EMOTIONAL MODULATION OF MEMORY

MECHANISMS UNDERLYING STRENGTH AND ACCURACY OF MEMORY

Erika Atucha Treviño

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Emotional modulation of memory. Mechanisms underlying strength and accuracy of memory

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CHAPTER 01

GENERAL INTRODUCTION

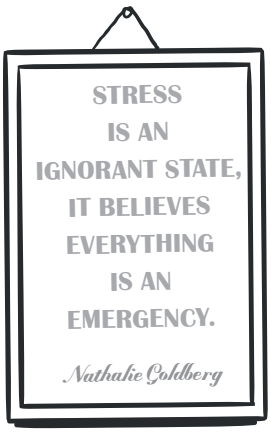
Emotional arousal and stress - induced effects on the brain influence our memory. On the one hand, emotionally arousing expe-riences are well remembered, which is a highly adaptive mechanism that helps us remembering significant life events (MGaugh, 2000; Roozendaal, 2002; McEwen, 1998). On the other hand, the overly good memory might lead to undesirable long - term consequences, like post - traumatic stress disorder (PTSD) and anxiety (Tsoory *et al.*, 2008; Parsons and Ressler, 2013). Such pathologies are currently treated with drugs that result in many adverse side effects. The two parts of this thesis investigate modulation of memory consolidation by manipulating stress hormones and neurotransmitter systems. The first part of this thesis will focus on experiments designed to investigate which aspects of an experienced event are better remembered as well as the underlying mechanisms. The reported experiments investigate how the consolidation of an event is enhanced, how it affects accuracy of memory,

and which molecular mechanisms underlie such enhancement. The second part of this thesis characterizes and tests two potential novel glucocorticoid drugs on memory. Glucocorticoids are commonly prescribed as anti - inflammatory / immunosuppressant drugs that present many side effects, including psychopathology (Judd *et al.*, 2014). Since, stress - related disorders are a major concern in our society, there is an increasing need for drugs that have higher specificity and less adverse effects. Using our knowledge of molecular mechanisms influencing memory, we might be able to leverage them as targets for therapeutic interventions. The following introductory sections define the key concepts discussed in this thesis and briefly review the related literature. First, stress and arousal will be introduced and the effects they exert on the body and the brain. Second, main memory consolidation related concepts will be introduced at the molecular and systems level. Finally, stress and arousal - induced effects on memory will be presented.

STRESS AND AROUSAL

Stress and Arousal as a Concept

Termed less than a century ago, stress has become commonly used and is recognized as being a burden on society (Selye, 1936). It has earned a negative connotation in modern society because we relate it to pressure, concerns, anxiety or tension (McEwen, 1998). Nevertheless, stress covers a broader concept which is not only limited to our psychological status of uncontrollability in a given situation, but also deals with 'threats' that result in physiological alterations that bring our body away from the equilibrium (Cannon, 1926; Korte *et al.*, 2005). Moreover, novelty and surprise components of such situations are associated with norepinephrine release which results in hyper vigilance and an alertness state (Berridge and Foote, 1991). In behavioral neuroscience it is often difficult to distinguish and define conditions for stress or arousal. Therefore, often in laboratory conditions the temporal physiological response of our body and brain is considered in order to cluster and categorize stress-induced phenotypes. Commonly, in rodents exposed to a given behavioral paradigm stress hormone levels are quantified as a measure for stress (Long and Ulrich, 1956; Lachuer *et al.*, 1991; Weinstock *et al.*, 1998).



Originally the term stress derives from *stringere* in Latin, a word that had been used in physics to refer to the internal distribution of a force exerted on a material body, resulting in strain. Selye defined what we call today stress as the non-specific response of the body to any demand (Selye, 1936). Later, he regarded it as an

alarm process that warns about disrupted homeostasis and helps to restore it. He named this process 'stress' (Selye, 1955). Today's definition of stress is restricted to the most extreme cases and, therefore, it has such a negative connotation. For example, Koolhaas and other experts mention that the term 'stress' should be restricted to conditions where an environmental demand exceeds the natural regulatory capacity of an organism (Koolhaas *et al.*, 2011).

Arousal is related to the significance of an event which activates the noradrenergic system. The release of norepinephrine from nerve endings acts on the heart, blood vessels, and respiratory centres inducing a global 'state-change' function. The locus coeruelus (LC), the main source of norepinephrine in the brain, projects extensively to the brain (cerebral cortex, hippocampus, thalamus, midbrain, brain stem, cerebellum and spinal cord) which gives rise to a global network (Aston-Jones *et al.*, 1984). LC exhibits regional and laminar specificity in its efferent projections (Morrison *et al.*, 1982), especially brain areas that are associated with attentional processing receive a dense noradrenergic innervation (Morrison and Foote, 1986).

Stress and arousal alter the body and brain state at various levels and generate a response. In 1926, Walter Cannon used the term *homeostasis* to refer to external factors that disrupt the equilibrium. Homeostasis conceptualized the physiology of stress as having two components: the general adaptation syndrome (GAS) initially is beneficial, whereas severe and prolonged stress may lead to a stage of exhaustion, in which the acquired adaptation is lost (Selye, 1956; Cannon, 1929). Maintenance of homeostasis in the presence of challenges requires adaptive responses, involving changes in the central nervous and neuroendocrine systems (Joëls and Baram, 2009; Ulrich and Herman, 2009). The adaptive processes that underlie the stress response have been termed *allostasis*. When this response becomes inappropriate, it leads to an excessive cost of adaptation, termed the *allostatic load* (McEwen, 2003).

This thesis will study effects of an aversive task on memory consolidation by manipulating the main hormones induced after arousal and stress. Therefore, in the following section the anatomy and physiology of the arousal and stress response will be described.

Anatomy and Physiology of the Stress Response

A stressful situation induces physiological changes in our body, such as an increase in heart rate, blood pressure, sweating and dilates pupils. Within the brain, a distributed neural network determines what is threatening and regulates the physiological and behavioural responses (McEwen, 2007). There are two major stress response systems: the autonomic nervous system and the hypothalamo-pituitary-adrenocortical (HPA) axis. Activation of the autonomic nervous system culminates in the release of catecholamines from the adrenal medulla, whereas activation of the HPA axis results in the release of glucocorticoids from the adrenal cortex. Released hormones promote the organism's ability to cope with stress by acting on target systems in the periphery and in the brain.

Activation of the autonomic nervous system results in the release of catecholamines, such as epinephrine and norepinephrine from the adrenal medulla and presynaptic nerve terminals (Smith and Vale, 2006; Ulrich-Lai and Herman, 2009). Catecholamines enable the 'flight or fight' response by inducing peripheral effects, i.e., an increase in heartrate, energy metabolism, blood pressure and respiration, and indirectly by effects on the brain. Glucocorticoids can cross the blood-brain barrier to exert their effect on the brain while peripherally released epinephrine is a polar substance that does not readily cross the blood-brain barrier (Weil-Malherbe *et al.*, 1959). However, epinephrine modulates brain function through activation of β-adrenergic receptors on ascending vagal nerve terminating in the nucleus of the solitary tract (NTS) (Introini-Collison *et al.*, 1992; Schreurs *et al.*,

1986; Williams and McGaugh, 1993; Clayton and Williams *et al.*, 2000). Thus, noradrenergic actions influence brain function via adrenergic activation of NTS.

From this nucleus, noradrenergic neurons project to the basolateral complex of the amygdala (BLA) and LC. The LC is the main. After the onset of a stressful event epinephrine will be released into the blood stream whereas in the brain the LC will be the main source of noradrenergic input to the BLA (Fallon, 1992). In the brain norepinephrine release as well as epinephrine-induced peripheral β-adrenoceptor activation in the vagal afferents will project to the NTS in the brainstem. Activation of the HPA-axis triggers a cascade of events that induce the release of glucocorticoids (cortisol in human and corticosterone in rats) from the adrenal glands (Miller and O'Callaghan, 2002).

First, corticotrophin-releasing factor (CRF) is released by the paraventricular nucleus (PVN) into the portal system. CRF induces the release of adrenocorticotropin hormone (ACTH) from the adrenal pituitary gland and subsequently stimulates the release of glucocorticoids from the adrenal cortex into the bloodstream (Axelrod and Reisine, 1984). In the periphery, glucocorticoids exert immunosuppressive actions and increase blood glucose levels which affect metabolic processes (McGaugh *et al.*, 1996). Glucocorticoid hormones are highly lipophilic and, thus, readily enter the brain (McEwen, 1979) where they bind directly to adrenal steroid receptors: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) (Reul and de Kloet, 1985).

MRs have a high affinity for the natural steroids corticosterone and aldosterone and are almost saturated during basal corticosterone levels whereas GRs become occupied by higher levels of corticosterone and cortisol (Reul and de Kloet, 1985).

LEARNING AND MEMORY

History of Memory Research

Learning is the acquisition of new information or knowledge, whereas *memory* is the retention of learned information (Crowder, 1976). Newly acquired knowledge is first encoded and initially stored in a temporary and fragile trace and over time becomes stabilized (Dudai, 2004). The notion that new memories are not immediately hardwired was proposed more than a century ago by Müller and Pilzecker (1900) (Lechner *et al.*, 1999), after a collection of experimental findings on newly acquired memories. These experiments based on sequences of non-related syllables (Ebbinghaus, 1885) showed that newly learned memories were easier to disrupt than older memories. It was thought that memories would follow Ribot's law of regression; hence the initial fragile memory traces would be stabilized over time (Lechner *et al.*, 1999). As a consequence of those experiments, they proposed the 'perseveration-consolidation hypothesis' of memory in 1900.

Late nineteenth century studies supported a localizationist view where functions of the nervous system were thought to be localized. This view was supported by Broca (1961) Fritsch and Hitzig (1870), Ferrier (1876), and others (Millet, 1998; Squire and Wixted, 2011). In the early twentieth century, several experiments led to the conclusion that memory was distributed throughout the cortex and that the contribution of memory was equivalent across regions (Lashley, 1929). Hebb (1949) and Hunter (1930) built upon this idea by adding a more modern point of view summarized as, 'memory storage is equally distributed, and in addition different areas store different features of the whole'. The study of patient H.M. revolutionized prior knowledge of memory consolidation. In 1957, Brenda Milner reported the effects of bilateral medial temporal lobe (including large portions

of the hippocampus, amygdala and surrounding cortex) resection, performed in order to relieve epilepsy, on memory of patient H.M. (Scoville and Milner 1957; Squire and Wixted, 2011). After the surgery, H.M. was diagnosed with partial retrograde amnesia since he could not remember autobiographical events acquired just before his surgery. Although memory for events further back in the past was unaffected, i.e., semantic memories, as well as procedural memories. On the other hand, he had severe anterograde amnesia, since he could not acquire new memories of people, events and places i.e., episodic memory. These findings and the multiple subsequent studies carried on led to the notion that memory is a distinct function separated from cognitive and perceptual abilities as well as the notion that there are different and anatomically distinct memory systems (Squire and Wixted, 2011).

Memories for events and places are called 'declarative memories' which can be divided into two different types: episodic and semantic memory, based on processing modes (Cohen and Squire, 1980). Episodic memory is the memory for autobiographical events; a record of a person's experience that holds temporally dated information and spatio-temporal relations (Tulving *et al.*, 1983). In other words, episodic memory allows an agent to imagine traveling back in time (Tulving, 2002). Semantic memory (Squire and Zola, 1998) refers to a structured record of facts, concepts and skills that have been acquired. Episodic memories can become semantic memories over time. As an example, we all know that Amsterdam is the capital of The Netherlands (semantic memory), but we have probably forgotten in which situation we acquired that particular information (episodic memory).

Memory Consolidation

Memory consolidation is the process by which initially labile memories become

more stable over time (McGaugh, 2000). Memory consolidation can be divided into two phases from a molecular perspective: short-term memory (protein synthesis independent) and long-term memory (protein synthesis dependent) (Kandel, 2004; McGaugh, 2000). Short-term memory is characterized by the capacity of holding a limited amount of information in mind in a very accessible state (Cowan, 2008). Moreover, short-term memory does not require protein synthesis (Schwartz *et al.*, 1971) but rather second messenger-mediated covalent modifications of previously synthesized proteins (Kandel and Schwartz, 1982). These posttranslational modifications modulate membrane properties of nerve cells and their synaptic connections (Kandel and Schwartz, 1982; Byrne *et al.*, 1987; Tweedie-Cullen *et al.*, 2009).

On the other hand, long-term memory consolidation requires mRNA and de novo protein synthesis that requires several hours or even days (Emptage and Carew, 1993; Izquierdo and Medina, 1998). It has been shown that long-lasting forms of behavioral and synaptic plasticity require protein synthesis (Davis and Squire, 1984; Bailey *et al.*, 1996; Kang and Schuman, 1996; Mayford *et al.*, 1996; Nguyen and Kandel, 1996; Schuman, 1999).

In general, long-term memory consolidation requires two main stages. First, transcriptional changes induce protein synthesis critical for establishing long-lasting modifications, possibly in order to replace degraded proteins as well as to increase levels of present proteins, or express novel proteins (Steward and Schuman, 2001). These changes induce structural modifications at the local network level (Squire and Alvarez, 1995; Kandel, 2004). Second, long-term memory consolidation requires systems-level processes that involve multiple brain regions (Nadel and Moscovitch, 1997; Frankland and Bontempi, 2005; Winocur *et al.*, 2010; Sutherland and Lehman, 2011). ***(See systems consolidation theories section).***

Box 1: microRNAs

MicroRNAs (miRNAs) are small, non-coding RNAs and about 22 nucleotides long. Some of the miRNA genes are expressed under the control of their own promoter, others are arranged in clusters and may be co-regulated (Ambros, 2004). Moreover, animal miRNA genes are often organized in clusters on the genome. The biogenesis of miRNAs starts with the transcription from the miRNA genes. The miRNA transcript (pre-miRNA) is cleaved by nuclear RNase III Drosha into precursor miRNA (pre-miRNA). This precursor has an approximate length of 70 nucleotides and contains an imperfect stem-loop/hairpin structure and a 2-nt 3' overhang.

The pre-miRNA can be exported by exportin (a transporter protein that recognizes the 2-nt 3' overhang) to the cytoplasm via a nuclear pore. In the cytoplasm pre-miRNA will be further processed by Dicer into mature miRNA (imperfect dsRNA duplex). The duplex is unwound by a helicase and the single strand mature miRNA is incorporated into the RNA-induced silencing complex (RISC) with Argonaute proteins (proteins that can bind to small non-coding RNAs, including miRNAs) and coordinate downstream gene-silencing by interacting with other protein factors.

The miRNA will bind to the 3' untranslated regions (UTR) of the target mRNA. Depending on the degree of complementarity between the miRNA and its target mRNA, the target mRNA will be degraded or translationally repressed. When the miRNA is perfectly complementary, binding will induce degradation, whereas non-perfect complementarity leads to inhibition of translation (He and Hannon, 2004; Joshi *et al.*, 2011; Christensen and Schratt, 2009) (See Figure 1). MiRNAs regulate synaptic plasticity including synaptic tagging as well as neuronal homeostasis. Despite efforts on miRNA target prediction (Fiore *et al.*, 2009), little is known about the specific functionality of most miRNAs (Tanzer and Stadler, 2004), including their interplay in regulating specifically their targets. Nevertheless, short non-coding RNA are strikingly well preserved sequences across species (Pasquinelli *et al.*, 2000). One example of miRNA function is the continuous dendritic spine regulation by two antagonistic pathways.

MiRNA-138 enhances RhoA pathway activation and spine shrinkage, whereas miRNA-134 inhibits LIMK1-mediated action polymerization reducing spine growth (Siegel *et al.*, 2011; Christensen and Schratt, 2009). Another downstream target of miRNA-134 is Creb mRNA. Creb is necessary for learning and long-term memory (Silva, 2008). In more detail, Creb is a transcription factor that modulates the transcription of certain genes by binding to cAMP response elements (Montminy and Bilezikijan, 1987). These elements are DNA sequences within promoter regions. An increase of calcium or cAMP can trigger the phosphorylation and activation of Creb. The transcription of several genes is regulated by Creb, such as c-fos, BDNF and tyroxine hydroxylase which enhance both short-and long-term memory (Suzuki *et al.*, 2011) (See Figure 2).

Protein synthesis and maintenance of memory

Formation of long-term memory requires protein synthesis and a large variety of molecules to orchestrate this process. Some of these molecular systems are messenger systems as signalling proteins, epigenetic changes, transcription factors and translation modulators. Second messenger systems such as 3'-5'-cyclic adenosine monophosphate (cAMP) have been shown to be involved in associative learning in the sea slug (*Aplysia*) (Kandel and Schwartz, 1982), fruitfly (*Drosophila melanogaster*) (Waddell *et al.*, 2000), honeybee (*Apis mellifera*) (Hammer and Menzel, 1995) as well as in rodents. cAMP induces a cascade that links neurotransmitter and protein synthesis necessary for long-term memory. Activation of cAMP signalling occurs after stimulation of adenylyl cyclases, stimulated by G-proteins after binding of an extracellular ligand (e.g. norepinephrine) (Wang and Storm, 2003). One of the main targets of cAMP is cAMP-dependent protein kinase (PKA) which is necessary for the consolidation of long-term memory (Kandel, 2004). Second messenger systems serve as an amplification system, and protein kinases, ion channels and transcription factors, like cAMP response element binding protein (CREB), have been confirmed to be 'neural substrate' of learning and memory (Kandel, 2005). Many experiments have investigated memory consolidation on hippocampus-dependent memory tasks and have focused on unravelling the underpinnings of memory consolidation in this structure (Bliss and Collingridge, 1993; Axmacher *et al.*, 2008).

