as deeper layers of the vEnt appear to be minimally influenced. Such modulation is thought to contribute to the regulation of spatial and contextual learning processes (Perez, 2020). Again, the rather long labeling window for activity in the FosTAP2 system might be related to this.

Additionally, extensively trained animals displayed reduced activity in the lateral thalamus and inferior colliculus compared to both saline-injected controls and YOH-treated mice. Traditionally considered a hub for (passively) relaying sensory information to the cortex, the thalamus is increasingly recognized as a critical region for stress research (Yoshii, 2021), encoding the salience, valence, and social relevance of sensory signals (Yang et al., 2025) . One could speculate that the increased pre-exposure to the training context in case of extensive training (sessions of 10 instead of 5 min) could have reduced the saliency of the OiC training episodes, potentially explaining this effect. Connecting the auditory brain stem to sensory, motor, and limbic systems, the inferior colliculus is a critical midbrain station for auditory processing, which is rather hard to connect to the OiC task. Future work should investigate this further.

Interestingly, both the YOH and extensive training groups exhibited largely comparable brain-wide activation patterns, which similarly differed from the saline control group, as consistent with their observed behavioral memory performance. This suggests that posttraining administration of YOH (i.e., in the memory consolidation phase) is similarly effective in increasing neuronal activity and plasticity in brain regions recruited during training as more prolonged training durations.

Lastly, we investigated miR-134, *Creb* and *Bdnf* mRNA expression across hippocampal subregions to elucidate the molecular underpinnings of altered hippocampal processing following the posttraining - administration of YOH. Prior research in our lab revealed that NE administration into the BLA following training on the dual-event inhibitory avoidance task in rats induced a down-regulation in miR-134 expression within the dDG (Atucha et al., 2025), which led to a local up-regulation of *Creb* and *Bdnf* mRNA levels, both essential molecular players in memory consolidation (Silva et al., 1998; Bramham and Messaoudi, 2005). In this earlier study, pharmacological down-regulation of miR-134 in the hippocampus after training was sufficient to enhance memory specificity, whereas pharmacological overexpression of miR-134 blocked the effect of NE administration into the BLA on enhancing memory specificity in the dual-event inhibitory avoidance task (Atucha et al., 2025). These effects were specific to conditions that required pattern separation; i.e., when rats were exposed to two learning events close in time as NE administration following training on a single-event inhibitory avoidance task did not induce a down-regulation in miR-134 within the hippocampus. Here, we tested whether the systemic administration of YOH following training on the OiC task, which also requires the separation of memory for two training events, was associated with a similar down-regulation of miR-134 and upregulation of *Creb* and *Bdnf* mRNA expression in the dDG in mice. We replicated the increase in *Creb* mRNA in the dDG, but we did not find any significant changes in *Bdnf* or miR-134 expression in the dDG. However, we did observe increased levels of miR-134 in the CA3 region, together with an increase in *Creb* mRNA in the CA1. These increases in hippocampal *Creb* mRNA are in line with its reported memory-enhancing role (Josselyn et al., 2001; Brightwell et al., 2007; Han et al., 2008; Zhou et al., 2009). Yet, our finding that YOH administration did not increase *Bdnf* levels within the dDG is at odds with earlier findings (Atucha et al., 2025). Potentially, the route of administration and thus level of noradrenergic manipulation, systemic vs intra-BLA, plays a role here, as NE can also exert direct effects on hippocampal neurons in our work. It is also possible that differences in the saliency of the two memory tasks could explain these contrasting findings, with the dual-event inhibitory avoidance task being notably more salient than the OiC task. Also the exact type of memory

targeted, i.e., a shock-context vs object-context association, is distinct. These reasons might equally account for the null effect of YOH on down-regulating miR-134 levels in the dDG after OiC training here. However, intriguingly we observed an increase of miR-134 specifically in CA3 area; the same area where we observed reduced neuronal activity during the retention test. One could speculate that this suggests that memory specificity in the OiC task is more tightly related to pattern completion processes rather than pattern separation, and proposes the CA3 as primary site of action for NE for this. Future research should assess whether these two phenomena (increase in posttraining miR-134 and reduction in postretention c-Fos) are directly related and how noradrenergic activity modulates them.

In summary, our findings demonstrate that both posttraining YOH administration and extensive training enhanced episodic-like memory specificity in the OiC task. This enhancement was not associated with significant changes in neuronal activity during the training/early consolidation phase in the dorsal hippocampus, but with reduced activation in the vCA1, vCA3, parasubiculum, vEnt , substantia nigra and thalamic subregions in both groups. Molecular analyses further indicated elevated levels of miR-134 in the CA3 and increased *Creb* expression in the dDG and CA1 in the YOH group. Following the retention test, reduced neuronal activation in the dorsal CA3 region was observed in both the YOH and extensive training groups. Taken together, the results highlight that whole-brain activation patterns were remarkably similar between the YOH and extensive training groups, indicating that posttraining noradrenergic activation produces comparable behavioral outcomes and whole-brain activation profiles to those achieved with extensive training. Moreover, it identifies the hippocampal CA3 as prime region of interest as well as some other new target brain regions that require future dedicated investigation.

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6. Supplements

Table S1. Object exploration times during the per training session and retention test.

	Training Session (s) mean ± SEM	Retention Test (s) mean ± SEM
Saline (*n* = 19)	12.5 ± 2.4	11.5 ± 2.1
Yohimbine 0.3 mg/kg (*n* = 20)	11.8 ± 2.2	11.8 ± 2.6
Yohimbine 1 mg/kg (*n* = 19)	12.1 ± 2.0	12.0 ± 1.5
Extensive training (*n* = 20)	16.8 ± 4.2	11.8 ± 2.1

Table S2. Whole-brain comparison of relative tdTomato$^+$ counts across 92 pre-defined regions

Region Number	Region ID	Hedges' g Yoh vs Saline	Hedges' g Ext vs Saline	Hedges' g Ext vs Yoh
1	Frontal pole, cerebral cortex	-0.44	-0.43	0.16
2	Primary motor area	-0.11	0.09	0.23
3	Secondary motor area	-0.24	0.13	0.28
4	Primary somatosensory area, nose	-0.17	0.25	0.31
5	Primary somatosensory area, barrel field	0.09	0.16	0.10
6	Primary somatosensory area, lower limb	-0.15	0.03	0.13
7	Primary somatosensory area, mouth	-0.13	0.13	0.18
8	Primary somatosensory area, upper limb	-0.02	0.01	0.03
9	Primary somatosensory area, trunk	-0.20	0.12	0.27
10	Supplemental somatosensory area	-0.15	0.24	0.37
11	Gustatory area	-0.12	0.14	0.17
12	Visceral area	-0.19	0.10	0.26
13	Dorsal auditory area	-0.09	0.01	0.07
14	Primary auditory area	-0.06	-0.04	0.00
15	Ventral auditory area	-0.07	0.06	0.06
16	Anterolateral visual area	-0.07	-0.11	-0.01
17	Anteromedial visual area	-0.25	-0.37	0.00
18	Lateral visual area	-0.09	-0.15	-0.07
19	Primary visual area	-0.12	-0.20	-0.07
20	Posterolateral visual area	-0.37	-0.32	-0.03
21	Posteromedial visual area	-0.21	-0.12	0.10
22	Anterior cingulate area, dorsal part	-0.20	0.02	0.12
23	Anterior cingulate area, ventral part	-0.13	0.00	0.07

Table S2. Continued

Region Number	Region ID	Hedges' g Yoh vs Saline	Hedges' g Ext vs Saline	Hedges' g Ext vs Yoh
24	Prelimbic area	-0.22	-0.06	0.12
25	Infralimbic area	-0.08	-0.04	0.08
26	Orbital area, lateral part	-0.07	-0.04	0.04
27	Orbital area, medial part	-0.35	-0.11	0.15
28	Orbital area, ventral part	-0.17	-0.09	0.09
29	Agranular insular area, dorsal part	-0.10	0.09	0.15
30	Agranular insular area, posterior part	-0.13	0.10	0.22
31	Agranular insular area, ventral part	-0.08	0.02	0.03
32	Retrosplenial area, lateral agranular part	-0.11	-0.13	0.02
33	Retrosplenial area, dorsal part	-0.11	-0.16	-0.02
34	Retrosplenial area, ventral part	0.08	0.05	-0.11
35	Temporal association areas	-0.02	0.02	-0.03
36	Perirhinal area	-0.09	0.09	0.07
37	Ectorhinal area	-0.04	0.13	0.12
38	Main olfactory bulb	-0.09	0.01	0.09
39	Accessory olfactory bulb	-0.06	0.10	0.16
40	Anterior olfactory nucleus	0.05	-0.02	-0.13
41	Taenia tecta	-0.37	-0.38	0.01
42	Nucleus of the lateral olfactory tract	-0.30	-0.41	-0.04
43	Dorsal peduncular area	-0.08	0.12	0.21
44	Piriform area	0.09	0.09	0.01
45	Piriform-amygdalar area	-0.23	-0.23	0.05
46	Postpiriform transition area	-0.22	-0.20	0.07
47	Cortical amygdalar area	-0.12	0.03	0.15
48	Amygdala	-0.09	0.06	0.19
49	Hippocampal formation	-0.08	-0.34	-0.24
50	Dorsal CA1	0.06	-0.05	-0.24
51	Dorsal CA2	-0.41	-0.29	-0.12
52	Dorsal CA3	-0.12	-0.34	-0.33
53	Ventral CA1	-0.58	-0.94	-0.24
54	Ventral CA2	-0.32	-0.41	0.03
55	Ventral CA3	-0.86	-0.66	0.05
56	Dorsal DG	-0.05	-0.39	-0.30
57	Ventral DG	-0.17	-0.20	0.02
58	Lateral entorhinal cortex	-0.09	-0.12	-0.06

Table S2. Continued

Region Number	Region ID	Hedges' g Yoh vs Saline	Hedges' g Ext vs Saline	Hedges' g Ext vs Yoh
59	Medial entorhinal cortex	-0.05	-0.27	-0.22
60	Ventral entorhinal cortex	-0.56	-0.53	0.04
61	Parasubiculum	-0.55	-0.51	0.06
62	Postsubiculum	-0.50	-0.38	0.04
63	Presubiculum	-0.07	-0.16	-0.09
64	Subiculum	0.02	0.03	0.00
65	Cortcial subplate	0.02	0.24	0.11
66	Striatum, dorsal & ventral	0.13	0.32	0.13
67	Lateral septal complex	-0.04	0.07	0.12
68	Dorsal Pallidum	-0.24	-0.49	-0.21
69	Globus Pallidus	-0.35	-0.07	0.09
70	Substantia innominata	-0.10	-0.31	-0.24
71	Magnocellular nucleus	-0.37	-0.46	-0.02
72	Ventral group of the dorsal thalamus	0.04	-0.35	-0.47
73	Lateral thalamus	-0.03	-0.63	-0.56
74	Geniculate group of the dorsal thalamus	-0.57	-0.56	0.05
75	Ventral thalamus	-0.22	-0.59	-0.29
76	Medial thalamus	-0.26	-0.53	-0.24
77	Geniculate thalamus	-0.12	-0.32	-0.15
78	Interlaminar thalamus	-0.14	-0.13	0.01
79	Hypothalamus	-0.06	-0.37	-0.33
80	Periventricular region	-0.21	-0.28	-0.06
81	Medial hypothalamus	-0.12	-0.21	-0.08
82	Lateral hypothalamus	-0.12	-0.05	0.06
83	Superior colliculus	-0.08	-0.21	-0.14
84	Periaqueductal gray	-0.17	-0.22	-0.04
85	Inferior colliculus	0.02	-0.42	-0.50
86	Pretectal area	-0.31	-0.16	0.06
87	Substantia nigra	-0.51	-0.71	-0.25
88	Pons	-0.14	-0.27	-0.09
89	Medulla	0.04	-0.20	-0.29
90	Fibre tracts	-0.06	-0.20	-0.16
91	Corpus callosum	-0.08	-0.11	-0.16
92	Amygdalar capsule	-0.04	-0.31	-0.31

Table S3. Object exploration times during the training session for the qPCR experiment.

	Training Session (s) mean ± SEM
Saline (*n* = 28)	7.1 ± 0.3
Yohimbine 1 mg/kg (*n* = 28)	7.0 ± 0.3

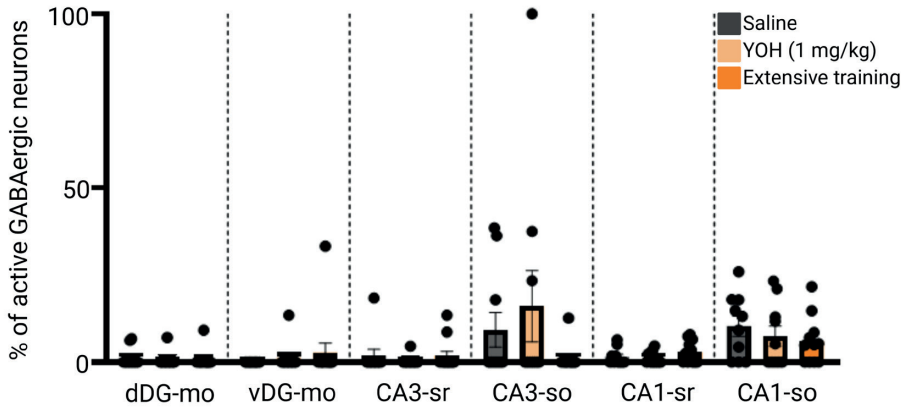
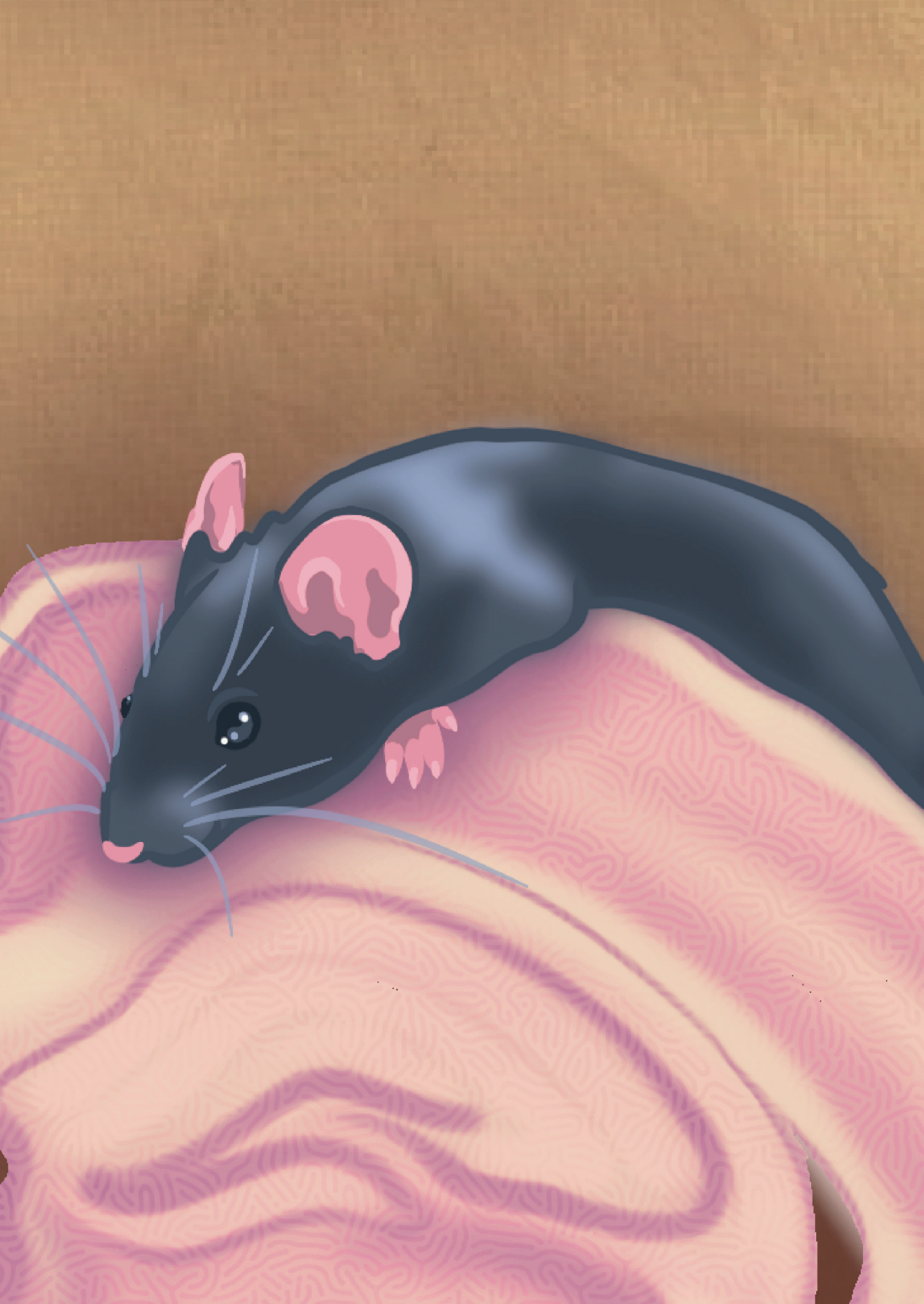


Figure S1. Effect of posttraining yohimbine administration and extensive training on the activity of GABAergic neurons in the hippocampus



CHAPTER 5

Summary and General Discussion

The primary focus of these studies was to investigate the effects of the stress hormones norepinephrine (NE) and corticosterone (CORT) on both the consolidation and retrieval of episodic-like specificity of memory. My aim was to examine these stress hormone effects not only at the behavioral level but also investigate the neuronal and molecular mechanisms underlying these stress hormone effects on memory specificity.

While it is widely accepted that memory for stressful or emotionally arousing experiences is stronger, the extent to which stress and emotional arousal also influence qualitative aspects of memory such as its specificity, accuracy, and vulnerability to incorporation of misinformation remains a subject of debate (Morgan et al., 2004; Porter et al., 2008; Hoscheidt et al., 2014). Human studies have reported conflicting results. On the one hand, stress exposure has been associated with enhanced, generalized, gist-based memory processing of the central aspects of an event, often accompanied by reduced specificity, diminished context-dependency, overconfidence in later recollection, and even an increase in false memories (Loftus, 1979; Payne et al., 2002; Talarico and Rubin, 2003; Sharot et al., 2004; Schwabe and Wolf, 2009; Rimmele et al., 2011). On the other hand, stress has also been reported to improve memory for details (Kensinger et al., 2007a, Kensinger et al., 2007b; Segal et al., 2012; Wiemers et al., 2013; Song et al., 2021). In contrast to human research, this topic had received relatively little attention in animal studies, largely due to the limitations of existing behavioral tasks in assessing memory quality aspects. However, a recently developed behavioral task for rats made it possible to examine the effects of stress and stress hormones on episodic-like specificity of memory (Atucha and Roozendaal, 2015; Roozendaal and Mirone, 2020). In this dual-event inhibitory avoidance task, rats are trained in two different inhibitory avoidance apparatuses with a brief interval, but only receive a footshock upon entering the dark compartment of the second context. Episodic-like specificity of memory for this task is approximated by contrasting later retention latencies in the prior Shock box, to those observed in the Non-Shock box. Interestingly, these studies indicated that whereas the two stress hormones NE and CORT both strengthen memory of the shock experience per se, they exert opposing effects on the episodic-like specificity of memory. CORT administration was found to induce a generalized strengthening of memory whereas NE was found to increase episodic-like memory specificity (Atucha and Roozendaal, 2015; Roozendaal and Mirone, 2020). Further research showed that NE administration directly into the basolateral amygdala after training on this task replicated the behavioral effects of systemic manipulations, and was found to enhance the consolidation of pattern-separated memories via post-transcriptional

regulation of gene expression by microRNA (miR)-134 within the dentate gyrus of the dorsal hippocampus (Atucha et al., 2025). A second task that allows for the investigation of episodic-like memory specificity is the object-in-context (OiC) paradigm, in which accurate memory performance similarly relies on the ability to differentiate between the two subsequent learning events, only this time being the object previously encountered in a specific context. In this task, posttraining administration of the noradrenergic stimulant yohimbine was also shown to dose-dependently enhance memory performance (i.e., specificity), whereas posttraining CORT administration impaired performance (Guo, 2024). However, significant gaps remained in understanding how the stress hormones NE and CORT influence the neural substrate of memory supporting memory consolidation and its retention.