Day and Sweatt (2010) proposed that an epigenetic modification, more precisely DNA methylation, might be the key to maintaining memory. DNA methylation is a biochemical process where a methyl group is added to the cytosine DNA nucleotides (Gold *et al.*, 1963). DNA methylation is a very well-known process involved in the development of an embryo which directs cell fate. Genes that are no longer needed during late stages of development

are permanently switched off by DNA methylation. By the time cells reach differentiation, DNA methyl transferase (Dnmt) expression is reduced, which could suggest that DNA methylation pattern in differentiated cells is stable. However, neurons in the mature mammalian brain express Dnmts (Goto *et al.*, 1994; Feng *et al.*, 2005) and these enzymes have been shown to be required in early stages of memory consolidation (Miller and Sweatt, 2007). Furthermore, DNA methylation might also be critical to maintain long-term memory (Miller *et al.*, 2010). In this article they showed that the methylation of a low calcium affinity protein phosphatase (calcineurin) was needed in order to express memory during contextual fear conditioning 30 days after training (Miller *et al.*, 2010). Therefore, they postulated that DNA methylation, might work as a self-perpetuating biochemical reaction critical for maintenance of memory (Day and Sweatt, 2010). DNA methylation-dependent gene transcription control would render the cell independent from external inputs i.e. methylation on specific genes would maintain a certain cellular transcriptional activity, rendering the cell more independent from the environment (Day and Sweatt, 2010; Moore *et al.*, 2013).

An alternative explanation that would orchestrate the initial memory would be synaptic tagging. Since synaptic strength at one set of afferents does not spread to other afferent synapses (Andersen *et al.*, 1977), as long as they are further apart than 50µm (Bonhoeffer *et al.*, 1989; Engert and Bonhoeffer, 1997; Schuman and Madison, 1994), there must be mechanisms to 'tag' and independently control their strength generated by synaptic activity.

The idea of synaptic tagging as a means to independently control the strength generated by synaptic activity was proposed by Frey and Morris (Frey and Morris, 1997). Newly synthetized proteins are stabilized on neurons containing the tag. Rather than nuclear control, tagging could be regulated at the synaptic

sites by local protein translational regulation mediated by synapse-associated polyribosome complexes (SPRCs) (Steward, 1983). Recently discovered small, non-coding RNAs named microRNAs (Lee *et al.*, 1993) are key post-transcriptional regulators of gene expression (Filipowicz *et al.*, 2008) **(See Box 1)**.

Figure 1 ·····/ MicroRNA Biogenesis

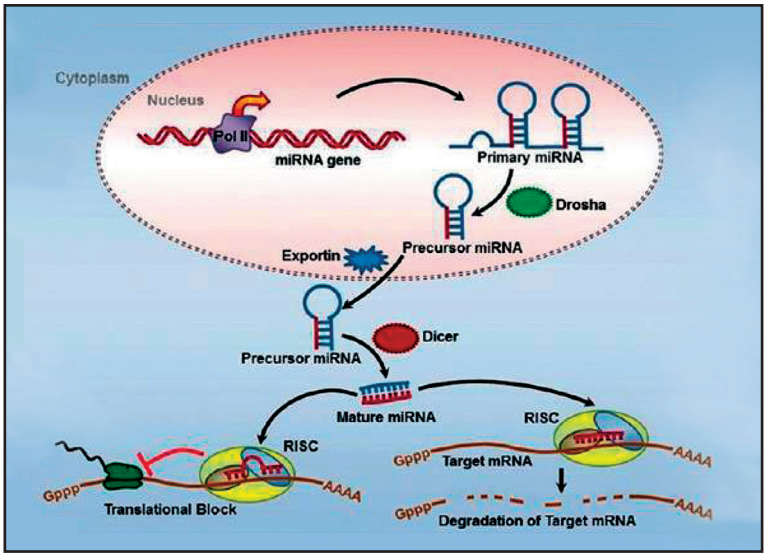


Figure 2

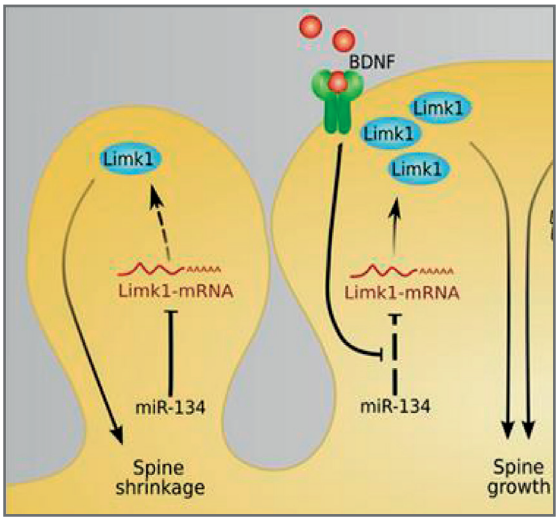
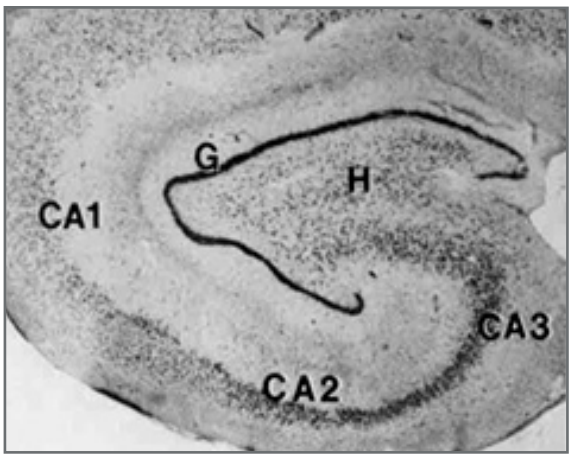


Figure 3



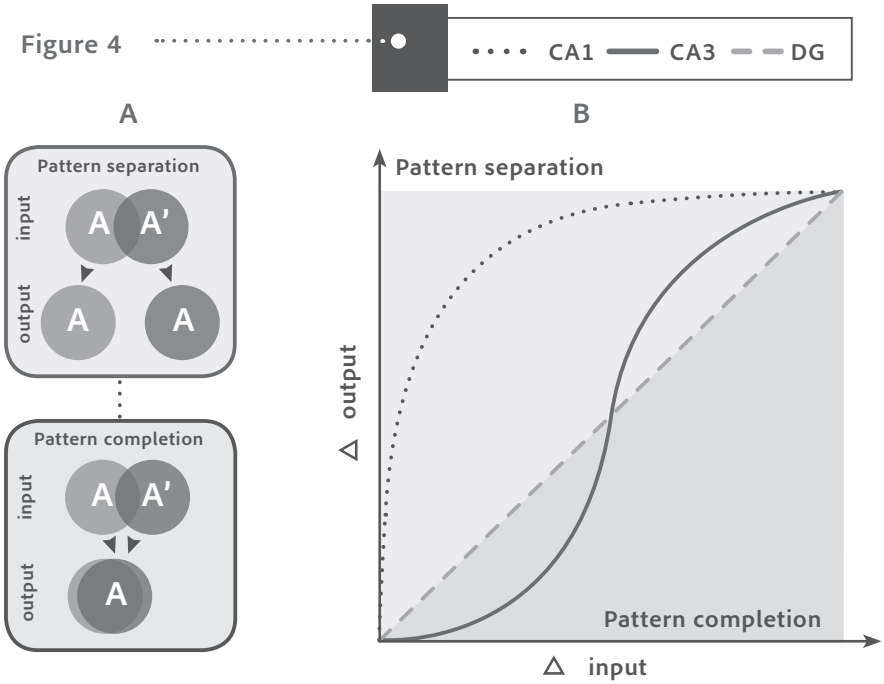
FIGURES:

1. The transcription of primary micro RNA from miRNA genes, is followed by cleavage to precursor mRNA by the nuclear RNase III Drosha. Afterwards the pre-miRNA is exported by exportin to the cytoplasm. Pre-miRNA is further processed by Dicer to the mature miRNA duplex in the cytoplasm. The duplex goes into in the RISC complex with Argonaut ribonucleases and unwinds. The mature miRNA strand (red strand) can cause degradation or blocking of the translation of the target mRNA (Joshi *et al.,* 2011).

2. MiR-134 inhibits the translation of Limk1 mRNA and thereby restricting dendritic spine growth. BDNF relieves the inhibitory effect of miR-134 on Limk1 mRNA translation by a yet unknown mechanism. The enhanced synthesis of Limk1 protein results in dendritic spine growth (Christensen and Schratt, 2009).

3. Subregions of the hippocampus: CA1, CA2, CA3, H (hilus) and G (granular layer) (Sloviter, 1994.)

Figure 4



Box 2: Pattern Separation

Pattern separation refers to a computational process by which a neural circuit decorrelates similar input into a more orthogonal output signal (Marr, 1971). In order words, pattern separation is a computational model that explains how the hippocampus might process discrimination between similar situations. The ability to discriminate between similar experiences is a crucial feature of episodic memory and has been ascribed to the dentate gyrus (DG) (Yassa and Stark, 2011). Computational models based on attractor networks to describe how hippocampal function have been proposed (Marr, 1971) and brought to a deeper understanding by McNaughton and Morris (McNaughton and Morris, 1987; Wills and Cacucci, 2014). In order to summarize theoretically what the brain might need to discriminate, these theories describe two processes: pattern separation and pattern completion. Pattern separation refers to the conversion of similar patterns of neural activity into distinct representations whereas pattern completion refers, ignoring the differences or generating an orthogonal representation.

FIGURE 4:

SCHEMATIC REPRESENTATION OF:

4A. Conceptual representation of pattern separation and pattern completion

4B. Output transfer functions in hippocampal subfields that represent dynamics of pattern separation and completion. The graph represents the non-linear output in relation to increments in input where the dissimilar coloured in light gray and similar in dark gray (adaptated from Yassa and Stark, 2011).

Systems level processes

- ***Hippocampus: hub of memory processes***

The hippocampus is necessary for the encoding, consolidation and retrieval of memory as well as for episodic memory. The hippocampus is part of the medial temporal lobe (MTL). It consists of three major subdivisions: the dentate gyrus (DG), the Ammon's horn (fields CA1, CA2 and CA3) and the subiculum **(Figure 3)** shows representative brain slice of the hippocampus, (Sloviter, 1994).

The DG is the first input region of the hippocampus via the perforant path, which originates mainly in layer II of the medial entorhinal cortex (Binicewicz, 2015). The classical 'trisynaptic circuitry' is a relay of synaptic transmission in the hippocampus which consists of three synapses. First, from the entorhinal cortex to granule cells within the DG, via the performant path. Second, from the DG to pyramidal neurons within the CA3 via mossy fibers, and third from the CA3 to CA1 via Schaffer collaterals (Amaral and Witter, 1989). In addition, there are anatomical connections from neurons from the entorhinal cortex directly to the CA3 pyramidal neurons, bypassing the DG (Steward and Scoville, 1976; Sloviter, 1994), **(see Figure 3)**. Moreover, the DG also receives information from the CA3, through interneuron and hilar mossy cell back-projections (Scharfman, 1991). The DG has a critical role in memory consolidation as well as in discriminating between similar events. The reason why the dorsal DG has such a central role might be attributed to its specific pattern of activity. Granular cells in the DG have a hyperpolarized resting membrane potential and are under high level of inhibitory control from hilar interneurons (Scharfman, 1991; Rolls and Webb, 2012). Immediate-early gene studies have confirmed 'sparse coding' of the DG, since only 1-5% for all granular cells showed to be active at a given time during behavioral activity, compared to a much higher percentage of CA neurons (Ramirez-Amaya *et al.*, 2006; Chawla *et al.*, 2005). Although the DG does not seem to be

very active, a single synapse from DG to CA3 can facilitate sparse coding. It has been shown that a single mossy fiber can induce an action potential in a CA3 neuron (Henze *et al.*, 2002).

- ***How do we discriminate between the similar?***

It has been shown that the hippocampus is necessary for memory accuracy of episodic events (Yassa and Stark, 2011). Without the ability to discriminate between similar information, previous information could be overwritten during the encoding of new information (Kheirbek *et al.*, 2012; Schmidt *et al.*, 2012). There is experimental evidence that the DG of the hippocampus acts as a critical mediator of discrimination due to its anatomical and network properties (Kesner *et al.*, 2000; Leutgeb and Moser, 2007). Neural coding in the DG has been investigated in an open field where gradual changes in the environment correlated with substantial changes in location and firing rate of place fields in granular cells, whereas activity of the CA3 neurons changed in a linear fashion (Leutgeb *et al.*, 2007). Behavioral studies have shown that brain-derived neurotrophic factor (BDNF) protein synthesis is required in the DG for achieving discrimination or pattern separated-like memory (Bekinschtain *et al.*, 2013). This study showed that BDNF in the DG facilitates pattern separated-like memories in a highly ambigious behavioural task whereas in a less demanding task did not have an effect. Therefore the ability to discriminate between similar situations is attributed to the DG.

Systems consolidation theories

Although the hippocampus plays a central role in the consolidation of memories, over time memories appear to be more distributed throughout cortical regions (Frankland *et al.*, 2004; Miller *et al.*, 2010). Neurological studies of patients with region-selective brain damage have driven systems level theories of memory consolidation. The reports from human patients suggest that the hippocampus and

its related structures might be actively engaged in the consolidation process for long periods of time (weeks, months or years) after the acquisition of that memory. The extent of the damage correlated with the degree of retrograde amnesia. H.M. retained memories of events dated back 11 years before the surgery (Corkin, 2002). Studies of patients with MTL-related lesions showed similar levels of retrograde amnesia (Squire and Alvarez, 1995; Squire and Zola, 1998). Moreover, patients with lesions restricted to the CA1 region of the hippocampus showed limited retrograde amnesia (Zola-Morgan *et al.*, 1986), whereas patients with more extensive MTL lesions showed extended retrograde amnesia (Squire *et al.*, 2004). These observations led to further experiments in rodents in order to investigate the involvement of the hippocampus in relation to retrograde amnesia and its molecular, cellular and anatomical underpinnings.

Systems consolidation processes might involve a gradual reorganization of the brain network in order to make memories more resistant to disruption. Experiments in rodents show contradicting findings in relation to the dispensability of the hippocampus in relation to recent and remote memories. This thesis will describe experiments with rodents designed to investigate the systems consolidation process on emotional memories. The recent time point will refer to the early stage of a consolidated memory whereas a remote time point will refer to a later stage of a consolidated memory.

The Standard Consolidation Model (SCM) claims that the hippocampus plays a time-limited role in consolidating new memories into long-term memories. The general consensus is that there is a transfer of information from the hippocampus to the cortex that depends on hippocampus-cortical interactions (Scoville and Milner, 1957; Squire and Bayley, 2007; Jarrard, 1993; Zola-Morgan and Squire, 1990). Experiments in rodents demonstrated that hippocampal lesions at recent time points impair memory, whereas hippocampal lesions at remote time points (about a month in laboratory experiments with rodents) do not have an effect (Kim

and Fanselow, 1992; Anagnostaras *et al.*, 1999). Frankland *et al.* (2004) showed that not only the memory had become hippocampus independent over time, but in fact several cortical regions showed higher levels of activity, in comparison to recent memory. Remote memories are likely to be stored in distributed networks, rendering memory resistant to focal disruption (Lashley, 1958).

The Anterior Cingulated Cortex (ACC) has been pointed as a critical brain region in maintaining long-term memory (Miller *et al.*, 2010) as well as other cortical regions (Frankland *et al.*, 2004). The prefrontal cortex is the anterior part of the frontal lobes and is involved in higher cognitive function. It is highly interconnected with other cortical regions as well as subcortical areas and organized in a topographical manner. The ACC is the frontal part of the cingulate cortex, it plays a role in a variety of functions such as reward anticipation (Kerr *et al.*, 2014), conflict monitoring (Kerns *et al.*, 2004), attention (Peterson *et al.*, 1999), cognition and emotion (Allman *et al.*, 2001). Pharmacological blockade of the ACC with lidocaine at remote time points after contextual fear conditioning impairs memory (Frankland *et al.*, 2004). Accordingly, freezing levels after contextual fear conditioning correlated with hippocampal activity at recent time points but not at remote time points (Restivo *et al.*, 2009), as in similar studies (Bontempi *et al.*, 1999; Frankland *et al.*, 2001; Nadel and Moscovitch, 1997; Zola-Morgan and Squire, 1990).

On the contrary, several studies contradict the SCM. On the one hand, Nadel and Moscovitch (1997) proposed the Multiple Trace Theory (MTT), whereby semantic memory undergoes systems consolidation but episodic and spatial memories continue to depend on the hippocampus. To illustrate this, a couple of studies showed that spatial memory remains permanently hippocampus dependent (Martin *et al.*, 2005; Broadbent and Clark, 2013). Another study demonstrated that H.M. suffered flat-graded retrograde amnesia since remote episodic memories did not become hippo-campus independent (Steinvorth *et al,* 2005).

Several studies fail to show MTT or SCT facts, Sutherland and Lehman named a new theory for memories that are established and maintained in a hippocampus-independent manner, Distributed Reinstatement Theory (DRT) (Sutherland and Lehman, 2011; Sutherland *et al.*, 2010). The main difference with the previous models (MTT and SCT) is that DRT is based on memories that do not depend on the hippocampus whereas MTT and SCT describe initially hippocampus-dependent memories. Flavour map experiments from the Morris lab are an example of episodic memory, which involves the hippocampus. Rats learn to associate a certain flavour to a specific location over several sessions of training. The memory of that map of flavours after extended training undergoes systems consolidation within hours (Tse *et al.*, 2011). In accordance with this experiment, extended training in fear conditioning (over 11 sessions) prevented retrograde amnesia effects on rodents with complete hippocampus damage (Lehman *et al.*, 2009). Furthermore, an interesting report on a taxi driver with bilateral hippocampus lesions showed that passage of time might not be determinant for a memory to become hippocampus independent but rather experiencing the same information over and over (Maguire *et al.*, 2006). In addition, several studies involving spatial contextual memory tasks show spared remote memory with hippocampus partial or complete lesions (Lehman *et al.*, 2007; Wiltgen *et al.*, 2010; Winocur *et al.*, 2007, 2013, Wang *et al.*, 2009; for a review see Wiltgen and Tanaka, 2013). Opposite to what MTT and SCT theories claim, DRT shows that what classically have been considered hippocampus-dependent memories can be hippocampus independent as long as they have been repeatedly experienced

/ STRESS AND MEMORY

EPINEPHRINE AND GLUCOCORTICOIDS

We tend to forget everyday experiences, but remember emotional events for longer periods of time. Adrenal hormones (epinephrine and glucocorticoids), released during and after an emotionally arousing experience, mediate stress effects on memory. Both animal and human studies indicate that memory traces are initially fragile after training and become consolidated over time (McGaugh, 2000). Memory consolidation can be influenced during this critical time window by manipulating stress hormone levels (Roozendaal *et al.*, 1997a; Joëls *et al.*, 2011). An early study showed that posttraining administration of epinephrine enhanced memory consolidation (Gold and van Buskirk, 1975). Stress hormones released during and after an emotional event as well as systemic administration of stress hormones (corticosterone or epinephrine) modulate memory consolidation in a dose and time-dependent manner (de Kloet *et al.*, 1999; McGaugh and Roozendaal, 2002; Joëls and Baram, 2009; de Quervain *et al.*, 1998; Sandi and Pinelo-Nava, 2007; Roozendaal *et al.*, 2008b). Furthermore, glucocorticoid hormones (corticosterone in rodents, cortisol in humans), facilitate the consolidation of memory of emotionally arousing experiences (de Kloet *et al.*, 1999; McGaugh and Roozendaal, 2002; Sandi and Pinelo-Nava, 2007; Abercrombie *et al.*, 2005; Okuda *et al.*, 2004; Schwabe *et al.*, 2011). GR agonists have been shown to enhance memory consolidation when administered into several brain regions such as the BLA, dorsal hippocampus or prefrontal cortex (Roozendaal *et al.*, 1997b; Roozendaal and McGaugh, 1997a; Roozendaal *et al.*, 1999; Roozendaal *et al.*, 2001; Miranda *et al.*, 2008a).

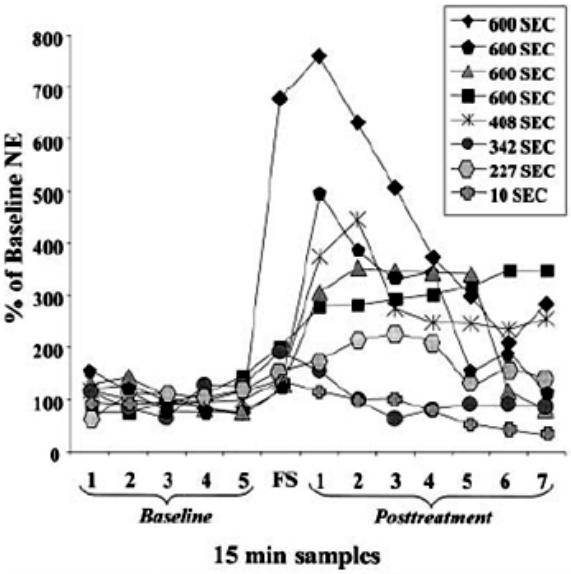
Although studies have focused mainly on negatively motivated experiences, it has been shown that stress hormones modulate memory of emotionally arousing experiences regardless of the valence of the experience in animals (Wichmann *et al.*, 2012) and in humans (Fastenrath *et al.*, 2014). In contrast, glucocorticoids do not enhance memory consolidation of emotionally neutral experiences in animals (Roozendaal and McGaugh, 2011; Okuda *et al.*, 2004; Sandi *et al.*, 1994) or in humans (Buchanan and Lovallo, 2001; Okuda *et al.*, 2004; Kuhlmann and Wolf, 2006).

Adrenergic-Glucocorticoid Interaction

Systemic injections of epinephrine enhance memory consolidation of inhibitory avoidance when administered shortly after training (Gold and van Buskirk, 1975; Gold *et al.*, 1977). Comparable results were described in subsequent experiments in different types of tasks with rodents (Introini-Collison *et al.*, 1991; Costa Miserachs *et al.*, 1994). Emotional arousal such as the delivery of a single footshock in an inhibitory avoidance training induces a significant increase of norepinephrine in the BLA that strongly correlates with a better memory (McIntyre *et al.*, 2002). In addition, blockade of the adrenergic system by administration of a β-adrenoceptor antagonist immediately after training blocks corticosterone-induced memory enhancement (Roozendaal *et al.*, 2006b; Quirarte *et al.*, 1997).

Glucocorticoid effects on memory consolidation depend on an interaction with arousal-induced noradrenergic activation in the brain (Roozendaal *et al*, 1999; 2002; 2006b). As an example, Okuda *et al.* (2004) showed that training-induced emotional arousal was critical in mediating glucocorticoid effects on memory consolidation. In this study they reported that systemic injections of corticosterone do not enhance memory consolidation if rats had extensive habituation prior to the training session. Thus, glucocorticoids enhance memory consolidation of emotionally arousing events rather than neutral events. Extensive research shows that hormones of the adrenal medulla (epinephrine) and adrenal cortex (glucocorticoids), enhance memory consolidation of emotionally arousing events rather than neutral events (Quirarte *et al.*, 1997; Okuda *et al.*, 2004; Roozendaal *et al.*, 2006a). Consistent with these findings, experiments in humans show that arousal is necessary for systemically administered glucocorticoids to modulate memory consolidation (Buchanan and Lovallo, 2001; Kuhlman and Wolf, 2006; van Stegeren *et al.*, 2010).

Figure 5



Norepinephrine in the Brain

FIGURE 5:

5. Effects of inhibitory avoidance training with footshock (FS) on norepinephrine levels in the amygdala. Each line represents the NE levels in relation to baseline per rat. Retention latencies interpreted as memory 24h after micro dialysis measurements (McIntyre *et al.*, 2002).

Noradrenergic projections are largely spread in the forebrain but the amygdala has been shown to be critical in regulating emotional arousal and stress effects on memory (McGaugh *et al.*, 1996). Among the different nuclei of the amygdala, the BLA is crucially involved in modulating stress effects on memory consolidation. On the contrary, the adjacent central nucleus of the amygdala does not seem to play a significant role in modulating memory consolidation (Roozendaal and McGaugh, 1997b; McGaugh, 2000). Posttraining infusions of norepinephrine into the BLA enhance memory consolidation (Roozendaal *et al.*, 1999). It has been shown that posttraining noradrenergic activation of the BLA

enhances memory of inhibitory avoidance training (Liang and McGaugh, 1983; Lalumiere, 2003) as well as object-in-context recognition memory (Barsegyan *et al.*, 2014) and spatial memory (Roozendaal *et al.*, 2009a; Almaguer *et al.*, 2005). Administration of a β-adrenoceptor antagonist immediately after training blocks corticosterone-induced memory enhancement (Quirarte *et al.*, 1997; Roozendaal *et al.*, 1999; Roozendaal *et al.*, 2006a). A micro dialysis study demonstrated that the amount of norepinephrine released in the BLA after inhibitory avoidance training correlates with retention latencies 24h later (McIntyre *et al.*, 2002) **(See Figure 5)**.

The notion that emotional memories can be better remembered than neutral memories has led to the memory modulation theory.

Memory Modulation

Memory modulation theory

Invasive studies in animals as well as non-invasive imaging studies corroborate a role for the amygdala in memory enhancement (Cahill *et al.*, 1994; Ferry *et al.*, 1999; Roozendaal *et al.*, 2009). The amygdala has a central role in modulating norepinephrine as well as peripheral epinephrine effects on memory consolidation (Liang *et al.*, 1986). The BLA is critical to modulate the effects of stress on memory consolidation rather than the central amygdala (McGaugh, 2000; Quirarte *et al.*, 1997; Roozendaal and McGaugh, 1997b; Roozendaal *et al.*, 1999), nevertheless the BLA is not the locus of memory consolidation (Cahill and McGaugh, 1998; Packard *et al.*, 1994). Packard *et al.* (1994), showed that posttraining administration of amphetamine in the hippocampus enhanced consolidation of spatial memory whereas the same drug in the caudate nucleus enhanced cued version of the water maze task. On the other hand, posttraining amphetamine infusions into the

Figure 6 ····· Memory modulation theory by BLA

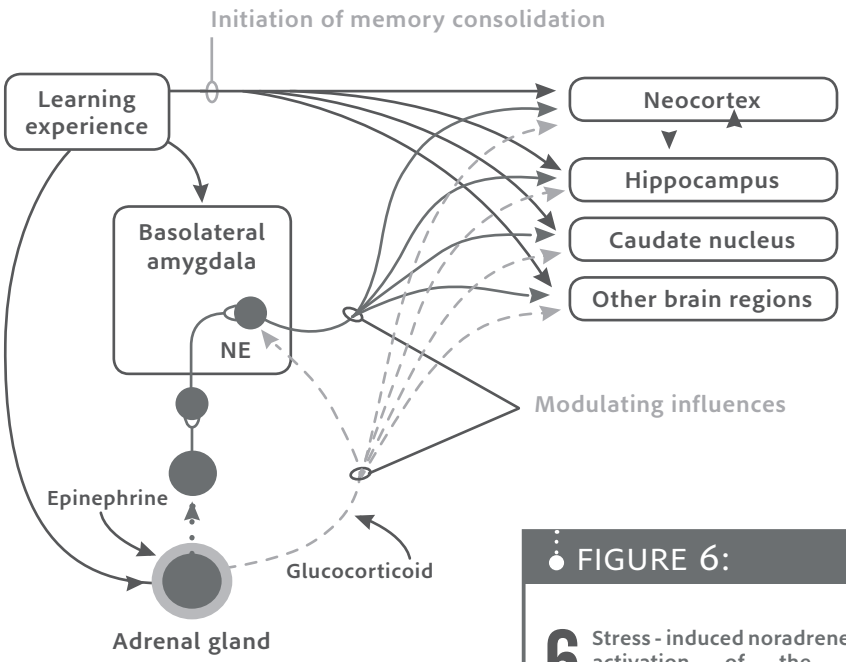


FIGURE 6:

6. Stress - induced noradrenergic activation of the BLA modulates synaptic plasticity in other brain regions altering the consolidation process as a consequence (adapted from McGaugh, 2000).

BLA enhanced consolidation of both versions of water maze task (Packard *et al.*, 1994). In addition, beta-adrenergic modulation induced amygdala and hippocampus responses on emotional memories (Strange and Dolan, 2004). Posttraining norepinephrine infusions specifically into the BLA enhanced memory consolidation (Lalumiere *et al.*, 2003; Roozendaal *et al.*, 2006b; Roozendaal *et al.*, 2008) whereas blockade of training-induced norepinephrine with β-adrenoceptor antagonists impaired memory consolidation (Hatfield and McGaugh, 1999). Stress hormones require an intact BLA in order to exert their actions (Roozendaal and McGaugh 1997b; Roozendaal *et al.*, 1999), since BLA lesions block the enhancing effects of emotional arousal or systemic administration of epinephrine (McGaugh *et al.*, 1996).

Overall, an emotional response to an event leads to a better remembering (Cahill and McGaugh, 1998) as they are better stored into long-term memory (LaBar and Cabeza, 2006). Emotional arousal induced release of norepinephrine and activation of β-adrenoceptors within the BLA is critical to mediate stress hormone modulation of memory consolidation (Roozendaal and McGaugh, 2002). Noradrenergic activation of the BLA will modulate efferent brain regions, including the hippocampus and cortical regions altering synaptic plasticity which will influence the consolidation process (McGaugh *et al.*, 1996; Roozendaal *et al.*, 1996; Setlow *et al.*, 2000; Roozendaal *et al.*, 2001; Ikegaya *et al.*, 1997; McIntyre *et al.*, 2005; McReynolds *et al.*, 2010; Holloway-Erickson *et al.*, 2012) **(See Figure 6)**.

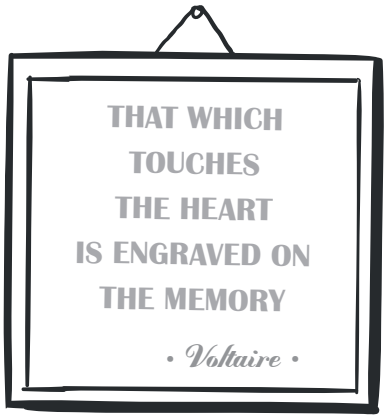
Amygdala - Insular cortex

The insular cortex (IC) is part of the arousal network as it has been shown in human neuroimaging studies that show the involvement of the IC in emotional learning and memory (Hermans *et al.*, 2011). The human insula is situated within the lateral sulcus, between the temporal and the frontal lobes and can be divided in 3 regions based on the cytoarchitecture: agranular, dysgranular and granular (Cecheto and Saper, 1987).

These include (1) the agranular insular cortex (AI) which surrounds the rhinal fissure and lacks agranular layer, (2) the dysgranular insular cortex (DI) which is located just dorsal to the rhinal fissure and contains a diffuse granular layer, and (3) the granular insular cortex (GI), situated just ventral to the secondary somatosensory cortex with a clear granular layer (Cecheto and Saper, 1987). Each subdivision has been associated with the processing of particular sensory information. For example, the agranular region is involved in nociception (Burkey *et al.*, 1999) and autonomic processing (Cecheto and Saper, 1987), the dysgranular region has been associated with gustatory processing (Yamamoto, 1984) and the granular

region has an important role in modulating visceral function (Cecheto and Saper, 1987).

In rats, the IC is mostly agranular and usually subdivided into dorsal and ventral parts. However, most animal studies investigating the role of the IC in emotional arousal effects on learning and memory have been limited to the formation and maintenance of taste memory (Bermudez-Rattoni *et al.*, 1997; Bermudez-Rattoni, 2004; Miranda *et al.*, 2008a; Stehberg *et al.*, 2011). Several studies show a critical involvement of the BLA in conditioned taste aversion (Miranda *et al.*, 2002; Miranda *et al.*, 2003; Miranda et al, 2008a) as well as a functional interaction between the BLA and the IC for taste memory (Bielauska and Roldan, 1996). More specifically, systemically administered corticosterone induced enhancement of taste aversion memory consolidation depends on the IC as well as in the BLA (Miranda *et al.*, 2008b). In addition, recent studies indicate that IC might play a more general role in emotional memory (Sullivan *et al.*, 2013; Hermans *et al.*, 2011).



Amygdala - Hippocampus

The BLA is critical in modulation of memory consolidation. Noradrenergic stimulation of the BLA via β-adrenergic receptors plays a necessary role in the enhanced hippocampal plasticity as a result of an emotional experience (Hatfield and McGaugh, 1999). Posttraining BLA lesions block memory consolidation

enhancing effects of glucocorticoid hormones (Quirarte *et al.*, 1997; Roozendaal and McGaugh, 1997b; Roozendaal *et al.*, 1999; Flavell *et al.*, 2012). A study that selectively blocked BLA and central amygdala regions after fear conditioning, showed that the BLA is critical in modulating memory consolidation and that the expression of fear conditioning through projections towards the central amygdala (Koo *et al.*, 2004). Furthermore, blockade of β-adrenoceptors in the medial septum blocks BLA influenced protein synthesis dependent phase of LTP, in the DG (Bergado *et al.*, 2007). Anatomical studies show that the amygdala sends extensive projections to cortical and subcortical regions (Ono *et al.*, 1985; McDonald and Jackson, 1987; Price, 2003).

Nevertheless, the memory modulation theory does not restrict to monosynaptic connections from the amygdala to efferent regions. Most of the evidence restricts to the hippocampus related plasticity since BLA is functionally (Hübner *et al.*, 2014) and anatomically (Pitkanen *et al.*, 2000) connected to the hippocampus. The BLA and the DG have a strong functional connection (Ikegaya *et al.*, 1996). Noradrenergic stimulation of the BLA is known to modulate protein levels in the DG (McIntyre *et al.*, 2005; Huff *et al.*, 2006). Furthermore, under stress BLA-hippocampus connectivity seems unidirectional; functional connectivity from BLA to hippocampus grows stronger (Ghosh *et al.*, 2013), regardless of the valence (Fastenrath *et al.*, 2014). Modulation of hippocampal long term potentiation by the amygdala seems to be the synaptic mechanisms that link emotions with memory (Abe, 2001).

Accuracy of emotional events

Glucose levels in the amygdala during encoding of emotional material have shown to strongly correlate with later episodic memory (Cahill *et al.*, 1996). Moreover, another study showed that amygdala activity can predict episodic memory recall (LaBar,

1998). It is well known that emotional events are vividly remembered due to specific neural and hormonal mechanisms (Cahill *et al.*, 2004; Phelps *et al.*, 2004; LaBar and Cabeza, 2006; de Quervain *et al.*, 2009; Roozendaal *et al.*, 2009b; Hermans *et al.*, 2014). Emotional events tend to be remembered for long periods of time in a detailed manner due to a modulating effect on the medial temporal lobe (MTL) memory system (Dolcos *et al.*, 2004b; Richardson *et al.*, 2004; Erk *et al.*, 2010; Fastenrath *et al.*, 2014).