Summary of main findings

In **Chapter 2,** I aimed to set up the dual-event inhibitory avoidance task for mice. The dual-event inhibitory avoidance task was originally designed for rats, yet mice have become the dominant species in neuroscientific research due to advances in genome-editing technologies that are more readily available in mice (Cryan et al., 2005). After optimizing training and testing parameters, I found that mice were capable of acquiring episodic-like memories in this task, displaying specifically longer retention latencies in the Shock box than in the other two boxes. My next aim was to replicate the previously reported effects of NE and CORT on episodic-like memory specificity and strength. I administered yohimbine and CORT immediately after training and assessed later memory retention. However, I did not observe the modulatory effects of yohimbine or CORT administration on episodic-like specificity of memory as previously reported for rats. Starting from the introduction of posttraining intraperitoneal injections, I observed testing order effects and substantially increased variability both within experimental groups and across experiments, precluding a thorough investigation of stress hormone effects on memory specificity. I further only found a weak memory-strengthening effect of yohimbine and was unsuccessful in replicating the memory-strengthening effect of CORT. Thus, these findings showed that whereas the dual-event inhibitory avoidance task is effective for studying stress hormone effects on the consolidation of memory specificity in rats, it is less suitable for such experiments in mice.

In **Chapter 3**, I used the dual-event inhibitory avoidance task to examine whether acute stress exposure shortly before retention testing affects episodic-like specificity of the recalled memory, and whether stress exposure interacts

with mitochondrial function in regulating memory specificity. To investigate this, I used both wildtype (WT) mice and a mouse line with a genetic deficiency in the *nuclear-encoded NADH dehydrogenase [ubiquinone] iron-sulfur protein 4* (*Ndufs4*), which shows an approximate 25% reduction in respiratory chain complex I (CI) activity. Both WT and *Ndufs4*$^{GT/GT}$ mice were trained on the dual-event inhibitory avoidance task, and retention was 48 hours later. Half of the mice were exposed to stress (forced swim) 30 minutes before the retention test. I additionally examined whether stress exposure affected mitochondrial activity in the hippocampus and prefrontal cortex (PFC), and analyzed neuronal activity patterns following retention using c-Fos immunofluorescence in excitatory and inhibitory neurons in both regions. Comparable to the findings of Chapter 2, I found that non-stressed WT mice were able to acquire the task and exhibited significantly longer retention latencies in the Shock box as compared to the Non-Shock and Novel boxes. Stress exposure in WT mice selectively shortened latencies in the shock context, such that latencies in all three text contexts became similar. Further, stress exposure in WT mice was associated with reduced complex I mitochondrial activity in the hippocampus. Non-stressed *Ndufs4*$^{GT/GT}$ mice also showed short retention latencies in all three test contexts, providing support for the view that reduced complex I mitochondrial activity, as found after stress exposure, is involved in the behavioral effect. Interestingly, stress exposure in *Ndufs4*$^{GT/GT}$ mice generally increased retention latencies across all boxes except novel box. As discussed in that chapter, we interpret this general increase in retention latencies in these mitochondrial activity deficient mice as a result of fatigue. Stress exposure in *Ndufs4*$^{GT/GT}$ mice did not further reduce complex I mitochondrial activity in the hippocampus, but increased complex IV mitochondrial activity in the PFC. Further, stress exposure reduced c-Fos activity in the hippocampus but increased c-Fos activity within the PFC. Both of these effects were more pronounced in *Ndufs4*$^{GT/GT}$ mice. Thus, whereas these findings could not reveal whether stress exposure prior to retention test specifically influenced episodic-like specificity of the recalled memory, the findings did reveal some very interesting findings of how stress exposure prior to retention testing influences mitochondrial activity within the hippocampus in regulating episodic-like memory and demonstrate how stress exacerbated the suboptimal functioning in *Ndufs4*$^{GT/GT}$ mice.

In **Chapter 4,** I investigated the effects of systemic yohimbine administration after training on the OiC task on episodic-like specificity of memory and its neural correlates in mice. As findings from Chapter 2 indicated that the dual-event inhibitory avoidance task is not the most suitable task to investigate the effect of drug administration on memory specificity, I used the OiC task here. An

advantage of the OiC task, as compared to the dual-event inhibitory avoidance task, is the relatively long duration of the training episodes (5 min in each context) that allows for more robust memory encoding and might therefore be less prone to disruptions due to stressful posttraining injections. Moreover, the task only contains a single retention test, precluding any testing order effects. The aim of my study was to investigate how posttraining systemic administration of yohimbine affects hippocampal processes in supporting the memory. Specifically, we aimed to determine whether noradrenergic activation following training on the OiC task alters hippocampal activity during the retention test and whether it alters the reactivation of neurons that were activated during or after the training. We used FosTRAP2xtdTomato mice, which allow the examination of neuronal activity associated with training-associated activity through tdTomato expression and c-Fos activity for retention-induced reactivation. The main focus of the study was to examine how yohimbine affected hippocampal activity during consolidation and retention test, but we also examined yohimbine effects on brain-wide neuronal activity using iDISCO+ combined with light-sheet microscopy. To test whether the observed effects in these experiments were specific to yohimbine or rather related to enhanced memory performance per se, we included a control group subjected to extensive training to match the memory performance of yohimbine-treated mice. Finally, I examined whether systemic yohimbine administration after training on the OiC task would similarly engage a miR-134-mediated consolidation process in the dorsal dentate gyrus. I also investigated changes in downstream targets of miR-134, analyzing cAMP response element-binding (*Creb)* and brain-derived neurotrophic factor (*Bdnf*) messenger RNA (mRNA) levels. I found that posttraining administration of yohimbine enhanced OiC memory to a similar degree as extensive training. Both experimental groups did not display any differential neuronal activity in the hippocampus during the encoding/early consolidation period, but displayed reduced neuronal activity in the hippocampal CA3 during the subsequent retention test, without changing the reactivation of training-activated neurons (i.e., the proposed memory engram). These results might reflect a suppression of pattern completion processes necessary for expressing memory specificity in the yohimbine and extensive training groups. Exploratory iDISCO+ results revealed that the yohimbine and extensive training generated quite similar brain-wide effects on neuronal activity during the encoding/early consolidation phase compared to the saline group, characterized by reduced activity within the hippocampal formation, the ventral entorhinal cortex, parasubiculum and certain subregions of thalamus. These similar activation patterns align with the similar behavioral memory performance of these groups. Lastly, I found that posttraining yohimbine treatment following the OiC task was associated with increased *Creb* mRNA levels in the

dorsal dentate gyrus and CA1 regions. Additionally, in the CA3 region, yohimbine treatment induced elevated levels of miR-134. Overall, these findings highlight that posttraining noradrenergic activation produced comparable behavioral outcomes and neural activation patterns to extensive training conditions, suggesting shared neural mechanisms.

In the following sections, I will further integrate findings across chapters to dissect its central messages.

Suitability of the dual-event inhibitory avoidance and object-in-context tasks to study episodic-like memory in mice

In Chapter 2, I first aimed to set up the dual-event inhibitory avoidance task for mice, and replicate the effects of systemic yohimbine and CORT administration on memory strength and episodic-like memory specificity. In previous studies, our laboratory had used this task to explore the impact of posttraining NE and CORT on episodic-like memory in rats (Atucha et al., 2015; Roozendal and Mirone, 2020; Atucha et al., 2025), with yohimbine generating strong memories with enhanced episodic-like specificity, but CORT including a generalized strengthening of memory (Roozendaal and Mirone, 2020). We successfully managed to set up the dual-event inhibitory avoidance task in mice, creating episodic-like memories as evidenced by specifically long retention latencies in the Shock box compared to the Non-shock box. We carefully determined the effective levels of CORT and yohimbine by testing several dosages referencing previous studies on various behavioral tasks in mice (Cai et al., 2006; Song et al., 2021; Brosens et al., 2023); yet, we only found a very weak memory-strengthening effect of yohimbine and were unable to replicate the memory-strengthening effect of CORT altogether. We observed no modulatory effects of either hormone on the specificity of the memory. The range of dosages tested for both compounds make it unlikely that an optimal dose was overlooked. Rather, with the introduction of posttraining intraperitoneal injections to evaluate the effects of stress hormones on memory consolidation we started to observe context order interactions in memory performance during testing that complicated data interpretation. In hindsight, the injection procedures was probably too stressful in mice, as they required restraint (Johnson et al., 2000), previously shown to be more stressful than intramuscular or subcutaneous injections (in rats) and to elevate plasma CORT levels (Meijer et al., 2006). It is likely that this relatively intense stressor posttraining interfered with the consolidation process of the relatively short (and less intense) training episode. Consequently, this task proved unsuitable for studying the impact of stress hormone manipulations during the posttraining consolidation phase on episodic-like memory specificity in mice.