The ability to discriminate between similar events is a crucial aspect of episodic memories and therefore the hippocampus. Furthermore, the ability to discriminate between similar experiences has been postulated to be dependent on pattern separation **(see Box 2)**. Pattern separation is a computational model proposed by Marr (Marr, 1971) that explains how similar representations (experiences) are stored in a distinct, non-overlapping fashion (Yassa and Stark, 2011). Different studies in humans as well in rodents have shown that increased norepinephrine levels, released due to an emotional event, lead to a better memory (McGaugh *et al.*, 2004; Strange and Dolan, 2004; van Stegeren, 2008). In accordance, emotional enhancement measured as increased salivary amylase enzyme levels facilitated accurate memories (Segal *et al.*, 2012). In contrast, other studies report a tendency of emotional memories to be remembered in a generalized manner (Loftus, 1979; Payne, 2002; Qin *et al.*, 2012). Although there is no explanatory evidence yet, this thesis will try to elucidate the mechanisms of emotional enhancement of accurate memories.

OUTLINE OF THE THESIS

The chapters in this thesis can be divided in two parts. The first part **(Chapters 2, 3, 4 and 5)** describes a series of studies aimed at understanding the effects of stress and emotional arousal in modulating the consolidation of memory in rodents. Stress hormones, such as adrenal catecholamines and glucocorticoids, are released in

response to emotionally arousing training and known to activate noradrenergic mechanisms within the BLA, which, in turn, regulates information storage processes in other brain regions. This work focuses on different novel aspects of emotion arousal-induced memory enhancement. Although stress-induced release of hormones and neurotransmitters and enhancement of memory has obvious adaptive value in evolutionary terms, persistent or incontrollable changes in stress hormone levels might result in maladaptive cognitive and behavioral changes, and ultimately psychopathology, like anxiety, depression or PTSD. Such pathologies, but also many other medical conditions, are often treated with drugs that target the glucocorticoid system. Although highly effective, the currently available drugs have many adverse side effects.

The second part of this thesis **(Chapters 6 and 7)** focuses on the characterization at the genetic, molecular and cognitive level of two newly developed glucocorticoid drugs with therapeutic potential. Finally **(Chapter 8)** will summarize and discuss the main findings in a general framework.

Part I:

Stress and emotional arousal induce the activation of two major stress hormone systems. On the one hand, stress rapidly activates the sympathetic nervous system, which induces the release of catecholamines such as epinephrine and norepinephrine from the adrenal medulla and sympathetic nerve endings. On the other hand, stress induces a more delayed activation of the HPA axis that culminates in the release of glucocorticoid hormones from the adrenal cortex. An impressive body of literature indicates that these stress hormones mediate the effects of stress and emotional arousal on enhancement of the consolidation of memory processes (Gold and Buskirk, 1975; McGaugh, 2000; Roozendaal *et al.*, 2009). Extensive evidence indicates that the BLA is essentially involved in

the association of environmental information with emotional significance by engaging stress-related hormones and neurotransmitters to modulate memory processes (Liang *et al.*, 1986; Roozendaal *et al.*, 1999; Ferry *et al.*, 1999; McIntyre *et al.*, 2002; Berlau and McGaugh, 2006; Roozendaal *et al.*, 2009a; Roozendaal and McGaugh, 2011). In turn, such BLA activation facilitates the consolidation of memory processing by coordinating the activation of a network of interacting brain regions and by regulating neural plasticity and memory processes that take place elsewhere in the brain (Roozendaal *et al.*, 1999; Roozendaal *et al.*, 2003; Roozendaal *et al.*, 2004; Nathan *et al.*, 2004; McReynolds *et al.*, 2010).

Prior research has indicated that the BLA mediates emotional arousal effects on memory consolidation by influencing information storage within a network of efferent brain regions (Roozendaal *et al.*, 2009a). Moreover, studies have indicated that each of these brain regions might have a highly specific role in regulating particular aspects of information processes. For example, the hippocampus is known to be specifically involved in regulating spatial and contextual aspects of memory (Jarrard, 1993; Stella *et al.*, 2012). Prior studies have indicated that glucocorticoids act directly within different parts of this network of interacting brain regions, including the BLA (Roozendaal *et al.*, 2002), hippocampus (Roozendaal *et al.*, 1999); dorsal striatum (Quirarte *et al.*, 2009) and prefrontal cortex (Roozendaal *et al.*, 2009b; Barsegyan *et al.*, 2010), to enhance the consolidation of emotionally arousing information. However, human imaging studies also consistently point to a prominent role for the insular cortex (IC) in the processing of emotionally salient information (Hermans *et al.*, 2011; Cloutman *et al.*, 2012). Moreover, imaging studies have indicated that the IC is part of the salience network (Hermans *et al.*, 2011). However, such imaging studies in humans obviously cannot investigate the mechanistic role of the IC in memory or related processes. Animal studies have largely ignored the IC as part of the brain network involved in regulating stress and emotional arousal effects on memory,

since the IC has been mainly associated with drug seeking and craving behavior (Naqui *et al.*, 2014) as well as taste memory (Guzman-Ramos and Bermudez-Rattoni, 2012). Moreover, little is known concerning the effects of glucocorticoids in the IC or other cortical regions in regulating memory consolidation. Therefore in **Chapter 2**, I will examine the effect of direct posttraining administration of glucocorticoids into the IC on memory consolidation of an emotionally arousing training experience. In the first part of this chapter, male Sprague-Dawley rats will be trained on an inhibitory avoidance task followed by immediate posttraining administration of the specific glucocorticoid receptor (GR) agonist RU 28362 into the IC. Retention performance is assessed 48h after the training trial. In the second part of this chapter, a modified two-phase inhibitory avoidance task (Roozendaal *et al.*, 2009; Malin and McGaugh, 2006) will be used to examine whether this brain region might be selectively involved in mediating glucocorticoid effects on memory of the contextual or aversive aspects of the training. Finally, I will assess whether the IC is normally recruited by emotionally arousing training and whether systemic glucocorticoid administration alters neuronal activity within this brain region. To this aim, corticosterone or vehicle will be administered systemically after an inhibitory avoidance training experience and the brains will be collected 30min later for determination of training-induced phosphorylation of ERK1/2 as a marker for neuronal activity.

Chapter 3 describes the characterization and validation procedures of a new behavioral task designed to investigate accuracy of memory. This new task is a modification of the classical inhibitory avoidance task, named inhibitory avoidance discrimination task, which serves to study discrimination. On this task, rats are subsequently trained in two very similar inhibitory avoidance apparatuses, but aversive stimulation of footshock is administered only in one of the contexts. On the retention test, rats are exposed to these two training apparatuses as well as a novel apparatus, they had not seen during the training trial. This task allows to investigate whether the rats

accurately remember in which of the two training contexts they received footshock and thus to distinguish between an episodic-like memory and memory for the features of the box (referred to as context memory in the literature).

In Chapter 4 the inhibitory avoidance discrimination task will be used to study the involvement of noradrenergic activation of the BLA in modulating accuracy of memory. Extensive evidence indicates that posttraining infusions of noradrenergic drugs administered into the BLA enhance memory consolidation of a wide variety of emotional experiences (Roozendaal *et al.*, 2009a; Roozendaal and McGaugh, 2011). However, with inhibitory avoidance, it is difficult to determine which aspects of the learning event are enhanced and particularly whether the noradrenergic activation also increases accuracy of memory. Therefore, this chapter combines posttraining administration of a memory-enhancing dose of norepinephrine with the inhibitory avoidance discrimination task to investigate whether noradrenergic activation of the BLA facilitates discrimination. As discrimination is known to depend on the DG of the hippocampus, this chapter will further investigate how noradrenergic activation of the BLA enhances memory accuracy by altering functioning of the DG. In order to study the neurobiological mechanism of discrimination, different pharmacological and molecular techniques, in combination of behavior will be employed. The focus will be on miRNA 134 within the DG and how this miRNA might regulate emotional arousal effects on memory accuracy and neural plasticity.

Despite extensive evidence indicating that noradrenergic activation of the BLA enhances memory consolidation (Roozendaal *et al.*, 2009a; Pena *et al.*, 2014; McReynolds *et al.*, 2010), the long-term fate of such memories is not generally considered. Although the term memory storage might have a static connotation, memories are continuously changing while they are being consolidated at the systems level. Emerging evidence indicates that memory traces initially depend on

the hippocampus but that over time the role of the hippocampus becomes reduced and that memory traces become more dependent on cortical regions (Frankland *et al.*, 2004; Wiltgen and Silva, 2007). This mechanism is referred to as systems consolidation and might have important consequences for remembering episodic aspects of the task (source memory). However, whether and how noradrenergic activation of the BLA shortly after training might not only alter the strength of a memory but also influence systems consolidation processes and 'transfer of memory' has not been studied. In **Chapter 5**, I will investigate the consequence of noradrenergic activation of the BLA shortly after training on the accuracy of both recent (2 days) and remote memories (28 days) using the inhibitory avoidance discrimination task and determine their dependence on the hippocampus.

Part II:

The second part of this thesis deals with the characterization of two novel glucocorticoid receptor modulators in search for novel therapeutic drugs. Glucocorticoids are the most commonly used anti-inflammatory / immunosuppressant drugs. Within the United States alone, 44.3 million prescriptions for oral glucocorticoids are written annually (Hsiao *et al.*, 2010). Overall, glucocorticoids are ubiquitous (Rosenfeld *et al.*, 1988) and show pleiotropic effects (Chen and Qiu, 1999) that as medication often result in many side effects such as increased vulnerability to stress, personality changes, severe neuropsychiatric consequences, changes in mood, cognition, memory or behavior that might occur during treatment or withdrawal (Judd *et al.*, 2014). Hence, there is a strong need for novel and more specific drugs that target the stress system, i.e., glucocorticoid agonists and antagonists, which maintain their clinical effect but with less negative side effects. Selective GR modulators are ligands that can act as agonist and/or antagonist, depending on endogenous glucocorticoid levels and other background factors (Schacke *et al.*, 2004; Spiga *et al.*, 2007; Datson *et al.*, 2011). Therefore, it is critical to select specific drugs that show beneficial effects without negative side effects.

Chapter 6 characterizes the novel GR modulator C108297 at the level of gene expression, receptor-binding specificity as well as diverse behavioural responses, including memory consolidation. Sustained increases in circulating glucocorticoid levels lead to disease conditions such as Cushing's disease and psychotic depression. Treatment with the classical GR antagonist RU486 (mifepristone) has beneficial effects for these conditions (Cohan, 2014; Golier *et al.*, 2012; van der Lely *et al.*, 1991; Debattista *et al.*, 2006; Nieman *et al.*, 1985). However, full GR antagonism with RU486 also blocks the negative feedback loop of the HPA axis, leading to increased endogenous glucocorticoid levels, which can result in an imbalance between activation of the GR and mineralocorticoid receptor (MR). Studies in animals and humans have indicated that the MR is importantly involved in mood and anxiety and that disruption of its function can lead to mood disorders (Mason and Pariante, 2006). Moreover, RU486 is not specific for the GR, but can also bind the progesterone receptor (PR), which might lead to undesired side effects (Allan *et al.*, 2006). This chapter will investigate the interaction profile of C108297 with GR and its downstream effectors in comparison to RU486 as well as regulation of gene expression in the hippocampus and effects on memory consolidation. Chapter 7 will investigate the effects of C118335, another novel GR modulator with more antagonistic properties than C108297, and low affinity for the PR (Hunt *et al.*, 2012), at the genetic, molecular and behavioral level.

Finally, Chapter 8 will summarize and discuss the main findings of this thesis and will provide conclusions and future perspectives.

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Involvement of the Insular Cortex in Regulating Glucocorticoid Effects on Memory Consolidation of Inhibitory Avoidance Training

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CHAPTER 02

ABSTRACT

Glucocorticoids are known to enhance the consolidation of memory of emotionally arousing experiences by acting upon a network of interconnected brain regions. Although animal studies typically do not consider the insular cortex (IC) to be part of this network, the present findings indicate that the IC is importantly involved in regulating glucocorticoid effects on memory consolidation of emotionally arousing inhibitory avoidance training. The specific glucocorticoid receptor (GR) agonist RU 28362 (3 or 10ng in 0.5µl) infused bilaterally into the IC of male Sprague-Dawley rats immediately after one-trial inhibitory avoidance training dose-dependently enhanced 48h retention performance. Moreover, training on the inhibitory avoidance task increased neuronal activity of the IC, as assessed by an increased number of cells expressing immunoreactivity for phosphorylated extra-cellular signal-regulated kinase 1/2 (pERK1/2). However, systemic administration of a memory enhancing dose of corti-

costerone (1mg/kg) after inhibitory avoidance training rapidly reduced the number of pERK1/2-positive cells in the IC, suggesting that glucocorticoid administration reduces overall neuronal activity of the IC. To investigate which components of the inhibitory avoidance training experience were influenced by the intra-IC glucocorticoid administration, in the last experiment rats were trained on a modified inhibitory avoidance task in which context exposure and footshock training occur on two sequential days. RU 28362 administration into the IC enhanced later retention when infused immediately after either the context or footshock training. Thus, these findings indicate that the IC mediates glucocorticoid effects on the consolidation of memory of different components of inhibitory avoidance training and suggest that the IC might be an important element of the rodent brain network involved in emotional regulation of learning and memory.

INTRODUCTION

Glucocorticoid hormones (stress hormones released from the adrenal cortex) are known to strengthen the consolidation of memory of emotionally arousing experiences (de Kloet *et al.*, 1999; Roozendaal, 2000; Joëls *et al.*, 2006; Sandi and Pinelo-Nava, 2007; de Quervain *et al.*, 2009; Roozendaal and McGaugh, 2011; Schwabe *et al.*, 2012). Most animal studies investigating glucocorticoid-induced enhancement of memory consolidation examined their effects on a network of interacting brain regions involved in emotional regulation of memory, including the basolateral amygdala (BLA), prefrontal cortex and hippocampus (Micheau *et al.*, 1984; Roozendaal and McGaugh, 1997a,b, 2011; Roozendaal *et al.*, 2008a, 2009b; Miranda *et al.*, 2008b). Although human neuroimaging studies generally support an involvement of these brain regions, as well as their functional interaction, in emotionally influenced learning and memory (Buchel *et al.*, 1998; Richardson *et al.*, 2004; Marschner *et al.*, 2008; Shin and Liberzon, 2010), they also consistently point to a key role for the insular cortex (IC) (Craig, 2009; Menon and Uddin, 2010; Shin and Liberzon, 2010; Hartley *et al.*, 2011; Ille *et al.*, 2011).

Extensive evidence indicates that the IC, which receives autonomic, visceral, and somatosensory inputs (Saper, 1982; Augustine, 1996), might be part of a 'salience network' involved in the detection of novel and salient information (Downar *et al.*, 2002; Seeley *et al.*, 2007; Menon and Uddin, 2010) that is collectively upregulated in response to an acute stressful event and after highly stressful experiences (van Marle *et al.*, 2010; Hermans *et al.*, 2011; van Wingen *et al.*, 2011).

Accordingly, increased anterior insula activity has been reported during the subjective awareness of both positive and negative emotions (Craig, 2009; Menon and Uddin, 2010) as well as during the encoding and recall of a broad spectrum of

emotionally salient learning tasks (Buchel *et al.*, 1998; Alvarez *et al.*, 2008, 2011; Marschner *et al.*, 2008; King *et al.*, 2009; Rasch *et al.*, 2009).

Contrasting the human literature, the IC never received a prominent position in animal research investigating stress hormone or emotional arousal effects on learning and memory. Although Bermudez-Rattoni and colleagues have reported several findings supporting the view that the IC might be involved herein (Bermudez-Rattoni *et al.*, 1991, 1997, 2005; Bermudez-Rattoni and McGaugh, 1991; Nerad *et al.*, 1996; Gutierrez *et al.*, 1999; Miranda and Bermudez-Rattoni, 2007), most animal studies have been limited to investigating its involvement in the formation and maintenance of taste memory (Berman and Dudai, 2001; Bermudez-Rattoni *et al.*, 2004; Shema *et al.*, 2007; Nunez-Jaramillo *et al.*, 2010; Stehberg *et al.*, 2011).

The present study investigated whether the IC is implicated in regulating gluco corticoid effects on the consolidation of memory of emotionally arousing inhibitory avoidance training. In the first experiment, we investigated whether the specific glucocorticoid receptor (GR) agonist RU 28362 administered into the IC immediately after one-trial inhibitory avoidance training enhances long-term retention of the training experience.

Next, we examined whether a systemic injection of corticosterone, the major endogenous glucocorticoid in rodents, given immediately after inhibitory avoidance training recruits the IC and induces changes in cellular activity within this brain region. Cellular activity was assessed by determining phosphorylation levels of extracellular signal-regulated kinase 1/2 (pERK1/2), a signaling cascade implicated in neuronal activity and synaptic plasticity (Thomas and Huganir, 2004; Peng *et al.*, 2010).

To gain a better understanding of the precise role of the IC in regulating glucocorticoid effects on inhibitory avoidance memory, in the last experiment we employed a modified inhibitory avoidance procedure (Malin and McGaugh, 2006; Medina *et al.*, 2007; Roozendaal *et al.*, 2009b) that allows investigating which components of the inhibitory avoidance experience (i.e., memory of the context or the footshock) were influenced by the glucocorticoid infusion.

MATERIALS AND METHODS

Subjects

Male adult Sprague–Dawley rats (280-320g at time of surgery) from Charles River Breeding Laboratories (Kisslegg, Germany) were kept individually in a temperature-controlled (22°C) colony room and maintained on a standard 12h light/12h dark cycle (07:00h-19:00h lights on) with *ad libitum* access to food and water.

Training and testing were performed during the light phase of the cycle between 10:00h and 15:00h. All procedures were in compliance with the European Community's Council Directive (86/609/EEC) and approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Surgery for Cannula Implantation

Animals, adapted to the vivarium for at least 1 week, were anesthetized with a subcutaneous injection of ketamine (37.5mg/kg of body weight; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion), and received the non-steroidal analgesic

carprofen (4mg/kg, s.c.; Pfizer). Oxygen (35%) mixed with ambient air was administered during surgery such that blood oxygenation levels would not drop below 90% (Fornari *et al.*, 2012a).

The skull was positioned in a stereotaxic frame (Kopf Instruments), and two stainless-steel guide cannulae (15mm; 23 gauge; Small Parts, Inc.) were implanted bilaterally with the cannula tips 2.0mm above the anterior IC. The coordinates were based on the atlas of Paxinos and Watson (2007): anteroposterior: +1.0mm from Bregma; mediola-teral: ±5.5mm from midline; dorsoventral: -4.8mm from Bregma; incisor bar: -3.3mm from interaural. The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (15mm long 00 insect dissection pins), inserted into each cannula to maintain patency, were removed only for the infusion of drugs.

After surgery, the rats received a subcutaneous injection of 3ml of saline to facilitate clearance of drugs and prevent dehydration, and were subsequently administered antipamezole hydrochloride (Antisedan, 0.25mg/kg, s.c.; Orion) to reverse anesthesia. The rats were allowed to recover for a minimum of 7 days before initiation of training and were handled three times for 1min each during this recovery period to accustom them to the infusion procedure.

Inhibitory Avoidance Apparatus and Procedures

For all experiments, rats were trained and tested in an inhibitory avoidance apparatus, consisting of a trough-shaped alley (91cm long, 15cm deep, 20cm wide at the top, and 6.4cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor (McGaugh *et al.*, 1988). The starting compartment

(30cm) was made of opaque white plastic and was well lit; the shock compartment (60cm) was made of dark, electrifiable metal plates and was not illuminated. Training and testing were conducted in a sound -and light- attenuated room.

For one-trial inhibitory avoidance training, the rats were placed in the starting compartment of the apparatus, facing away from the door, and were allowed to enter the dark (shock) compartment. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.35mA; 1s) was delivered. The rats were removed from the shock compartment 15s later and, after drug treatment, returned to their home cages. For the modified, two-phase inhibitory avoidance procedure (Malin and McGaugh, 2006; Roozendaal *et al.*, 2009b), on the first day (context training), the rat was placed into the starting compartment, facing away from the door, and allowed to freely explore the inhibitory avoidance apparatus for 3min. On day 2 (shock training), each rat was placed directly into the dark compartment, facing away from the starting compartment, with the retractable door closed. The rat then received an inescapable footshock (0.75mA; 1s) and immediately afterward was removed from the training apparatus. For both one-trial and two-phase inhibitory avoidance, retention was tested 48h after training by placing the rat into the starting compartment of the inhibitory avoidance apparatus and measuring the latency to re-enter the former shock compartment with all four paws (maximum latency of 600s). Longer latencies were interpreted as indicating better retention. Shock was not administered on the retention test trial.

Local Drug Infusion into the Insular Cortex

The specific GR agonist RU 28362 (11β, 17β-dihydroxy-6, 21-dimethyl-17β-pregna-4, 6-trien-20yn-3-one, 3 or 10ng; a generous gift of Aventis, Frankfurt, Germany) was first dissolved in 100% ethanol and subsequently diluted in 0.9% saline to reach a

final ethanol concentration of 0.5%. Receptor binding studies have shown that this compound has selective and high affinity for GRs (Teutsch *et al.*, 1981). Bilateral infusions of RU 28362 or an equivalent volume of vehicle (0.5% ethanol in saline) into the IC were given immediately after one-trial inhibitory avoidance training or after either the context or footshock components of the modified, two-phase inhibitory avoidance task by using 30 gauge injection needles connected to a 10µl Hamilton microsyringe with polyethylene (PE-20) tubing. The injection needle protruded 2.0mm beyond the tip of the cannula and a 0.5µl injection volume was infused over a period of 50s by an automated syringe pump (Stoelting Co). The injection needles were retained within the cannulae for an additional 20s after drug infusion to maximize diffusion and to prevent backflow of drug into the cannulas. The infusion volume was based on previous findings indicating that infusion of this volume into the IC (Miranda *et al.*, 2008a; Roozendaal *et al.*, 2010), but not the cortex dorsal to the IC (Bermudez-Rattoni *et al.*, 2005), modulates memory consolidation. To control for time -and site- specificity, additional groups of rats received delayed infusions of RU 28362 or vehicle into the IC 3h after the training trial or immediate post-training infusions into the somatosensory cortex, located approximately 1mm dorsal to the IC. The use of post-training drug administration provides direct support for the view that the treatment affects memory consolidation processes and that retention performance is, thus, not confounded by possible effects on attentional, motivational, or sensory-perceptual mechanisms at the time of training or test (McGaugh, 1966).

Systemic Corticosterone Treatment

Corticosterone (1mg/kg, Sigma-Aldrich) or vehicle, in a volume of 2ml/kg body weight, was given subcutaneously immediately after the training trial. Corticosterone was dissolved in 5% ethanol in saline. This dose of corticosterone is known to enhance memory consolidation of different types of training (Hui *et al.*, 2004; Okuda *et al.*, 2004; Miranda *et al.*, 2008a).

Cannula Placement Verification

Rats were deeply anesthetized with an overdose of sodium pentobarbital (≈100mg/kg, i.p.) and perfused transcardially with a 0.9% saline solution followed by 4% formaldehyde. Following decapitation, the brains were removed and immersed in fresh 4% formaldehyde. At least 24h before sectioning, the brains were submerged in a 25% sucrose (wt/vol) solution in water for cryoprotection. Coronal sections of 50µm were cut on a cryostat, mounted on gelatin-coated slides, and stained with cresyl violet. The sections were examined under a light microscope and determination of the location of injection needle tips in the IC was made according to the atlas plates of Paxinos and Watson (2007), by an observer blind to drug treatment condition. Rats with injection needle placements outside the IC or with extensive tissue damage at the injection needle tips were excluded from analysis.

Immunohistochemistry

Thirty minutes after training and systemic corticosterone treatment, rats were perfused transcardially with ice-cold 0.01M phosphate-buffered saline (PBS), pH 7.4, followed by ice-cold 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. The brains were removed, post-fixed overnight at 4°C, and then transferred to a 25% sucrose solution in 0.1M PB for 3-6 days at 4°C. Frozen coronal sections at the level of the anterior IC (0.2–2.5mm anterior to Bregma) were cut at a thickness of 20µm on a cryostat and collected in Tris-buffered saline (TBS) with 0.1% sodium azide and phosphatase inhibitors (20mM sodium fluoride and 2mM sodium orthovanadate). Every eighth section was used for quantification. Free-floating sections were rinsed in 0.3% Triton X-100 in TBS with phosphatase inhibitors. To block non-specific binding, sections were incubated with TBS containing phosphatase inhibitors, 0.3% Triton X-100 and 5% normal donkey serum (nds, Jackson Immuno Research) for 1h. Subsequently, sections

were incubated with a cocktail of primary antibodies for pERK1/2 (rabbit anti-phospho-p44/42 MAP kinase, 1:1000; cell signaling #9101) and NeuN (mouse anti-NeuN, 1:100; Millipore Mab 377) or pERK1/2 and CaMK II (mouse anti-calcium/cal modul independent protein kinase II, 1:400; Millipore #05-532) in TBS containing phosphatase inhibitors, 0.3% Triton X-100 and 1%nds for 48h at 4°C. Sections were then rinsed several times with TBS and incubated with a cocktail of specific fluorochrome-conjugated antibodies (Alexa Fluor 488 donkey anti-rabbit 1:500 and Alexa Fluor 594 donkey anti-mouse 1:1000 or Alexa Fluor 633 goat anti-rabbit 1:500 and Alexa 488 donkey anti-mouse 1:500; Invitrogen) for 2h in the dark. Sections were rinsed again in TBS, incubated with Hoechst dye (0.1µg/ml, Invitrogen) for 30min and mounted on gelatin-coated slides, using non-fading mounting medium for coverslipping and stored in the dark for further analysis. Images of each section were acquired with TissueFaxs®, Zeiss Axio Observer Z1 Microscope System (Tissue-Gnostics GmbH, Vienna, Austria). The number of pERK1/2 immunopositive nuclei was quantified with Image J 1.43m software (Girish and Vijayalakshmi, 2004; Papadopulos *et al.*, 2007). Cell counts were determined at two levels within the anterior IC (2.5-1.7mm and 1.6-0.7mm anterior to Bregma), according to the standard atlas plates of Paxinos and Watson (2007), and expressed as number of pERK1/2-positive nuclei per mm^2. Quantitative analysis of cell counts was performed blind to treatment condition. Double-labeling was examined with a Leica SP2 AOBS confocal microscope.

Statistics

Data are expressed as mean ± SEM. Inhibitory avoidance training and retention test latencies were analyzed with One-Way ANOVAs. Further analysis used Fisher's *post-hoc* tests to determine the source of the detected significances. To determine whether learning had occurred, paired *t*-tests were used to compare the training and retention latencies. Quantitative measures of pERK1/2 immunoreactivity

in the IC were analyzed with Two-Way-ANOVAs, using Training (training vs. no training) and Corticosterone treatment (vehicle vs. corticosterone) as independent factors. The analyses were followed by Fisher's *post-hoc* tests, when appropriate. Individual comparisons with home-cage control groups were performed with Student *t*-tests for independent samples. For all comparisons, a probability level of < 0.05 was accepted as statistical significance. The number of rats per group is indicated in the figure legends.

RESULTS

GR Agonist Administration into the Insular Cortex Enhances Memory Consolidation of Inhibitory Avoidance Training

This experiment examined whether the GR agonist RU 28362 infused into the IC enhances the consolidation of memory of inhibitory avoidance training. For that, bilateral infusions of RU 28362 (3 or 10ng in 0.5µl) or vehicle were administered into the IC immediately after one-trial inhibitory avoidance training and retention of the training was tested 48h later. Control groups received delayed infusions of the GR agonist into the IC 3h after the training trial to determine whether the GR agonist enhances retention by influencing time-dependent processes underlying memory consolidation.

Average step-through latencies for all groups during training, before footshock or drug treatment, were 9.9 ± 0.8s (mean ± SEM). One-Way ANOVA for training latencies revealed no significant differences between groups [$F_{(2,31)}= 0.17$, $p = 0.84$, data not shown]. Forty-eight hour retention latencies of rats administered vehicle into the IC immediately after training were significantly longer than their latencies during the training trial (paired *t*-test: $p < 0.05$), indicating that the rats

retained memory of the inhibitory avoidance experience. As is shown in **Figure 1A**, rats treated with the GR agonist immediately after training had significantly longer retention test latencies as compared with rats that received vehicle [$F_{(2,31)}$= 3.59, $p < 0.05$]. Fisher's *post-hoc* tests revealed that the lower dose of RU 28362 (3ng) enhanced retention ($p < 0.05$, compared with vehicle), whereas retention latencies of animals given the higher dose (10ng) approached, but failed to reach, significance ($p = 0.07$).

As is shown in **Figure 1B**, the GR agonist administered into the IC 3h after the training did not significantly alter retention latencies [$F_{(2,16)}$=0.26, $p = 0.78$], indicating a time-limited involvement of the IC in mediating glucocorticoid effects on the consolidation of inhibitory avoidance memory.

Figures 1C,1D shows cannula placement within the IC. All injection needle tips of rats included in the analysis were localized within the granular and dysgranular subdivisions of the IC. To control for site specificity, other groups of rats received immediate posttraining infusions of vehicle or the GR agonist (3 or 10ng in 0.5µl) into the somatosensory cortex, approximately 1mm above the IC **(see Figure1C)**. One-Way ANOVA for 48h retention latencies revealed no significant GR agonist effect [$F_{(2,12)}$ = 0.29, p = 0.75] **Figure 1E**, indicating that the memory-modulatory effects of RU 28362 are localized within the IC.

Systemic Corticosterone Administration Immediately After One-Trial Inhibitory Avoidance Training Reduces pERK1/2-Immunoreactivity in the Insular Cortex

The findings described above indicate that direct pharmacological activation of GRs in the IC after inhibitory avoidance training enhances the consolidation of

Figure 1

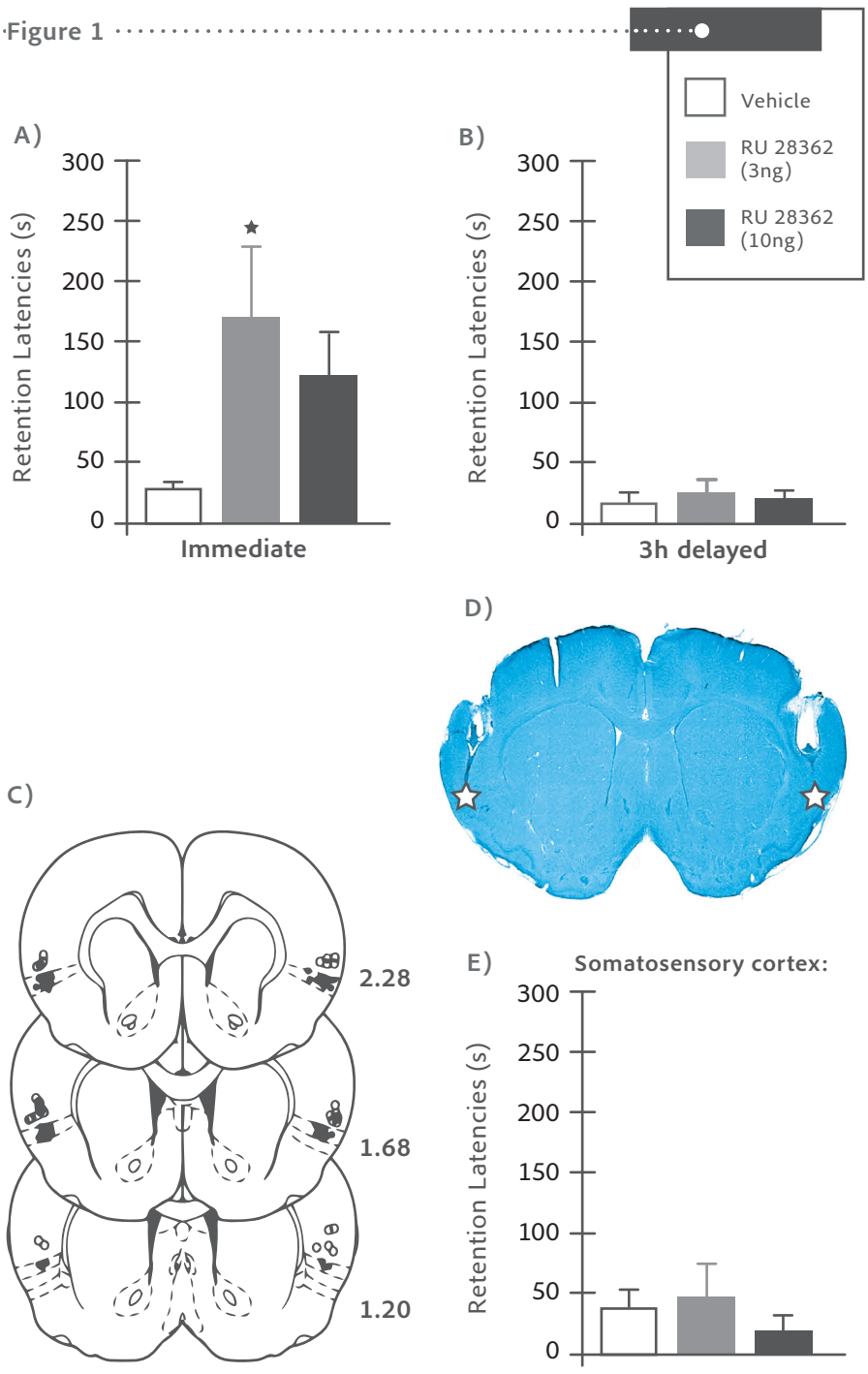

A)
Retention Latencies (s)
300
250
200
150
100
50
0
Immediate
B)
Retention Latencies (s)
300
250
200
150
100
50
0
3h delayed
Vehicle
RU 28362 (3ng)
RU 28362 (10ng)
C)
2.28
1.68
1.20
D)
E)
Somatosensory cortex:
Retention Latencies (s)
300
250
200
150
100
50
0

FIGURE 1:

GLUCOCORTICOID RECEPTOR AGONIST ADMINISTRATION INTO THE INSULAR CORTEX ENHANCES MEMORY CONSOLIDATION OF INHIBITORY AVOIDANCE TRAINING

1A Step-through latencies (mean ±SEM) in seconds on the 48h inhibitory avoidance retention test of rats given bilateral infusions of the glucocorticoid receptor agonist RU28362 (3 or 10ng in 0.5µl) into the IC immediately after training. ★ $p<0.05$ as compared with the vehicle-treated group (n = 11-12 per group).

1B Step-through latencies (mean ± SEM) in seconds on the 48h inhibitory avoidance retention test of rats given the glucocorticoid receptor agonist RU28362 (3 or 10ng in 0.5µl) into the IC 3h after training (n = 6-7 per group).

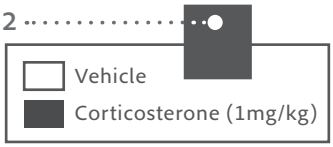
1C Location of injection needle tips within the IC of all rats included in the immediate infusions groups (black circles) and 15 rats with infusion needle tips in the somatosensory cortex as a control for site specificity (open circles). Adapted from Paxinos and Watson (2007).

1D Representative photomicrograph illustrating placement of cannulae and needle tips within the insular cortex.