However, the observation of episodic-like memory specificity in non-injected mice suggested that the task was potentially suitable for testing the effects of other types of manipulations on episodic-like memory specificity. This was confirmed by our findings in Chapter 3, in which we investigated the effects of stress exposure on episodic-like specificity of memory recall as well as the modulatory nature of mitochondrial dysfunction. As such, the dual-event inhibitory avoidance task serves as a valuable addition to existing behavioral paradigms for specifically teasing apart memory strength vs precision aspects of episodic-like memory, particularly when paired with complementary molecular or cellular techniques investigating the mechanistic underpinnings.

Yet, the dual-event inhibitory avoidance task appeared incompatible with posttraining manipulation of stress hormone signaling (by intraperitoneal injection). Therefore, in Chapter 4 we decided to shift to the OiC task to further investigate the effects of posttraining noradrenergic activation on memory specificity and underlying neuronal mechanisms. The overall concepts of the two tasks are very similar. In both tasks, the animals are trained in two contexts close in time, and for each training context they need to form an association with a specific stimulus (shock or object). Both tasks offer flexibility in adjusting the training parameters, either by modifying the shock intensity or altering the duration of the training period. This adaptability enables one to manipulate the level of memory specificity observed in the control group, which should have specific memory in case of targeting impairments in the experimental group and non-specific memory in case of targeting improvements. Based on their similarities, it is very likely that posttraining noradrenergic effects on episodic-like memory found in both tasks are brought about by their influence on some shared neural mechanism of separating memory for the two training episodes (enhancing memory specificity) (Atucha et al., 2025). Previous findings from our lab had already indicated the compatibility of the OiC task with drug manipulations in mice and replicated the behavioral effects of posttraining administration of yohimbine and CORT on memory performance (i.e., specificity) (Guo, 2024).

An advantage of the OiC task compared to the dual-event inhibitory avoidance task is that both training events hold equal significance/saliency. Firstly, this allows for complete counterbalancing of the order in which the two events are presented. In most cases, training on the two contexts in the dual-event inhibitory avoidance task was done in a fixed sequence in which animals were first trained in the Non-Shock box and then in the Shock box. Therefore, these findings do not completely rule out the possibility that the drug administration might have preferentially

enhanced the memory of the event occurring closest to the time of administration. However, it has been shown, in rats, that it is possible to train animals first in the Shock box and subsequently in the Non-Shock box (Atucha et al., 2025). However, it remains unclear whether the same would hold true in our mouse model. Nevertheless, because training in the Shock box is inherently more salient than training in the Non-Shock box, it cannot be ruled out that stress hormone administration preferentially enhanced memory for the more salient event in this task. This could result in the dominant memory of the Shock box overshadowing the less salient memory of the Non-Shock box (Mather et al., 2016). Importantly, these alternative explanations do not apply to the OiC task, as both training events are equally salient and the training context used for testing is counterbalanced across mice. One limitation of the OiC task is that the training and test procedures do not provide a direct measure of memory strength for the objects themselves. In contrast, the dual-inhibitory avoidance paradigm allows such assessments of both strength and specificity of memory by comparing retention latencies in the Shock box per se versus differences in retention latencies in the Shock box with those in the other contexts. In a recent experiment, this limitation of the OiC task was overcome by testing separate groups of trained animals with a completely novel object on a standard object recognition task (Gulmez-Karaca et al., 2025), but the assessment of memory strength in the OiC would then require additional animals.

Effects of stress (hormone manipulation) on episodic-like memory specificity depend on the memory phase

Effects of posttraining manipulation of noradrenergic activity on episodic-like memory specificity

Posttraining yohimbine administration has previously been shown to enhance memory specificity in both the dual-event inhibitory avoidance (Mirone and Roozendaal, 2020) and OiC task (Guo, 2024). In Chapter 4, I replicated these findings of enhanced episodic-like memory in the OiC task following posttraining administration of yohimbine.

Neural activity during the encoding/early consolidation phase: Previous research of our lab (Guo, 2024) had implicated the increased recruitment of a hippocampal pattern separation process in mediating these behavioral findings, reporting on positively correlated activity (assessed by c-Fos immunohistochemistry posttraining) between the dorsal dentate gyrus and CA3 in the yohimbine-treated group, suggestive of facilitated pattern separation (Yassa and Stark, 2011; Kesner, 2013); a correlation that was absent in the vehicle-treated control group. Moreover,

it reported on an increased number of activated cells in the CA1 posttraining in yohimbine-treated mice, which was proposed to be related to the separation of memories of the two training events , supporting the storage of these two different memories into two non-overlapping neuronal ensembles (Guo, 2024). Here, I observed significantly correlated activity between the dDG and CA3 of the dorsal hippocampus in the yohimbine-treated group, whereas a similar correlation was present in the saline controls. I did not find increased activity in the dorsal CA1. Most likely, differences in the experimental method to assess neuronal activity posttraining, i.e., analyzing endogenous c-Fos expression in mice sacrificed posttraining vs labeling activated neurons in FosTRAP2xtdTomato mice, is responsible for this.

Here, I chose to use the FosTRAP2xtdTomato mice as they allowed me to additionally assess the effects of posttraining administration of yohimbine on neural activity supporting later memory retention; a process yet uninvestigated in our lab. Moreover, this approach allowed me to specifically address the reactivation rates of encoding-activated (so-called 'engram') neurons, previously reported essential for supporting memory performance (Josselyn et al., 2015; Choi et al., 2018; Karaca et al., 2021). However, the TRAP system comes with the downside that it is rather insensitive; overall tdTomato expression— tagging neurons activated (i.e., expressing c-Fos) during the memory encoding and consolidation phase—was notably low, much lower than when assessed by traditional c-Fos immunohistochemistry. This aligns with previous findings from our lab (Gulmez-Karaca et al., 2025). Moreover, the labeling of neuronal activity happens over a more extensive time window in these transgenic mice (up to 5–6 hours following 4-OHT injection), potentially making it unsuitable to capture relevant transient changes in neuronal activity across encoding and consolidation time frames (Guenthner et al., 2013). Still, we observed greater reactivation rates of engram neurons than could be expected by chance, indicating statistically significant reactivation beyond random expectation. However, we did not observe any effects of yohimbine on the reactivation rate of training-activated neurons in the hippocampus, reproducing earlier findings with this method (Gulmez-Karaca et al., 2025).

Yohimbine administration likely exerts effects beyond the (dorsal) hippocampus. This interpretation aligns with neuroimaging evidence indicating that emotional arousal and activation of the locus coeruleus (LC) produce widespread changes in neuronal activity across the brain (Zerbi et al., 2019). However, it remains unclear whether and how these alterations relate to the observed memory effects. Here, we analyzed neuronal activity at the whole-brain systems level using iDISCO+ and light-sheet microscopy, and showed a notable downregulation of neuronal

activity in the ventral CA1 (vCA1) and ventral CA3 (vCA3) regions specifically in both the yohimbine and extensive training groups compared to the saline-treated group during the training/early consolidation phase. This outcome aligns with human studies where yohimbine administration was observed to initially reduce hippocampal activity relative to placebo (Krenz et al., 2021); an effect proposed to be necessary to reorganize the systems consolidation process, allowing prolonged hippocampal involvement (Krenz et al., 2021). Interestingly, along with vCA1 and vCA3 downregulation we observed that posttraining yohimbine administration and extensive training induced a moderately strong reduction in the number of tdTomato^{+} cells in other memory-related brain regions such as the ventral entorhinal cortex (vEnt) and parasubiculum (paraSub). Previous work has shown primarily activating effects of NE on the entorhinal cortex, affecting both synaptic transmission and intrinsic excitability, contrasting our findings. NE released from the locus coeruleus modulates neuronal activity in the vEnt, with prominent effects in the superficial layers. In layers II and III, NE decreases neuronal excitability, primarily through activation of α2-adrenergic receptors that enhance potassium conductance and, in some cases, alter calcium channel activity (Sara, 2009). In parallel, NE suppresses excitatory synaptic transmission by reducing postsynaptic responses mediated by NMDA(N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (Atzori et al., 2016; Yam et al., 2018). These effects are largely restricted to the superficial layers, as deeper layers of the vEnt appear to be minimally influenced. Such modulation is thought to contribute to the regulation of spatial and contextual learning processes (Perez, 2024). These observations may help explain the downregulation detected in these regions, as they likely reflect the interplay between excitatory and inhibitory processes influenced by NE. It is also possible that the FosTRAP2 system, which has a long labeling window and limited temporal specificity, captured this downregulation rather than more transient activity changes.

Molecular mediators: Previous work indicated that NE administration into the basolateral amygdala (BLA) following training on the dual-event inhibitory avoidance task resulted in downregulation of miR-134 and increased mRNA levels of its target genes, *Creb* and *Bdnf*, in the dDG (Atucha et al., 2025). We were unable to replicate these findings, which may be due to differences in experimental design—specifically, the use of systemic rather than intra-basolateral amygdala drug administration. Posttraining, targeted intracranial infusions offer highly precise manipulation of specific brain regions. Although this approach requires surgical implantation of cannulas provides several methodological advantages. These include postmortem verification of cannula placement and improved

pharmacokinetic control over drug or hormone delivery (Atucha et al., 2025). Overall, intra-BLA infusions enable the anatomical specificity needed to directly link NE's actions within the amygdala to distinct behavioral outcomes, thereby producing more reliable and interpretable results. Systemic injections affect the entire organism, making it impossible to isolate the specific contribution of the target region. Another important factor is that in the previous study, NE was administered into the BLA of one hemisphere while the contralateral hemisphere received a vehicle injection, allowing within-subject comparisons controlling for inter-individual variability. Such a design is not feasible with systemic yohimbine administration, which may have introduced greater variability between subjects and reduced the likelihood of detecting significant effects.