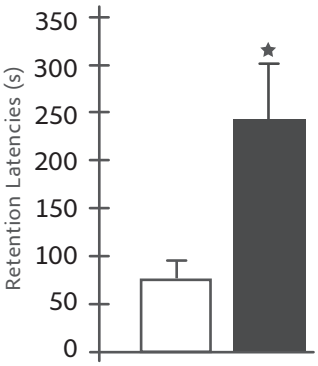
1E Step-through latencies (mean ±SEM) in seconds on the 48h inhibitory avoidance retention test of rats given bilateral infusions of the glucocorticoid receptor agonist RU28362 (3 or 10ng in 0.5µl) into the somatosensory cortex, approximately 1mm above the IC, immediately after training (n = 4-6 per group).

memory of this experience in a dose -time and site- specific manner. However, these findings do not indicate whether circulating glucocorticoids normally act upon the IC in regulating memory consolidation. Therefore, the next experiment examined whether a systemic injection of corticosterone after inhibitory avoidance training changes neuronal activity of the IC. Rats received a subcutaneous injection of corticosterone (1mg/kg) or vehicle immediately after one-trial inhibitory avoidance training (or without training) and were sacrificed 30min later. Brains were processed to investigate training -and drug- induced changes in the number of pERK1/2-positive cells at two locations within the anterior IC. ERK1/2, a member of the mitogen activated protein (MAP) kinase family, is considered to be phosphorylated by elevated neuronal activity and synaptic plasticity (Thomas and Huganir, 2004; Peng *et al.*, 2010). Home-cage control groups did not receive any training or drug treatment. Other rats received the same post-training injection of corticosterone or vehicle and their retention was tested 48h later.

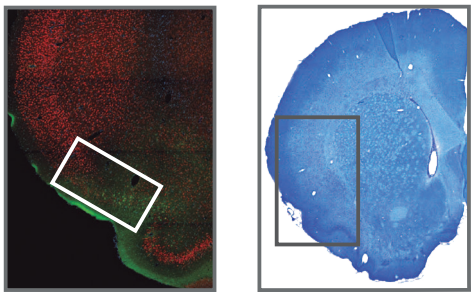
Figure 2



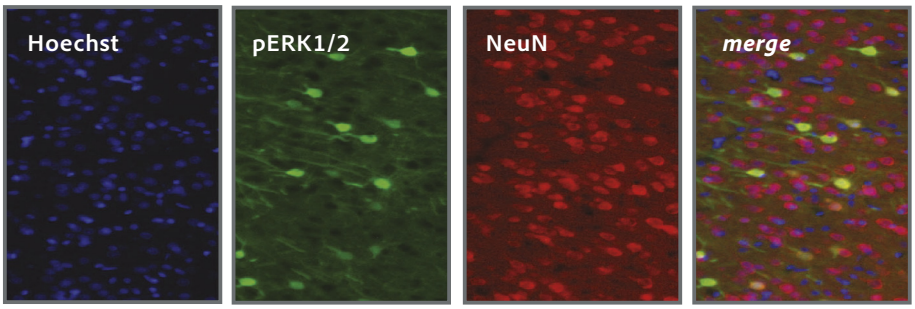
A)



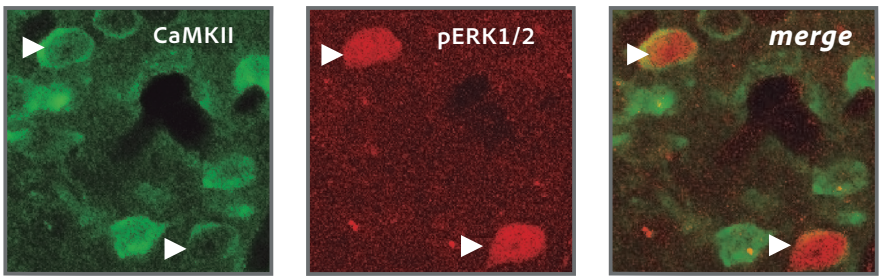
B)



C)



D)



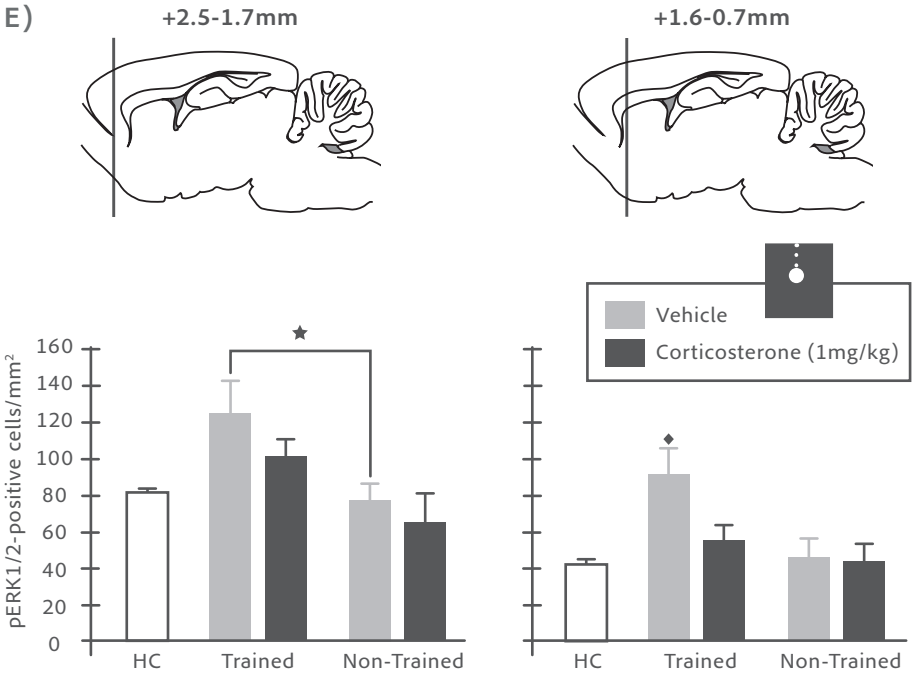


FIGURE 2:

SYSTEMIC INJECTION OF A MEMORY-ENHANCING DOSE OF CORTICOSTERONE AFTER ONE-TRIAL INHIBITORY AVOIDANCE TRAINING REDUCES PERK1/2-IMMUNOREACTIVITY IN THE INSULAR CORTEX

2A Step-through latencies (mean ±SEM) in seconds on a 48h inhibitory avoidance retention test of rats given a subcutaneous injection of corticosterone (1mg/kg) or vehicle immediately after training. ★ $p < 0.05$ (n = 8-9 per group).

2B Example of a Nissl-stained coronal section depicting the region that was acquired for the analyses of immunostaining and the distribution pattern of pERK1/2 expression in the insular cortex. The white square indicates the region analyzed for cell counts.

2C Triple localization of Hoechst (blue), pERK1/2 (green) and the neuronal marker NeuN (red) in the insular cortex. Merging the three channels demonstrates that all pERK1/2-positive cells exhibit immunoreactivity for NeuN.

2D Dual localization of pERK1/2 (red) and CamKII (green) immuno reactivity in the insular cortex. Merging of the red and green channels demonstrates that pER1/2-positive cells also showed immunoreactivity for CamKII. Arrows point to cells with combined pERK1/2 and CamKII immunoreactivity.

2E Number of pERK1/2-positive cells (mean ±SEM) at two levels within the insular cortex as assessed 30min after inhibitory avoidance training and systemic injection of corticosterone (n = 4) or vehicle (n = 4). Non-trained rats received an injection of corticosterone (n = 5) or vehicle (n = 5) but were not trained on the inhibitory avoidance task. Home-cage (HC) control rats (n = 6) did not receive any training or systemic injection. ★ $p < 0.05$ as compared with non-trained vehicle-treated rats. ♦ $p < 0.01$ as compared with al lot her groups.

As is shown in **Figure 2A**, post-training injection of this dose of corticosterone significantly enhanced 48h retention latencies ($p < 0.05$). **Figure 2B** shows the pattern of pERK1/2 expression in the IC. Immunoreactivity for pERK1/2 was found in somata as well as fibers **(Figure 2C)**. Qualitative double-labeling indicated that the vast majority of pERK1/2-positive cells in the IC also showed immunoreactivity for the neuronal marker NeuN **(Figure 2C)** or CaMKII **(Figure 2D)**, a marker for glutamatergic pyramidal cells (McDonald *et al.*, 2002).

These observations strongly suggest that pERK1/2 immunoreactivity within somata was mainly expressed in glutamate-rich pyramidal cells. Because most pERK1/2 immunoreactivity was concentrated in the superficial layers (II-III) of the agranular and dysgranular IC, cell counts were restricted to these regions, at two levels (2.5-1.7mm and 1.6-0.7mm anterior to Bregma), according to the standard atlas plates of Paxinos and Watson (2007). **Figure 2E** shows the number of pERK1/2-positive cells at these two levels of the IC of rats treated with corticosterone or vehicle immediately after inhibitory avoidance training or of rats that were not trained. Across all groups, the number of pERK1/2-positive cells at the more rostral level appeared to be higher than that at the more caudal level. At the rostral level, Two-Way ANOVA indicated a significant training effect [$F_{(1,14)} = 11.63$, $p < 0.01$] but no corticosterone effect [$F_{(1,14)} = 1.95$, $p = 0.18$] or interaction effect between these two parameters [$F_{(1,14)} = 0.53$, $p = 0.48$]. Fisher's *post-hoc* analyses indicated that rats that were trained on the inhibitory avoidance task and subsequently treated with vehicle had more pERK1/2-positive cells than non-trained vehicle-treated rats ($p < 0.05$).

Corticosterone administration after inhibitory avoidance training did not significantly alter the number of pERK1/2-positive cells as compared to vehicle-treated rats. When compared to home-cage controls, student *t*-test analyses indicated that rats treated with either vehicle ($p < 0.05$) or corticosterone ($p <$

0.05) immediately after training had more pERK1/2-positive cells, whereas the number of pERK1/2-positive cells of non-trained rats administered vehicle or corticosterone did not differ from those of home-cage controls.

On the other hand, Two-Way ANOVA for the number of pERK1/2-positive cells at the more caudal level revealed significant effects of training [$F_{(1,14)} = 21.66$, $p < 0.01$], corticosterone treatment [$F_{(1,14)} = 10.55$, $p < 0.01$] and the interaction between them [$F_{(1, 14)} = 6.64$, $p < 0.05$, **Figure 2E**]. Fisher's *post-hoc* analyses indicated that trained rats that were subsequently injected with vehicle had significantly more pERK1/2-positive cells than rats that were administered corticosterone after training ($p < 0.01$) or non-trained rats treated with either vehicle or corticosterone ($p < 0.01$). When compared to home-cage controls, the group that received vehicle immediately after training was the only one that showed increased cell counts ($p < 0.0001$). These findings indicate that training on the inhibitory avoidance task increases the number of pERK1/2-positive cells within this area of the IC, and that post-training administration of a memory-enhancing dose of corticosterone resulted in a significant decrease in the number of pERK1/2-positive cells (as compared to vehicle-treated trained rats), which did not differ significantly from home-cage controls or non-trained rats.

GR Agonist Infusions into the Insular Cortex Enhance Memory for Both the Context and Footshock Components of Inhibitory Avoidance Training

The findings described above indicate that the IC is an important target structure for glucocorticoids in regulating memory consolidation of inhibitory avoidance training. However, with one-trial inhibitory avoidance it is not possible to investigate the relative

involvement of a brain region in memory consolidation of the contextual information independently from that of the footshock. To address this issue, a modified two-phase inhibitory avoidance training procedure had been developed in which context training and footshock training occur on two sequential days (Malin and McGaugh, 2006; Medina *et al.*, 2007; Roozendaal *et al.*, 2009b). In the next experiment, we used this modified inhibitory avoidance training procedure to investigate whether GR agonist administration into the IC enhances memory consolidation of contextual information, the footshock experience, or of both components of training.

Figure 3 shows 48h retention latencies of rats administered the GR agonist (3 or 10ng in 0.5µl) into the IC after either the context or footshock component of inhibitory avoidance training. One-Way ANOVAs for retention latencies revealed significant group effects of rats given intra-IC infusions of RU 28362 immediately after either context [$F_{(2,37)} = 3.50$, $p < 0.05$] or footshock training [$F_{(2,37)} = 4.04$, $p < 0.05$]. Retention latencies of rats given intra-IC infusions of the 3 and 10ng doses of RU 28362 immediately after context training (both, $p < 0.05$), or infusions of the 3ng dose of RU 28362 after foot-shock training ($p < 0.01$) were significantly longer than those of their respective vehicle controls. Importantly, as is shown in **Table 1**, both doses of RU 28362 administered into the IC after either context or shock training to animals that did not receive the other component of training did not enhance 48h retention latencies [context: $F_{(2,15)} = 0.70$, $p = 0.51$; footshock: $F_{(2,15)} = 0.88$, $p = 0.44$], indicating that the expression of the enhanced memory depends on the learning about both context and footshock.

Figure 3

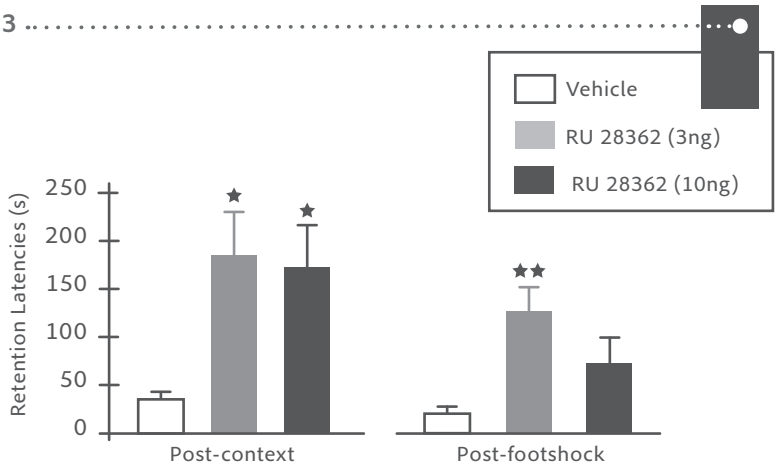


FIGURE 3:

GLUCOCORTICOID RECEPTOR AGONIST INFUSIONS INTO THE INSULAR CORTEX ENHANCE MEMORY CONSOLIDATION OF BOTH THE CONTEXT AND FOOTSHOCK COMPONENTS OF INHIBITORY AVOIDANCE TRAINING

3. Step-through latencies (mean ± SEM) in seconds on the 48h inhibitory avoidance retention test of rats given bilateral infusions of the glucocorticoid receptor agonist RU 28362 (3 or 10ng in 0.5µl) into the insular cortex immediately after either context or shock training on the two-phase modified inhibitory avoidance task (n = 12 - 14 per group). ★ $p < 0.05$, ★★ $p < 0.01$ as compared with the corresponding vehicle-treated group.

Table 1	Vehicle	RU 28362	RU 28362	*p*
Context exposure alone	4.2 ± 0.9	3.1 ± 0.3	3.2 ± 0.9	***0.51***
Footshock exposure alone	8.7 ± 4.2	19.0 ± 5.2	13.0 ± 8.3	***0.44***

Data are presented as mean ± SEM
Number of animals per group is shown in parentheses

DISCUSSION

The aim of this study was to investigate whether the IC is involved in regulating glucocorticoid effects on memory consolidation of inhibitory avoidance training. Our findings indicate that the specific GR agonist RU 28362 infused bilaterally into the IC after inhibitory avoidance training induced dose -and time- dependent enhancement of the consolidation of memory of this training experience. Moreover, training on the inhibitory avoidance task increased neuronal activity of the IC, as assessed with immunoreactivity for pERK1/2. Unexpectedly, systemic injection of a memory-enhancing dose of corticosterone after inhibitory avoidance training rapidly reduced pERK1/2 expression in the IC. Lastly, we show that post-training activation of GRs in the IC enhanced the consolidation of memory of both the footshock and contextual components of inhibitory avoidance training. These findings provide evidence that the IC is an important target structure for glucocorticoids in regulating the consolidation of different components of inhibitory avoidance memory.

It is well established that glucocorticoid hormones, via GR activation, enhance memory consolidation of emotionally arousing training experiences (de Kloet *et al.*, 1999; Sandi and Pinelo-Nava, 2007; Roozendaal *et al.*, 2009a; Schwabe *et al.*, 2012). However, most studies investigating glucocorticoid effects on the formation and stabilization of memory, and its molecular underpinnings, examined glucocorticoid actions on a network of interconnected brain regions such as the BLA and hippocampus (Revest *et al.*, 2005; Sandi and Pinelo-Nava, 2007; Roozendaal *et al.*, 2009b; Groeneweg *et al.*, 2011). Surprisingly little is known concerning glucocorticoid effects on the IC or other cortical regions in regulating learning and memory. Our finding that RU 28362 infused into the IC immediately after inhibitory avoidance training enhanced later retention is in line with other recent evidence indicating that corticosterone or GR agonist administration

into the IC also enhances the consolidation of memory of conditioned taste aversion (Miranda *et al.*, 2008a) and object recognition training (Roozendaal *et al.*, 2010). More generally, findings from human and, to a lesser extent, animal studies indicate that the IC is involved in memory formation of emotionally arousing experiences. Human neuroimaging studies reported activation of the IC during the encoding of aversive conditioning paradigms (Buchel *et al.*, 1998; Alvarez *et al.*, 2008, 2011; Marschner *et al.*, 2008). In rats, lesions of the IC, made before training, disrupted memory of emotionally arousing water-maze spatial (Bermudez-Rattoni *et al.*, 1991; Nerad *et al.*, 1996) and inhibitory avoidance training (Bermudez-Rattoni and McGaugh, 1991). Although such neuroimaging studies in humans and permanent lesion studies in animals obviously cannot determine whether the memory effects were attributable to specific influences on memory consolidation, our findings using immediate post-training or delayed drug delivery provide compelling evidence for the view that the IC is involved in time-dependent processes underlying the consolidation of memory (Bermudez-Rattoni *et al.*, 1991, 2005; Gutierrez *et al.*, 1999; Miranda and McGaugh, 2004; Roozendaal *et al.*, 2010).

To investigate whether inhibitory avoidance training normally recruits the IC, we examined the pattern of pERK1/2 expression in this cortical region 30min after inhibitory avoidance training. ERK is a rapidly activated protein that has been implicated in neuronal activity as well as neuroplasticity and memory consolidation (Atkins *et al.*, 1998; Schafe *et al.*, 2000; Thomas and Huganir, 2004). Exposure to the inhibitory avoidance training procedure increased the number of pERK1/2-positive cells within the IC of rats treated with vehicle. Qualitative immuno-staining indicated that most pERK1/2-positive cells were glutamate-rich pyramidal cells. These findings are consistent with the evidence that ERK1/2 is activated in neurons in response to excitatory glutamatergic activity (Fiore *et al.*, 1993; Xia *et al.*, 1996), and suggest that the elevated pERK1/2 immunoreactivity

reflects an increased neuronal activity within this region (Fujita *et al.*, 2010). Such findings are in agreement with the above-mentioned human neuroimaging studies indicating enhanced IC activity during affective processing and the encoding of emotionally arousing information (Buchel *et al.*, 1998; Alvarez *et al.*, 2008, 2011; Marschner *et al.*, 2008; King *et al.*, 2009; Rasch *et al.*, 2009; Hermans *et al.*, 2011). The pattern of pERK1/2 expression in the present study was similar to that of Kobayashi *et al.* (2010) reporting that novel sucrose stimulation increased the number of pERK1/2-positive cells in the IC, when compared to sucrose-experienced rats. As in our study, they found that pyramidal neurons, but not GABAergic interneurons, in the upper layers of the IC were most susceptible to ERK1/2 phosphorylation. However, the difference between naïve and sucrose-experienced rats was only found in the gustatory region -dysgranular and granular subdivisions- of the IC (Kobayashi *et al.*, 2010), whereas in the present study most pERK1/2 immunoreactivity was concentrated in the agranular and dysgranular regions, and not in the gustatory region. Other studies also indicated increased pERK1/2 levels in the IC in response to an unfamiliar taste stimulus (Berman *et al.*, 1998, 2000), and that such activation is necessary for taste learning (Berman *et al.*, 1998). These findings suggest that ERK1/2 activation in the IC may contribute to the detection of novelty and/or memory formation (Berman *et al.*, 1998; Bermudez-Rattoni, 2004; Kobayashi *et al.*, 2010).

A more puzzling observation is that a memory-enhancing dose of corticosterone administered immediately after inhibitory avoidance training resulted in a rapid decrease in the number of pERK1/2-positive cells within the IC. Corticosterone administration to non-trained rats did not induce such a reduction. The selective influence of corticosterone on trained, and not naïve, rats is consistent with prior evidence indicating that RU 28362 infused into the IC interacts with training-induced noradrenergic activation in enhancing memory consolidation (Roozendaal *et al.*, 2010). However, the direction of the corticosterone effect

on pERK1/2 immunoreactivity appears to contrast prior evidence indicating that glucocorticoid effects on memory consolidation involving other brain regions require an upregulation of pERK1/2-MAP kinase activity. Previously, we reported that a memory-enhancing dose of RU 28362 infused into either the BLA or medial prefrontal cortex after inhibitory avoidance training rapidly elevated pERK1/2 levels in the other brain site (medial prefrontal cortex or BLA, respectively) (Roozendaal *et al.*, 2009b). Moreover, stress and corticosterone are known to interact with training-associated glutamatergic activity within the hippocampus to increase the expression and enzymatic activity of the MAP kinase pathway (Revest *et al.*, 2005; Gutierrez-Mecinas *et al.*, 2011). A blockade of pERK1/2 signaling with a MEK inhibitor infused into any of these brain regions prevented glucocorticoid effects on memory consolidation. Thus, although we only examined pERK1/2 immunoreactivity in somata, and not in fibers, these findings suggest that glucocorticoids, at least at a 30min time interval, might exert an opposite influence on the pERK1/2-MAP-kinase pathway, and possibly neuronal activity, in the IC as compared to the BLA or hippocampus. Emerging evidence supports the view that glucocorticoids can activate or suppress synaptic plasticity and neuronal activity in a highly time-dependent and brain region-specific manner (Joëls *et al.*, 2011).

Our finding that corticosterone-induced enhancement of inhibitory avoidance memory is associated with a reduced pERK1/2 expression, and possibly neuronal activity, of the IC is in agreement with that of a recent functional magnetic resonance imaging study in humans showing that the combined oral administration of cortisol and the noradrenergic stimulant yohimbine shortly before the encoding of emotionally arousing pictures led to a strong deactivation of the IC, along with the hippocampus and orbitofrontal cortex (van Stegeren *et al.*, 2010). Moreover, the magnitude of this deactivation correlated with enhanced recall of the material assessed 1 week later. Highly comparable, another study

reported that human participants who responded with a large increase in cortisol when confronted with a psychosocial stressor, as opposed to low-responders, also showed deactivation of a network of limbic regions that includes the right anterior insula (Pruessner *et al.*, 2008). The crucial question is whether and how such overall deactivation of the IC might contribute to the enhanced consolidation of memory processing. Pruessner *et al.* (2008) interpreted the deactivation of limbic regions in their study as a stress-mediated regulatory influence on hypothalamic-pituitary-adrenal (HPA) axis activity. However, no findings are available indicating whether or not the IC per se is involved in HPA-axis regulation. Alternatively, based on the involvement of frontal areas, including the anterior IC, in emotional regulation, attention, and focusing (Dolcos *et al.*, 2004, 2007), van Stegeren *et al.* (2010) suggested that a reduced BOLD signal in these areas could reflect either a loss of top-down inhibition, and therefore activation (disinhibition) of other brain regions, or an increased signal-to-noise ratio, resulting in a shift of attentional processing from a peripherally to a centrally focused view. In line with this hypothesis, others have proposed that the anterior IC is part of a 'salience network' which detects salient stimuli (events) and triggers appropriate control signals to regulate behavior and homeostatic state (Downar *et al.*, 2002; Seeley *et al.*, 2007; Menon and Uddin, 2010). It is possible, therefore, that a reduced neuronal activity of the IC after exogenous glucocorticoid administration induces a 'reallocation of resources', thereby increasing the detection of the most relevant stimuli and enhancing the consolidation of memory of these experiences (Cahill and van Stegeren, 2003; van Stegeren *et al.*, 2010).

Such a potential role of the IC, as part of the salience network, in novelty and salience detection fits well with our finding that local activation of GRs with RU 28362 enhanced the memory of exposure to both the contextual and aversive components of inhibitory avoidance training. Similarly, findings of human neuroimaging studies indicated activation of the IC in fear conditioning studies

during the presentation of either specific conditioned stimuli or context (Buchel *et al.*, 1998, 1999; Alvarez *et al.*, 2008; Marschner *et al.*, 2008). A broad involvement of the IC in regulating the consolidation of memory of emotionally salient experiences fits also well with other findings of animal studies indicating that post-training pharmacological manipulation of IC activity modulates memory consolidation of training on many different kinds of learning tasks, including spatial water-maze (Gutierrez *et al.*, 1999), conditioned taste aversion (Miranda and McGaugh, 2004; Miranda *et al.*, 2008a,b; Shema *et al.*, 2009) and object recognition (Bermudez-Rattoni *et al.*, 2005; Roozendaal *et al.*, 2010).

The extensive network of connections between the IC and other cortical regions, including the prefrontal, cingulate, perirhinal and entorhinal cortices (Mufson *et al.*, 1981; Augustine, 1996; Shi and Cassell, 1998b; Hoistad and Barbas, 2008), might account for such a general modulatory influence on memory. There are also dense reciprocal connections between the IC and BLA (McDonald and Jackson, 1987; Shi and Cassell, 1998a,b). Extensive evidence indicates that the BLA influences memory consolidation of emotionally arousing training experiences by regulating neuroplasticity and information storage processes in other brain regions, including the hippocampus and dorsal striatum (McGaugh, 2002; Roozendaal and McGaugh, 2011). Therefore, it is possible that the IC and BLA share a functional commonality and cooperate in regulating memory consolidation. Indeed, some findings provide evidence for a necessary interaction between the IC and BLA in strengthening the consolidation of memory of emotionally arousing training. Post-training infusion of a cAMP analog into the IC is known to enhance memory consolidation of both conditioned taste aversion and inhibitory avoidance training. However, concurrent blockade of noradrenergic transmission in the BLA with the ß-adrenoceptor antagonist propranolol prevents this memory enhancement (Miranda and McGaugh, 2004). Findings of human studies indicating an increased connectivity between the amygdala and IC during the encoding of emotionally

arousing material (Rasch *et al.*, 2009) support a functional interaction between the BLA and IC in regulating memory consolidation.

In summary, the present findings provide evidence for the view that the IC is importantly involved in regulating glucocorticoid effects on memory consolidation of emotionally arousing inhibitory avoidance training. These findings indicate that the IC deserves a prominent position in animal research investigating the neural basis of emotional regulation of learning and memory.

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The Inhibitory Avoidance Discrimination Task to Investigate Accuracy of Memory

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  &

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CHAPTER 03

ABSTRACT

The present study was aimed at developing a new inhibitory avoidance task, based on training and/or testing rats in multiple contexts, to investigate accuracy of memory. In the first experiment, male Sprague-Dawley rats were given footshock in an inhibitory avoidance apparatus and, 48h later, retention latencies of each rat were assessed in the training apparatus (Shock box) as well as in a novel, contextually modified, apparatus. Retention latencies in the Shock box were significantly longer than those in the Novel box, indicating accurate memory of the training context. When the noradrenergic stimulant yohimbine (0.3mg/kg, sc) was administered after the training, 48h retention latencies in the Shock box, but not Novel box, were increased, indicating that the noradrenergic activation enhanced memory of the training experience without reducing memory accuracy. In the second experiment, rats were trained on an inhibitory avoidance discrimination task: They were

first trained in an inhibitory avoidance apparatus without footshock (Non-Shock box), followed 1min later by footshock training in a contextually modified apparatus (Shock box). Forty-eight hour retention latencies in the Shock and Non-Shock boxes did not differ from each other but were both significantly longer than those in a Novel box, indicating that rats remembered the two training contexts but did not have episodic-like memory of the association of footshock with the correct training context. When the interval between the two training episodes was increased to 2min, rats showed accurate memory of the association of footshock with the training context. Yohimbine administered after the training also enhanced rats' ability to remember in which training context they had received actual footshock. These findings indicate that the inhibitory avoidance discrimination task is a novel variant of the well-established inhibitory avoidance task suitable to investigate accuracy of different aspects of memory.

INTRODUCTION

Inhibitory avoidance is a commonly used behavioural task to investigate learning and memory processes in rodents (Gold, 1986; McGaugh and Roozendaal, 2009). During inhibitory avoidance training, rats typically receive a single aversive footshock after stepping from a lighted compartment into a darkened compartment in a straight alley. Retention of the training is tested usually 24 or 48h later by measuring rats' latency to enter the former shock compartment when they are placed in the lighted compartment. Longer retention test latencies are interpreted as indicating better memory. Even though inhibitory avoidance training consists of a single trial only, the brain processes underlying task adquisition are complex. Rats must encode different pieces of information in order to acquire a correct association between a particular location within the apparatus and the aversive stimulus of footshock (Liang, 2001; Malin and McGaugh, 2006; Roozendaal and McGaugh, 2011; Fornari *et al.*, 2012b).

During the past decades we have learned much regarding the involvement of specific brain regions and neurochemical processes in different aspects of inhibitory avoidance memory (Izquierdo *et al.*, 2006; Roozendaal and McGaugh, 2011). However, with these inhibitory avoidance training and test procedures it is difficult, if not impossible, to make any inference with respect to the accuracy or specificity of what has been learned. Better memory, i.e., longer retention test latencies, on the inhibitory avoidance task as induced, for example, by posttraining drug manipulation could indicate that the rat developed a more accurate or detailed representation of the training experience. On the other hand, it is also possible that the memory enhancement is associated with reduced accuracy. Particularly in the field of stress research on cognitive processes there is currently much interest in unraveling how stress and emotional arousal affect both the strength and accuracy of memory processing (Heuer and Reisberg, 1990; Payne *et al.*,

2002; Segal *et al.*, 2012; Hoscheidt *et al.*, 2014; Leal *et al.*, 2014). Moreover, recent developments in in vivo neuroimaging, octogenetics and electrophysiological techniques indicate the necessity of having refined behavioural tasks that allow investigating the neural substrates underlying specific aspects of information processing and memory (Rauch *et al.*, 2006; Laxpati *et al.*, 2014).

The present study was undertaken to establish and validate an inhibitory avoidance discrimination task to investigate accuracy of both contextual and episodic-like aspects of memory. In the first series of experiments the inhibitory avoidance training procedure was left unchanged, but retention latencies of each rat were assessed in the training apparatus in which they had received footshock as well as in another, contextually modified, inhibitory avoidance apparatus they had not seen during the training. This allows investigating whether the brief context exposure during training is sufficient for rats to develop an accurate memory of the training context. In the second series of experiments, rats were subsequently trained in two distinctly different inhibitory avoidance apparatuses with a short delay, but were given footshock in only one of these contexts. On the retention test, the rats were tested in these two training contexts as well as in a novel context.

This training and test procedure allows investigating whether rats remember the two contexts they visited during the training as well as have specific episodic-like memory of the association of footshock with the correct training context. Our main objective was to develop a study protocol which is highly ambiguous and induces poor discrimination in control animals, such that it can be examined and induces poor discrimination in control animals, such that it can be examined to what extent memory-enhancing drug treatment also increases the accuracy of memory. As norepinephrine, normally released during emotionally arousing training, is known to enhance the consolidation of inhibitory avoidance memory (Liang *et al.*, 1986; Introini-Collison *et al.*, 1991; Ferry *et al.*, 1999; McIntyre *et al.*,

2005), we investigated on both tasks how posttraining systemic administration of a memory-enhancing dose of the noradrenergic stimulant yohimbine might affect the accuracy and/or strength of the memory.

MATERIALS AND METHODS

Subjects

Adult male Sprague-Dawleyrats (330-370g at the time of behavioral experiments) from Charles River Breeding Laboratories (Kisslegg, Germany) were housed individually in a temperature controlled (22°C) vivarium room and maintained on a 12h/12h light/dark cycle (lights on: 7:00h-19:00h) with *ad libitum* access to food and water. Rats were handled three times for 1min each prior to training. Training and testing were performed during the light phase of the cycle, between 10:00h-15:00h. All experimental procedures were in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the Institutional Animal Careand Use Committees of the University of Groningen and Radboud University Nijmegen, The Netherlands.

Inhibitory Avoidance Discrimination Task

Inhibitory avoidance apparatus and contextual modifications:

For all experiments, rats were trained and tested in one or more inhibitory avoidance apparatuses. The basic features of each apparatus were identical and consisted of a trough-shaped alley (91cm long, 15cm deep, 20cm wide at the top, and 6.4cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor (McGaugh *et al.*, 1988). The starting

compartment (31cm) was made of opaque white plastic and was well lit; the dark (i.e., shock) compartment (60cm) was made of two electrifiable metal plates and was not illuminated. As shown in **Figure 1**, one apparatus **(Box A)** did not have any contextual modifications. Footshock was always delivered in this apparatus only. Two other apparatuses **(Boxes B and C)** served as non-shock, safe training or test environments and had some distinct contextual modifications. Box B had four vertical white stripes (2cm wide) taped on the 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. Box C had two white circles (3.5cm diameter) taped on each wall of the dark compartment, and the gap between both plates was closed with tape. All three inhibitory avoidance apparatuses were located next to one other within a sound -and light- attenuated room.

Procedures of the inhibitory avoidance task:

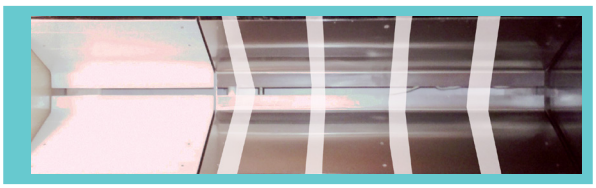
In the first series of experiments, rats were trained in Box A, as with the classical inhibitory avoidance training procedure, and subsequently tested for retention, 48h after training, in that same apparatus and a novel apparatus. The addition of a novel test environment during the retention test permits to determine whether the rats accurately remembered the context in which they had received footshock. Moreover, we examined in this experiment what the best method might be for assessing retention latencies of individual rats on both apparatuses. For training, the rats were placed into the starting compartment of Box A (Shock box), facing away from the door, and were allowed to freely explore the apparatus. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.50mA for 1s) was delivered. Rats were removed from the dark compartment 20s later and returned to their home cages until retention testing 48h later. For retention testing, the rats were placed, in a pseudo-random fashion, into the starting compartment of either Box A or a novel apparatus (Box B) and their latencies to enter the dark compartment with all four paws (maximum

latency of 600s) were measured. Shock was not administered on the retention test trial. Either immediately or 24h after testing in the first apparatus (see Results), the same rats were placed into the starting compartment of the other apparatus and their latencies to enter the dark compartment were measured. Immediately after the training or testing of each animal, the apparatuses were wiped clean with a 10% ethanol solution.

Figure 1

BOX A)

BOX B)



BOX C)

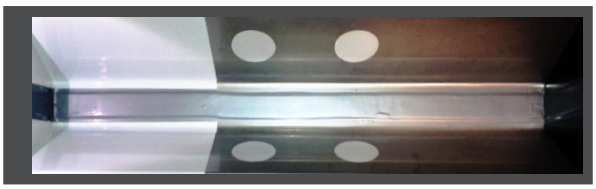


FIGURE 1:

INHIBITORY AVOIDANCE APPARATUS AND CONTEXTUAL MODIFICATIONS

BOX A. did not have any contextual modifications. Footshock was delivered in this apparatus only. Box B and C had some distinct contextual modifications and served as non-shock safe training and/or test contexts.

BOX B. had four vertical white stripes taped on the 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.