Neural activity following the retention test: Interestingly, whereas we did not find any effect of yohimbine administration or extensive training on neuronal activity in the dorsal hippocampus during the encoding/early consolidation phase, they both were associated with significantly reduced retention-induced neuronal activity in the dorsal CA3 region. Moreover, whereas we observed significant correlations between neuronal activity in the dorsal CA3 and CA1 in the saline control and extensive training groups, these were absent in the yohimbine-treated group. The CA3 region has a highly interconnected structure, where most excitatory synapses link CA3 neurons to one another. This recurrent organization is thought to enable pattern completion, allowing the network to reconstruct complete memories from partial or degraded inputs (Marr, 1971; McNaughton and Morris, 1987). Our findings may therefore suggest that noradrenergic activation suppresses CA3 pattern completion at retrieval, allowing for the recall of more detailed or specific memory representations. This is supported by prior work showing that the selective inhibition of the engrams in CA3 enhanced specificity of the retrieval performance (Kim and Kim, 2024). Future studies should exploit selective inhibition techniques (e.g., using chemogenetics) to specifically target engrams within the CA3 region during the retention test of the OiC task to test whether this promotes specific memory recall. This approach could offer a more comprehensive understanding of the role of CA3 in mediating NE and training effects on memory specificity and pattern completion.

Effects of stress exposure prior to retention testing on episodic-like specificity of recalled memory

Most studies examining the effects of stress and glucocorticoids on episodic-like memory specificity have focused on posttraining manipulations, primarily targeting the consolidation phase of memory. However, memory recall is also

known to be influenced (i.e., impaired) by prior stress (hormone) exposure, and it remains completely unclear whether such exposure also influences the episodic-like quality of the recalled memory. We investigated whether acute stress before retention testing on a dual-event inhibitory avoidance task impacts episodic-like memory specificity. Additionally, we assessed whether this interacts with mitochondrial function.

My findings in WT mice are in line with prior reports on impaired memory recall following stress exposure (de Quervain et al., 1998; Kuhlmann et al., 2005; Park et al., 2008; Wolf, 2009). Whereas WT mice expressed an accurate memory for the training context associated with footshock, dissociating it from the other contexts, stressed WT mice failed to do so, and displayed low retention latencies in the Shock box that were similar to those in the other contexts under control conditions. If the stress exposure would have impaired only memory specificity, one would expect increased latencies in the other contexts; however, this was not observed. In another study, it has been shown that corticosterone administration before retention testing selectively impaired retrieval of contextual memory in rats tested in the original training context, while having no effect in a different context or after weak, non-contextual training (Roozendaal et al., 2004). These findings are similar to ours, and suggest that corticosterone specifically disrupts the retrieval of context-associated memories. Similarly, in both an auditory-cue fear conditioning task (Roozendaal et al., 2006) and a contextual fear conditioning task (Atsak et al., 2012), which depend on correctly associating cues with their corresponding environments and discriminating between safe and aversive contexts and, corticosterone administration shortly before the retention test was associated with reduced freezing behavior in the previous cued context. Moreover, freezing behavior was selectively reduced in this conditioned context but remained unchanged in a novel, safe context. These findings suggest that acute stress (hormone) exposure influences memory specificity primarily during the consolidation phase.

At the neural level, we observed that stress exposure prior to the retention test in WT mice selectively reduced mitochondrial CI activity in the hippocampus, while leaving activity in the PFC unaffected. Moreover, we observed that stress exposure reduced c-Fos expression in the hippocampus and increased expression in the PFC following the retention test. These effects were strongest in *Ndufs4*$^{GT/GT}$ mice. Interestingly, these mice displayed short retention latencies across all test contexts under control conditions, failing to distinguish the shock box during the retention test, whereas stressed *Ndufs4*$^{GT/GT}$ mice showed strong, generalized memory, as evidenced by longer retention latencies across all boxes. Impaired

mitochondrial function reduces the amount of energy available for appropriate brain functioning. Previous studies have outlined how stress can contribute to and intensify mitochondrial dysfunction (Picard and McEwen, 2018). Stressors trigger and exacerbate mitochondrial dysfunction, including the activation of hormonal receptors and increased production of reactive oxygen species (ROS). This is particularly relevant for mitochondrial CI function, which may serve as a key factor in heightened stress susceptibility and stress-induced metabolic reprogramming in the brain (Emmerzaal et al., 2020).

Future directions

The findings presented in this thesis put forward several advancements in our understanding of how stress exposure and stress hormone administration influence episodic-like memory specificity as well as methods to investigate this. The dual-event inhibitory avoidance task was successfully adapted for mice, and it can be used to assess both memory strength and memory specificity—provided that no posttraining intraperitoneal injections are administered. An important advantage of this task is its ability to dissociate memory specificity from memory strength, offering a useful framework for examining how environmental or genetic factors differentially affect these two aspects of memory. Overall, based on our findings, if the goal is to investigate the effects of stress hormones on episodic-like memory specificity in mice, the OiC task represents the more suitable paradigm.

In the thesis we found that stress exposure prior to the retention test reduced mitochondrial CI activity in the hippocampus. Prior research reported that the effects of glucocorticoid administration on memory retrieval are mediated by the activation of CB1 receptors located on mitochondrial membranes of GABAergic interneurons (Hebert-Chatelain et al., 2016; Skupio et al., 2023). Interestingly, we also examined whether our stress manipulation affected the activity of GABAergic neurons within the hippocampus and found no such effect. Nonetheless, the present findings highlight the need for future research to determine whether the stress-induced reduction in CI activity is mediated by glucocorticoid-induced changes in endocannabinoid signaling or related pathways. Selectively blocking mitochondrial CB1 receptors would allow a more direct investigation of the link between stress exposure, mitochondrial function, and altered memory retrieval.

Further, we also found that yohimbine administration elevated *Creb* mRNA levels in the dorsal blade of the dentate gyrus and CA1 following OiC training, and

increased miR-134 levels in the CA3. However, we were unable to replicate previous findings of elevated miR-134 levels in the dentate gyrus, a result that could have provided insight into pattern completion and separation in this type of task. Given the similarity of the OiC task and dual-event inhibitory avoidance task, it is very likely that we simply missed detecting changes in this miR-134 in the present study. Previously, it was found that directly manipulating miR-134 levels with an antagomir administration into the hippocampus was sufficient to alter episodic-like specificity of memory on the dual-event inhibitory avoidance task. Therefore, we propose employing a similar strategy to administer an antagomir to selectively silence miR-134 in the hippocampus and subsequently assess both behavioral and immunohistochemical outcomes. This approach would enable a more direct investigation of whether miR-134 contributes to (the effects of NE on) pattern completion and pattern separation processes on the OiC task (Atucha et al., 2025).

Lastly, our findings demonstrated that both posttraining yohimbine administration and extensive training enhance episodic-like memory specificity in the OiC task, and elucidated some of the neural mechanisms associated. Our immunohistochemical analysis demonstrated that both yohimbine treatment and extensive training significantly reduced recall-induced neuronal activation in the CA3 region, which is implicated in pattern completion. This reduction suggests that diminished pattern completion may play a role in enhancing memory specificity; however, further studies are needed to validate this hypothesis. Chemogenetic approaches represent a suitable method for investigating this phenomenon. Selective inhibition or excitation of the CA3 region prior to retention testing could provide a valuable means to elucidate its contribution of episodic-like memory specificity. The enhancement of episodic-like memory specificity was not associated with significant changes in neuronal activity during the training/early consolidation phase in the dorsal hippocampus as assessed with the TRAP system.

Future studies could assess neuronal activity at multiple time points or use fiber photometry to monitor hippocampal activity in real time following NE administration. Fiber photometry measures calcium-dependent fluorescence signals through an implanted optical fiber, providing high temporal resolution and enabling detection of dynamic changes in neuronal activity within and between brain regions across distinct memory phases (Girven and Sparta, 2017). Because fiber photometry has limited spatial resolution, recordings should focus on a specific hippocampal subregion—such as the DG, CA3, or CA1—with CA3 being particularly relevant in this context. This approach would allow direct measurement of hippocampal activity fluctuations during the retention test as animals interact

with different objects, offering more precise temporal resolution than the c-Fos TRAP system.

A final suggestion for future work would be the study of the neural correlates of the modulatory effects of glucocorticoids on memory specificity. Prior research has indicated that glucocorticoids contribute to a stronger, yet more generalized episodic-like memory, which may carry clinical relevance considering the critical role for generalized fear memories in several stress-related mental disorders. This is particularly relevant in the context of PTSD, known to be characterized by enhanced HPA-axis feedback regulation (Albrecht, 2017; Vukojevic et al., 2014; Yehuda et al., 2015). Many PTSD patients show increased GR sensitivity and stronger HPA-axis feedback, resulting in lower circulating corticosterone levels (Pitman et al., 2012; Yehuda, 2009). Furthermore, epigenetic modifications (i.e., *NR3C1*-methylation) established during early life have been shown to shape glucocorticoid signaling and influence vulnerability to stress-related disorders (Turecki and Meaney, 2016; Weaver et al., 2017), which might be related to its effects on memory. Giving the wide distribution of glucocorticoid receptors throughout the brain (de Kloet et al., 1993), it is plausible that CORT may exert similarly broad effects as observed for noradrenergic activation. It would be of interest to determine which effects are shared (presumably mediating enhanced memory strength) and where in the brain they dissociate (mediating specificity vs generalization) to get a better grasp on potential targets for intervention.

Conclusion

This thesis demonstrates that the dual-event inhibitory task is an effective paradigm to assess episodic-like memory specificity in mice provided the absence of posttraining i.p. injections, revealing that stress exposure prior to memory recall impairs memory strength but not necessarily episodic-like memory specificity. Mitochondrial dysfunction was found to amplify stress effects on memory retention performance. This may carry clinical relevance for human pathologies implicating compromised mitochondrial function, as for example observed in major depressive disorder. Understanding the mechanisms of memory specificity and generalization could inform treatments, such as exercise to modulate cortisol signaling or fluoxetine to preserve hippocampal involvement during memory retrieval, reducing overgeneralization risks. The OiC task might however be a more robust behavioral paradigm to assess the effects of stress hormones in memory specificity and detaildness. I showed that posttraining yohimbine administration

enhanced OiC memory to levels comparable to extensive training. Neither condition was associated with differential dorsal hippocampal neuronal activity during the encoding/early consolidation phase, but both conditions were linked with suppressed CA3 activity during the retention test, without altering memory engram reactivation. Exploratory iDISCO+ analysis revealed similar brain-wide neuronal activity patterns in the yohimbine and extensive training groups, with reduced activity in the (ventral) hippocampal formation (extending beyond the hippocampus) during encoding/early consolidation. Finally, yohimbine administration elevated *Creb* mRNA levels in the dorsal blade of the dentate gyrus and CA1 following OiC training, and increased miR-134 levels in the CA3. These findings provide novel insights into the effects of posttraining noradrenergic activation on hippocampal subregions, integrating behavioral, cellular, molecular, and brain-wide activation perspectives.

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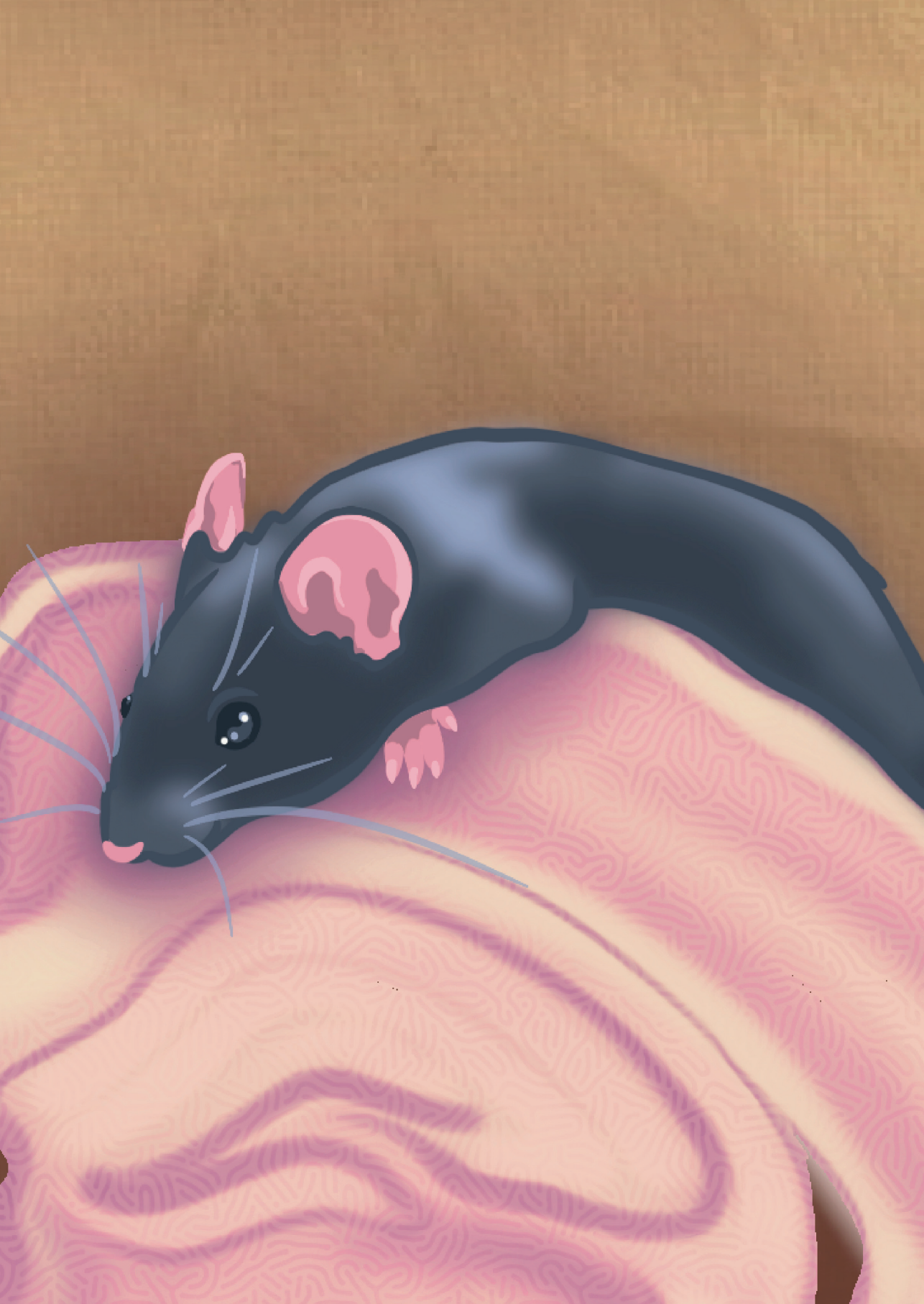

Appendix

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Samenvatting

Stressvolle of emotionele gebeurtenissen worden vaak goed onthouden. Veel onderzoek laat zien dat de versterking van deze herinneringen het resultaat is van een samenspel tussen twee stresshormonen: noradrenaline (NA) en cortisol (bij muizen corticosteron). Deze stresshormonen zorgen er niet alleen voor dat wij adequaat kunnen reageren op direct gevaar, maar ook op toekomstige uitdagingen, bijvoorbeeld door blijvende veranderingen in gedrag, leren en geheugen te veroorzaken. Zowel NA als cortisol zorgen ervoor dat herinneringen worden versterkt, maar beide hormonen hebben ook een effect op de kwaliteit van deze herinneringen. Hoe dit precies gebeurt is nog grotendeels onduidelijk, en studies in mensen hebben tegenstrijdige resultaten opgeleverd. In sommige studies leidde stress tot een verhoogde precisie en gedetailleerdheid, terwijl andere studies juist een generalisatie van de herinnering rapporteerden, en een onterecht gevoel van betrouwbaarheid. Een verkeerde verwerking van stressvolle gebeurtenissen speelt een belangrijke rol bij het ontstaan van diverse psychiatrische stoornissen zoals posttraumatische stressstoornis en fobieën. Door te begrijpen hoe stress en emotionele opwinding zowel de sterkte als kwaliteit van herinneringen kan veranderen, hopen we beter inzicht te verkrijgen in hoe ons geheugen functioneert onder stressvolle omstandigheden, en hoe dit een rol kan spelen bij het ontstaan van stress-gerelateerde psychiatrische stoornissen.

In dit proefschrift beschrijf ik een reeks experimenten met muizen waarin ik onderzocht heb hoe het stresshormoon NA de specificiteit van episodisch-achtige herinneringen beïnvloedt. Uit eerder onderzoek bleek dat een verhoging van NA direct na het aanleren van een taak de specificiteit van herinneringen aan deze taak verhoogt. Mijn doel was om te achterhalen via welke mechanismen NA deze specificiteit beïnvloedt en welke hersenprocessen daarbij betrokken zijn. Daarnaast wilde ik onderzoeken of blootstelling aan stress voorafgaand aan het ophalen van een episodisch-achtige herinnering ook een effect heeft op de specificiteit van die herinnering.

In **hoofdstuk 2** beschrijf ik de dual-event inhibitory avoidance taak voor het onderzoeken van episodisch-achtig geheugen bij muizen. Deze leertaak was oorspronkelijk ontworpen voor gebruik bij ratten. Het doel van mijn onderzoek was om deze leertaak ook geschikt te maken voor muizen en daarna te onderzoeken of stresshormoontoediening een effect heeft op de specificiteit van het geheugen. In de dual-event inhibitory avoidance taak worden muizen tijdens het trainen in twee verschillende boxen gezet, die ieder bestaan uit een licht en donker compartiment.

In de eerste box (de non-shock box) kunnen de dieren deze omgeving verkennen en gebeurt er verder niets, maar in de tweede box (de shock box) krijgen ze in het donkere compartiment een korte elektrische schok. Tijdens een geheugentest de volgende dag, worden de muizen in de non-shock box, shock box en een nieuwe box gezet die ze nog nooit eerder hebben gezien. De tijd die verstrijkt voordat de muizen van het lichte naar het donkere compartiment (waar normaliter de voorkeur voor bestaat) lopen wordt gebruikt als maat voor hun geheugen. Muizen bleken goed in staat een precieze herinnering voor deze taak te vormen; ze vermeden (vooral) het donkere compartiment van de box waarin ze de schok hadden gekregen. Hiermee konden we bevestigen dat muizen in staat zijn episodisch-achtige herinneringen te vormen in deze opstelling.

Daarna probeerde ik de eerder gevonden effecten van NA en corticosteron op geheugenspecificiteit in ratten te repliceren door yohimbine (een stof die de afgifte van NA in de hersenen stimuleert) en corticosteron na training op deze leertaak toe te dienen. Ik slaagde er echter niet in hun effecten op het geheugen te repliceren. Ik vond maar een zeer zwak geheugenversterkend effect van NA, en geen van corticosteron. Daarnaast hadden beide hormonen geen effect op de episodisch-achtige specificiteit van het geheugen. De injecties die nodig zijn om beide hormonen toe te dienen zijn zeer stressvol voor muizen, en veroorzaakten extra variatie in de resultaten en testvolgorde effecten, waardoor het moeilijk was de invloed van stresshormonen goed te onderzoeken. Daarmee bleek dat deze taak weliswaar geschikt is voor ratten, maar minder robuust bij muizen wanneer stressvolle injecties (na het trainen) nodig zijn.

In **hoofdstuk 3** onderzocht ik of de werking van mitochondriën — de energiefabriekjes van een cel — invloed heeft op geheugenspecificiteit, en of acute stress voorafgaand aan de geheugentest dit effect versterkt. Hiervoor gebruikte ik zowel normale muizen als muizen met een genetische afwijking die hun mitochondriale functie verstoort. Ze hadden een defect in hun *nuclear-encoded NADH dehydrogenase [ubiquinone] iron-sulphur protein 4* (*Ndufs4*) gen. Beide muislijnen werden getraind op de dual-event inhibitory avoidance taak en hun geheugen testten we twee dagen later. Net voorafgaand aan de test, werd de helft van de muizen blootgesteld aan stress, om te bepalen welke invloed acute stress heeft op de specificiteit van de opgehaalde herinneringen. De resultaten toonden aan dat stress in normale muizen zorgt voor een verslechtering van het ophalen van het geheugen, zonder duidelijk effect op de specificiteit ervan. Mitochondriële disfunctie zorgde ook voor een verslechtering van het geheugen. Stress in dieren met mitochondriële disfunctie liet niet een verdere verslechtering

van het geheugen zien maar zorgde er vooral voor dat de dieren minder bewogen in de boxen, mogelijk veroorzaakt door een tekort aan energie. Stress verlaagde bovendien de mitochondriële activiteit in de hippocampus; een gebied in het brein dat essentieel is voor de episodische kwaliteit van herinneringen. Dit gebeurde bij zowel de normale als genetisch-aangepaste muizen. Verder verlaagde stress de neuronale activiteit na de geheugentest in de hippocampus, maar verhoogde die in de prefrontale cortex; een hersengebied dat vooral een rol lijkt te spelen in de opslag en het oproepen van minder-specifieke herinneringen. Dit werd vooral waargenomen in muizen met mitochondriële disfunctie. Dit toont aan dat stress en mitochondriële problemen elkaar versterken en samen de kwaliteit van herinneringen beïnvloeden. Dit kan een mechanisme zijn waarmee mitochondriële afwijkingen een risicofactor vormen voor stress-gerelateerde stoornissen.

Omdat de dual-event hibitory avoidance taak uit hoofdstuk 2 beperkt bruikbaar bleek in muizen, maakte ik in **hoofdstuk 4** gebruik van de object-in-context (OiC)-taak — een alternatieve en meer robuuste taak om episodisch-achtig geheugen te meten bij muizen. Deze taak bestaat net als de dual-event inhibitory avoidance taak uit twee episodes waarin de muizen in verschillende boxen worden gezet, alleen zien ze daar nu telkens een ander object. Tijdens de geheugentest wordt getoetst of de muizen onthouden hebben in welke box ze welk object gezien hebben. Ik onderzocht hoe toediening van yohimbine na training het geheugen voor deze taak beïnvloedt en hoe dit gepaard gaat met een verandering in activiteit van de hippocampus. De resultaten hiervan vergeleek ik met die van een situatie waarin de muizen langer getraind werden op de taak, waardoor hun prestatie op de geheugentest vergelijkbaar was aan die van muizen die yohimbine toegediend hadden gekregen. Ik vond dat yohimbine de specificiteit van het geheugen in de OiC-taak verbeterde, en dat de langere training in eenzelfde verbeterde geheugenspecificiteit resulteerde. Beide condities veroorzaakten ook vergelijkbare effecten in de hippocampus: geen verschillen in activiteit tijdens het leren van de taak, maar minder activiteit in het CA3-gebied van de hippocampus tijdens de geheugentest. Deze resultaten wijzen mogelijk op een afgezwakt proces van 'pattern completion' (patroonherkenning of patroonaanvulling); een cognitief proces waarbij de hersenen onvolledige informatie invullen om een volledig, coherent beeld of begrip te creëren op basis van eerdere ervaringen en opgeslagen kennis. Op moleculair niveau zag ik dat yohimbine toediening leidde tot een verhoging van *Creb*-mRNA in delen van de hippocampus. *Creb* is een eiwit dat een belangrijke rol speelt bij neuronale plasticiteit en geheugenvorming. Verder heb ik ook onderzocht of yohimbine toediening tot veranderingen in activiteit leidde in andere hersengebieden door de breintjes transparant te maken en ze intact in

beeld te brengen met een zogenaamde light-sheet microscoop. Dit was inderdaad het geval en ik zag vooral veranderingen in hersengebieden die deel uitmaken van de hippocampale formatie: de ventrale hippocampus, maar ook de ventrale entorhinale cortex en het parasubiculum. Deze gebieden werken samen met de hippocampus in het vormen van episodisch en specifiek geheugen. Ook deze effecten van yohimbine waren vergelijkbaar aan de effecten van langere training op de taak.

Samenvattend laten mijn resultaten zien dat geheugenspecificiteit wordt beïnvloed door stress, mitochondriële functie en noradrenerge signaalverwerking. Het lijkt dat stress met name tijdens of vlak na een leertaak de specificiteit van het geheugen kan beïnvloeden, terwijl stress vlak voor het oproepen van het geheugen wel zorgt voor een algemene verslechtering van het oproepen van deze herinnering maar geen duidelijk direct effect lijkt te hebben op de specificiteit hiervan. Deze inzichten kunnen bijdragen aan een beter begrip van stress-gerelateerde stoornissen, waarin verstoorde geheugenprocessen een centrale rol spelen. Ze bieden bovendien aanknopingspunten voor behandelingen die de generalisatie van specifieke herinneringen verminderen, zoals lichaamsbeweging of bepaalde medicaties die het geheugen gericht ondersteunen.

Summary

Emotionally arousing or stressful experiences induce strong and lasting memories. A large body of literature shows that this strengthening of memory involves synergistic actions of both norepinephrine (NE) and glucocorticoid hormones (corticosterone in rodents, cortisol in humans (CORT)). These two major stress-hormone systems not only enable an individual to respond acutely to danger or threat, but also prepare the individual for future challenges by inducing long-term behavioral changes, including alterations in learning and memory. The strenghtening of emotional memories is a highly adaptive phenomenon, but memories can be subject to further modifications, affecting memory specificity, detailedness, and contextualization. Understanding the mnemonic modifications produced by stress and emotional arousal might provide important insight into the operational constraints of our episodic, autobiographical memory system under stressful circumstances. Moreover, it might enhance insight into the neurobiological mechanisms underlying stress-related psychopathology, as aberrant memory processing of stressful events can have a maladaptive outcome. Particularly, strong, yet poorly contextually embedded, memories are believed to be a major risk factor for the development of psychopathological conditions such as posttraumatic stress disorder (PTSD) and phobias.

Interestingly, the existing literature indicates that NE and CORT, when administered posttraining, exert a hormone-specific impact on memory quality. Besides their strengthening effects, NE was found to enhance memory specificity whereas CORT was found to induce memory generalization. This thesis presents a series of experiments in mice tailored towards exploring the neurobiological basis of the impact of NE on episodic-like quality of memory. Moreover, I investigated whether stress experienced prior to memory retrieval also modulates memory specificity and whether these effects rely on available energy resources (i.e., interacting with mitochondrial dysfunction).

In **Chapter 2**, I aimed to set up the dual-event inhibitory avoidance task for mice. The dual-event inhibitory avoidance task was originally designed for rats, yet mice have become the dominant species in neuroscientific research due to advances in genome-editing technologies that are more readily available in mice. In this task, mice were trained in two different inhibitory avoidance apparatuses with a brief interval, but only received a footshock upon entering the dark compartment of the second context. During a later retention test, the time taken to enter the dark compartments of the Shock box, Non-Shock box, and a Novel box the animal

has never been exposed to, was measured. After optimizing training and testing parameters, I found that mice were capable of acquiring episodic-like memories in this task, displaying specifically longer retention latencies in the Shock box than in the other two boxes.

My next aim was to replicate the previously reported effects of NE and CORT on episodic-like memory specificity and strength. I administered yohimbine (a noradrenergic stimulant) and CORT immediately after training and assessed later memory retention. However, I did not observe the modulatory effects of yohimbine or CORT administration on episodic-like specificity of memory as previously reported for rats. Starting from the introduction of posttraining intraperitoneal injections, I observed testing order effects and substantially increased variability both within experimental groups and across experiments, precluding a thorough investigation of stress hormone effects on memory specificity. I further only found a weak memory-strengthening effect of yohimbine and was unsuccessful in replicating the memory-strengthening effect of CORT. Thus, these findings showed that whereas the dual-event inhibitory avoidance task is effective for studying stress hormone effects on the consolidation of memory specificity in rats, it is less suitable for such experiments in mice.

In **Chapter 3**, I aimed to examine whether mitochondrial activity regulates episodic-like specificity of memory and whether acute stress exposure prior to retention testing interacts with this in influencing episodic-like specificity of memory. I additionally tested whether stress (hormone) effects on the specificity of the recalled memories also involved changes in mitochondrial function. To investigate this, I used a mouse line with a genetic deficiency in the *nuclear-encoded NADH dehydrogenase [ubiquinone] iron-sulphur protein 4* (*Ndufs4*), as well as wildtype control mice, which were trained on the dual-event inhibitory avoidance task and memory specificity (and memory strength) was tested 48 h later. Thirty minutes prior to the retention test, half of the *Ndufs4*$^{GT/GT}$ and wildtype mice was exposed to a forced swim stress to examine whether this would affect memory specificity of the recalled memory. After the retention test, I examined whether the stress exposure had reduced mitochondrial activity within both the hippocampus and prefrontal cortex, important memory regions. I also investigated the pattern of neuronal activity in both brain regions through immunofluorescence for the neuronal activity marker c-Fos in both excitatory and inhibitory neurons. Behavioral results showed that mitochondrial dysfunction impaired episodic-like specificity of memory. Stress exposure in control mice selectively shortened latencies in the shock context, reflecting impaired memory recall, whereas stress exposure in

Ndufs4$^{GT/GT}$ mice generally increased latencies in both training contexts, but not the novel context.

Stress and impaired mitochondrial activity were both related to lower complex I mitochondrial activity in the hippocampus compared to controls conditions. Stress exposure in *Ndufs4*$^{GT/GT}$ mice significantly increased mitochondrial complex IV activity in the PFC. Further, stress exposure was found to reduce c-Fos activity in the hippocampus, but to increase c-Fos activity within the PFC. Both of these effects were more pronounced in *Ndufs4*$^{GT/GT}$ mice. Our findings are consistent with prior literature demonstrating that mitochondrial dysfunction has a substantial impact on memory performance. Reduced mitochondrial function within the hippocampus has previously been associated with impairments in hippocampus-dependent memory. Moreover, the results revealed important insights into how stress exposure prior to retention testing influences hippocampal mitochondrial activity in the regulation of episodic-like memory, and showed that stress further exacerbates the suboptimal mitochondrial functioning observed in *Ndufs4*$^{GT/GT}$ mice. Our findings also provide new perspectives on suboptimal mitochondrial function, which is increasingly recognized as a key contributor to stress-related psychopathologies.

We concluded in Chapter 2 that the object-in-context (OiC) task is better suited for investigating the effects of systemic yohimbine administration after training on episodic-like memory specificity and its underlying neural correlates in mice. In **Chapter 4,** we used the OiC task to investigate how posttraining systemic administration of yohimbine affects hippocampal processes in supporting the memory. Specifically, we aimed to determine whether yphimbine administration altered hippocampal activity during the retention test and whether it affected the reactivation of neurons that were activated during/shortly after the training (i.e., the engram-cells). We have utilized the FosTRAP2xtdTomato transgenic mouse line which enabled me to tag the activated neurons during training/early consolidation (by expressing tdTomato), and overlay these with neurons activated during the later retention test, assessed by immunohistochemistry. My main region of interest was the hippocampus since it has been extensively implicated in pattern separation and episodic-like specificity of memory. Furthermore, as previous findings had indicated that NE administration into the basolateral amygdala (BLA) enhanced memory specificity on the dual-event inhibitory avoidance task by stimulating a miR-134-regulated consolidation process with the dorsal dentate gyrus (DG), I additionally tested for miR-134 levels in the hippocampus posttraining, as well as the expression of its target genes. I also used iDISCO+ and whole-brain neuronal activity analysis to determine whether posttraining yohimbine administration

produced changes in neuronal activity beyond the hippocampus and to compare these effects with those observed in the extensive-training group, as control group with similarly improved memory specificity.

I confirmed that posttraining administration of yohimbine enhanced OiC memory to a similar degree as extensive training. Both experimental groups did not display any differential neuronal activity in the hippocampus during the encoding/early consolidation period, but displayed reduced neuronal activity in the hippocampal CA3 during the subsequent retention test, without changing the reactivation of training-activated neurons. These results might reflect a suppression of pattern completion processes necessary for expressing memory specificity in the yohimbine and extensive training groups. I also found that posttraining yohimbine treatment following the OiC task was associated with increased *Creb* mRNA levels in the dorsal DG and CA1 regions. Additionally, in the CA3 region, yohimbine treatment induced elevated levels of miR-134. Exploratory iDISCO+ results showed that both posttraining yohimbine administration and extensive training produced similar brain-wide neuronal activity patterns during the encoding/early consolidation phase compared to saline. Both groups showed a moderately strong reduction in the number of tdTomato+ cells in several subregions of the hippocampal formation, specifically the ventral CA1 and CA3, as well as the ventral entorhinal cortex and parasubiculum. These results provide new insights into the involvement of additional brain regions and indicate that posttraining noradrenergic activation produces behavioral outcomes and whole-brain activation patterns comparable to those observed with extensive training, suggesting shared neural mechanisms.

Concluding, the findings presented in this thesis put forward several advancements in our understanding of how stress exposure and stress hormone administration influence episodic-like memory specificity as well as methods to investigate this. We have trialed dual-event inhibitory avoidance task to be only compatible with mice provided no posttraining i.p. injections are implemented, as demonstrated by its successful application alongside a stress manipulation. Then, our findings with *Ndufs4*$^{GT/GT}$ mice indicated that stress exposure intensifies the shortcomings associated with mitochondrial dysfunction. This may carry clinical relevance for human pathologies implicating compromised mitochondrial function, as for example observed in major depressive disorder. Understanding how memory specificity and generalization is affected by these factors could inform treatments, which could for example exploit exercise as means to reduce cortisol signaling or fluoxetine to preserve hippocampal involvement during memory retrieval, reducing overgeneralization risks. Lastly, our findings demonstrated that both posttraining

yohimbine administration and extensive training enhance episodic-like memory specificity in the OiC task, potentially by suppressing pattern completion. However, further studies are needed to validate this hypothesis.

Research data management

This thesis research has been carried out under the institute research data management policy of the Donders Institute for Brain, Cognition and Behavior. This research followed the applicable laws and ethical guidelines. Research Data Management was performed according to the FAIR principles. The information below details how this was achieved.

Ethical approval

This thesis is based on the results of animal studies, which were conducted in accordance with the European, Dutch and local regulations on the basis of the General ethic approval, the local Animal Welfare Body approved the protocols 2018-0014-034, 2018-0014-013, 2018-0014-011, 2018-0014-012, 2018-0014-005 and 2018-0014-028.

Accessibility

The table below details where the data and research documentation for each chapter can be found on the Radboud Data Repository (RDR). Chapter 2 archived as a Data Sharing Collection as open access and Chapter 3 and 4 data achieved as Data Acquisition Collection as closed access, both remain available for at least 10 years after termination of the studies.

Chapter	Subject to privacy	Way of Anonymization	Storage
2	No	N.A.	All data files are shared under open access conditions in the in Radboud Data Repository with regular back-up (di.dcmn. DSC_iadt_t0000867a_876)
3 and 4	No	N.A.	All data files are shared under closed access conditions in the at Radboud Data Repository with regular back-up (collection indentifier:di. dcmn.DAC_4070_695)

PhD Portfolio

Professional courses and workshops

Subject	Course provider	Year	EC
Laboratory Animal Science	Radboudumc	2017	3.0
Donders Graduate School Introduction	Radboud University	2018	1/3
Neurobiology (NWI-BB034B)	Radboud University	2018	6.0
Achieving your Goals	Radboud University	2018	1.5
Statistics for PhD using SPSS	Radboud University	2018	2.0
Image Analysis with FIJI	RIMLS	2018	1.0
Scientific Integrity for PhD candidates	Radboud University	2020	1/3
Scientific Writing	Radboud University	2021	3.0
Grant writing and Presenting for funding committees	Radboud University	2021	1.0
Career Guidance for International PhD's	Radboud University	2021	1.0
Donders Graduate School Day	Radboud University	2018 2023	1/3
Lectures and others			12
			31.5

Symposia and Congresses

Subject	Location	Year
Donders Discussions	Nijmegen, the Netherlands	2018,2019,2021
Donders Session/Lectures	Nijmegen, the Netherlands	2018,2019,2022
Dutch neuroscience meeting	Lunteren, the Netherlands	2018,2019
FENS Forum of Neuroscience	Online forum	2020
Dutch neuroscience meeting	Online	2021
Stress-NL meeting	Amsterdam	2019
Stress-NL meeting	Utrecht	2022

List of publications

Bahtiyar, S., Karaca, K. G., Henckens, M. J., & Roozendaal, B. (2020). Norepinephrine and glucocorticoid effects on the brain mechanisms underlying memory accuracy and generalization. Molecular and Cellular Neuroscience, 108, 103537.

Bahtiyar, S., Karaca, K. G., Henckens, M. J., & Roozendaal, B. (2025). Exploring stress hormone effects on memory specificity and strength in mice using the dual-event inhibitory avoidance task. Learning & Memory, 32(1), a053956.

Gulmez Karaca, K., **Bahtiyar, S.**, van Dongen, L., Wolf, O. T., Hermans, E. J., Henckens, M. J., & Roozendaal, B. (2025). Posttraining noradrenergic stimulation maintains hippocampal engram reactivation and episodic-like specificity of remote memory. Neuropsychopharmacology, 1-10.

About the author

Sevgi Bahtiyar was born on 5th of December 1990 in Istanbul, Turkey. In 2009, she started her bachelor study at Koc University, Istanbul with a major in psychology. She performed her research project under the supervision of Dr. Tilbe Goksun. It was during this time that she opened the new door to brain and cognition research. In 2015, she was admitted to a 2-year research Master program at Radboud University, Nijmegen, the Netherlands. She performed her research project under the supervision of Prof. Dr. Benno Roozendaal. In her last year, she applied for the Donders Top Talent, a highly competitive personal grant awarded by the Donders Graduate School to conduct a self-written PhD project at the Donders Institute.

In 2017, she earned her Master of Science degree and received the prestigious Top Talent grant, which provided her the opportunity to remain at the Donders Institute to continue her research activities under the mentorship of Prof. Benno Roozendaal and Dr. Marloes Henckens. She carried out her PhD project on the stress hormone effects in regulating episodic-like memory strength and specificity. During her PhD training, she supervised eight bachelor and master students during their internships. She also actively played a role as mentor for internship students in the Cognitive Neuroscience department of the Radboudumc for 2.5 years. Most of the scientific studies she performed during her PhD are described in this thesis.

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Donders Graduate School for Cognitive Neuroscience

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 de 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.

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