BOX C. had two white circles taped on each wall of the dark compartment, and the gap between both plates was closed with tape. The colored frames refer to the diagrams in Figures 2 and 3.

Procedures of the inhibitory avoidance discrimination task:

In the second series of experiments, rats were trained subsequently in two inhibitory avoidance apparatuses within a single training session, but footshock was delivered only in one of these two contexts. On the 48h retention test, their latencies to enter the dark compartment of these two boxes were determined as well as those into that of a novel apparatus they had not seen before. This experimental design was aimed at investigating whether rats, on a retention test, would remember the two apparatuses they had visited during training and discriminate in which of these two contexts they had received actual footshock. Rats were initially placed into the starting compartment of either Box B or C (Non-Shock box) and could explore this apparatus for 20s without any footshock being delivered. Afterwards, the rats were taken from that apparatus and, after a brief delay of either 1 or 2min, placed into the starting compartment of Box A (Shock box). After entering the dark compartment of Box A, the sliding door was closed and a single inescapable footshock of 0.5mA was delivered for 1s. Rats were removed from the dark compartment 20s after termination of footshock and returned to their home cages. The order of training on these two boxes was always the same. Rats were first trained in the Non-Shock box followed by the Shock box because they are less likely to explore a new environment shortly after shock delivery. During the 48h retention test, the rats were tested in the previously seen Non-Shock and Shock boxes and, additionally, in a Novel box they had not seen before. The order of retention testing in these three contexts was randomized. On all three inhibitory avoidance apparatuses, the rat was placed into the starting compartment and their latency to enter the dark compartment with all four paws (maximum latency of 600s) was measured. After the rat entered the dark compartment of the first test environment, it was immediately taken from that apparatus and without delay placed into the starting compartment of the second, and then third box. Shock was not administered on the retention test trial. Immediately after the training or testing of each animal, the apparatuses were wiped clean with a 10% ethanol solution.

Systemic Drug Administration

Some groups of rats received an immediate posttraining injection of a memory-enhancing dose of the noradrenergic stimulant yohimbine (0.3mg/kg; Sigma-Aldrich), a selective α_2-adrenoceptor antagonist. Yohimbine was dissolved in sterile 0.9% saline and administered subcutaneously in a volume of 2ml/kg immediately after the training session. Control animals received a saline injection only. The drug dose was selected on the basis of previous findings (Roozendaal *et al.*, 2006). Drug solutions were freshly prepared before each experiment.

Statistics

Data are expressed as the mean ± SEM. Retention test latencies were analyzed with one, two or three-way ANOVAs with latencies of individual animals in the different test environments (Shock, Non-Shock and Novel boxes) as repeated measure. *Post hoc* comparisons used unpaired and paired *t*-tests to determine the source of the detected significances, when appropriate. Training and retention latencies of each rat were compared with paired *t*-tests. For all comparisons, aprobability level of < 0.05 was accepted as statistical significance. The number of animals per group is indicated in the figure legends.

RESULTS

Testing Accuracy of Inhibitory Avoidance Memory

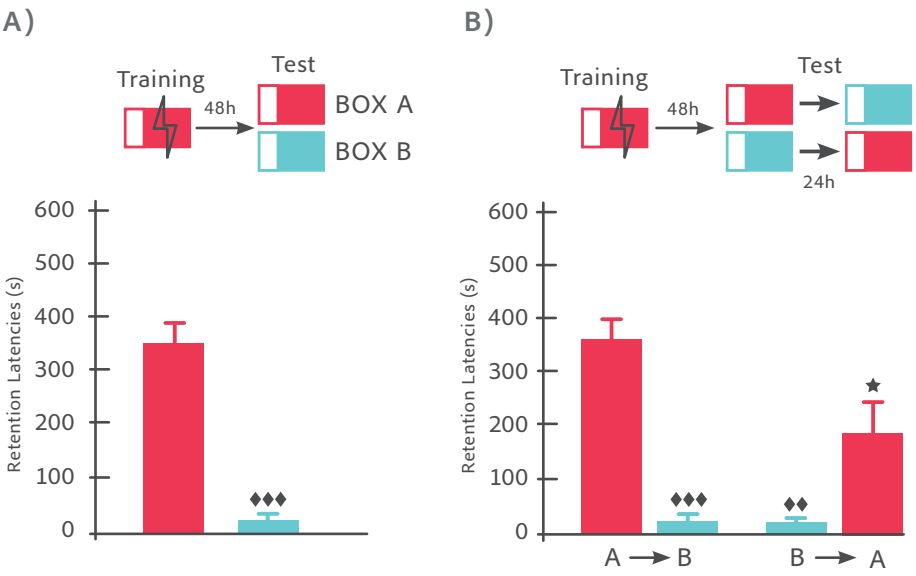
The first experiment investigated whether rats after inhibitory avoidance training develop accurate memory of the context in which they received footshock. Therefore, rats were trained on the one-trial inhibitory avoidance task (Box A).

Initial latencies to enter the dark compartment during training, before footshock, were 13.9 ± 2.1s (mean ± SEM). Forty-eight hours later, half of the rats were tested in the same apparatus (Box A) and the other half in a distinctly different novel context (Box B). As shown in **Figure 2A**, retention latencies of rats tested in the Shock box (Box A) were significantly longer (350.4 ± 49.4s) than those of rats that were tested in the novel context (Box B) (10.6 ± 1.7s; $p < 0.0001$). Moreover, retention latencies of rats tested in Box A were significantly longer than their entrance latencies in that apparatus during the training trial (paired *t*-test: $t_7 = 6.84$; $p = 0.0005$), whereas retention latencies of rats tested in Box B did not differ from their prior training latencies in Box A (paired *t*-test: $t_8 = 0.59$; $p = 0.57$). Thus, these findings indicate that rats showed accurate memory of the context in which they received footshock.

Twenty-four hours after the first retention test, the same animals were tested in the other inhibitory avoidance apparatus. Thus, rats that had been tested in Box A were now tested in Box B, and rats that had been tested in Box B were now tested in Box A. Again, we found that rats had significantly longer retention latencies when tested in Box A as compared to Box B ($p < 0.01$; **Figure 2B**). However, retention latencies in Box A were now significantly shorter than those during the first retention session $p < 0.05$. In a next experiment, another group of animals was trained in Box A and 48h later tested for retention in both inhibitory avoidance apparatuses, but now without any delay. Rats were tested, in a randomized fashion, in either Box A or B and immediately afterwards in the other context. As shown in **Figure 2C**, retention latencies in Box A were significantly longer than those in Box B ($p < 0.0001$). Most importantly, retention latencies in Box A and B were now independent of the order of testing [repeated-measures ANOVA: $F_{(1,16)} = 0.0005$; $p = 0.98$]. These findings indicate that repeated testing of the same animals in both inhibitory avoidance apparatuses without a delay is an accurate method for assessing discrimination in individual rats. Therefore, for all further experiments retention latencies in the different test environments were determined without delay.

As we are particularly interested in investigating how posttraining drug treatment might affect accuracy of memory, we examined whether noradrenergic activation after inhibitory avoidance training would enhance memory of the training in a context-specific manner. Rats were trained on the inhibitory avoidance task (Box A) and given a posttraining subcutaneous injection of either saline or memory-enhancing dose of the noradrenergic stimulant yohimbine (0.3mg/kg). Rats were tested 48h later for retention in both Box A and B. The order of retention testing in these two contexts was randomized and without any delay. As shown in **Figure 2D**, yohimbine increased retention latencies in the Shock box ($p < 0.05$ vs. saline) without influencing retention latencies in the Novel box ($p = 0.84$ vs. saline). Moreover, repeated-measures ANOVA indicated that successive testing did not affect retention latencies [$F_{(1,15)} = 1.57$; $p = 0.23$]. Thus, these findings indicate that yohimbine enhanced memory of the training experience without inducing any generalization across contexts.

Figure 2 ····· **Accuracy of inhibitory avoidance memory**



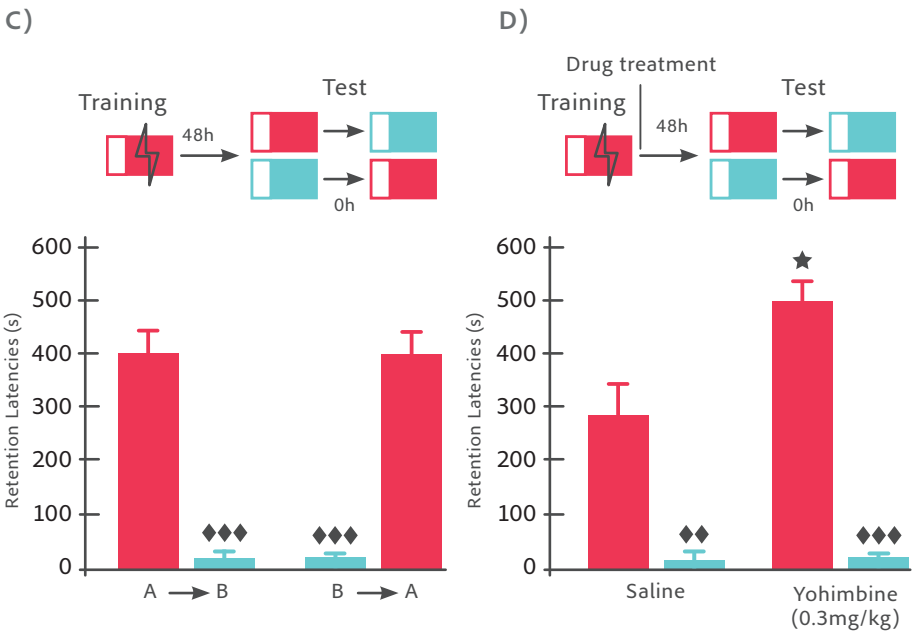


FIGURE 2:

ACCURACY OF INHIBITORY AVOIDANCE MEMORY

Inhibitory avoidance retention latencies (mean + SEM) in seconds. The different test procedures are shown as schematic for each of the experiments.

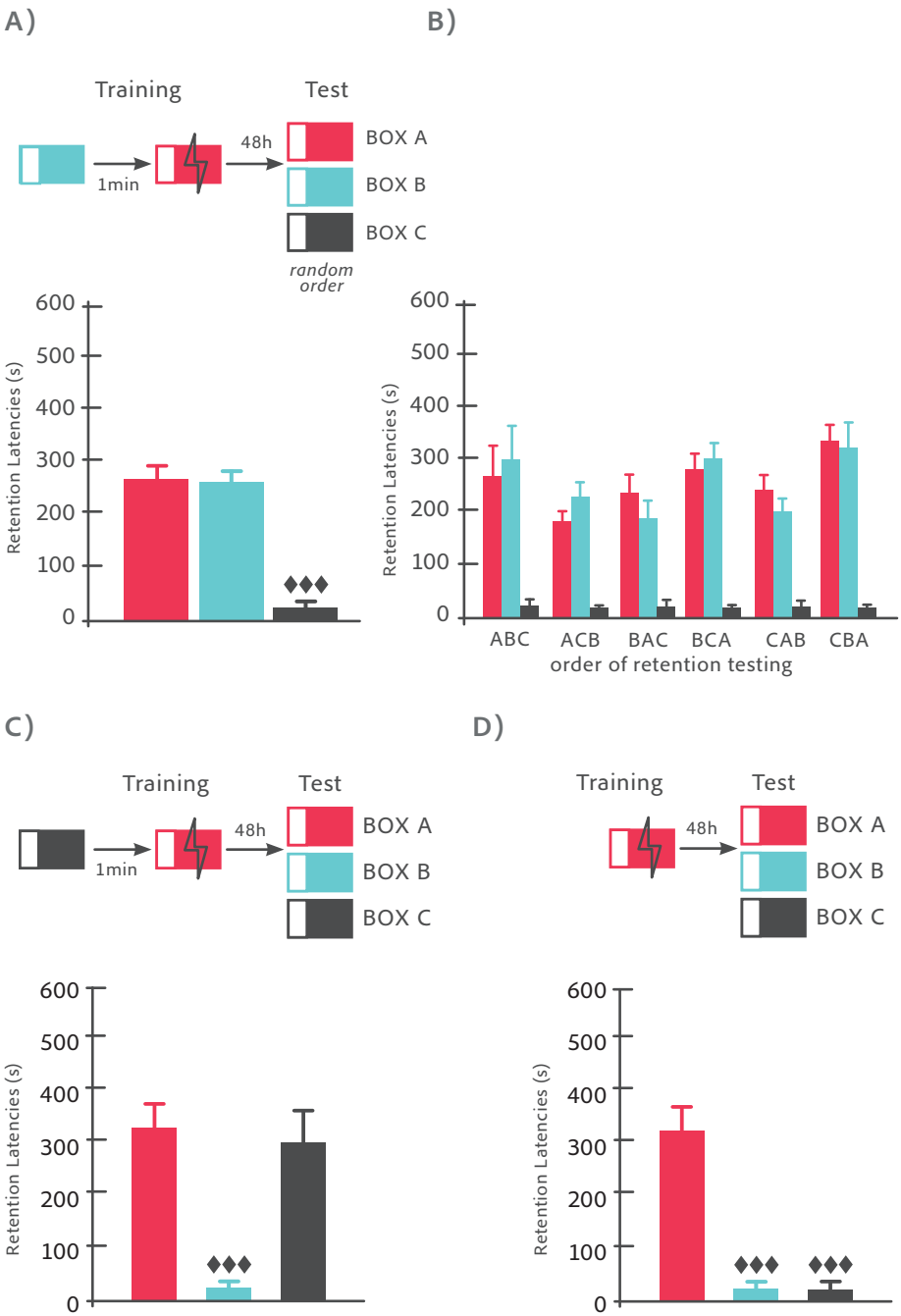
2A Rats were given footshock in Box A (red) and 48h later half of the rats were tested for retention in that same apparatus and the other half in a novel apparatus (Box B, blue). Retention latencies in Box A were significantly longer than retention latencies in Box B. ♦♦♦, $p < 0.0001$ versus Box A. N = 8-9 rats per group.

2B Twenty-four hours later the same rats were tested in the other apparatus. Retention latencies in Box A were now significantly shorter than during the first retention test. A→B, rats were first tested in Box A and 24h later in Box B; B→A, rats were first tested in Box B and 24h later in Box A. ★, $p < 0.05$, versus Box A during first retention test. ♦♦, $p < 0.01$; ♦♦♦ $p < 0.0001$ versus Box A.

2C Rats were trained in Box A and 48h later half of the rats were tested for retention in the same apparatus and the other half in a novel apparatus (Box B). They were immediately afterwards tested in the other apparatus. Retention latencies were now independent of the order of testing. A→B, rats were first tested in Box A and then in Box B; B→A, rats were first tested in Box B and then in Box A. ♦♦♦ , $p < 0.0001$ versus Box A. N = 9 rats per group.

2D Yohimbine (0.3m/kg, sc) administered immediately after inhibitory avoidance training enhanced 48h retention latencies in Box A without affecting retention latencies in Box B. ★, $p < 0.05$ versus the saline group. ♦♦, $p < 0.01$; ♦♦♦ , $p < 0.0001$ versus Box A. N = 8-9 rats per group.

Figure 3 ·····/ Accuracy of inhibitory avoidance discrimination memory



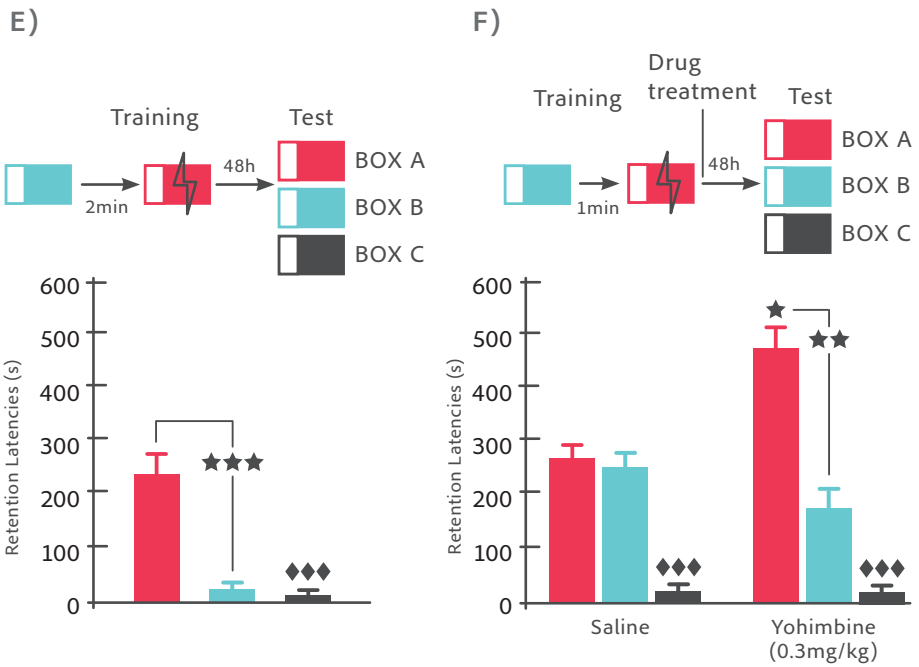


FIGURE 3:

ACCURACY OF INHIBITORY AVOIDANCE DISCRIMINATION MEMORY

Inhibitory avoidance retention latencies (mean + SEM) in seconds. The different test procedures are shown as schematic for each of the experiments.

3A Rats were trained in Box B (Non-Shock box, blue) without footshock followed 1min later by footshock training in Box A (Shock box, red). On the 48h retention test rats were sequentially tested in all three inhibitory avoidance apparatus in a random order and without delay. Retention latencies in the previously visited Box A and Box B did not differ from each other but were both significantly longer than those in Box C (Novel box, grey). ♦♦♦, $p < 0.0001$ versus Box A and Box B. N = 30 rats.

3B The order of retention testing in the three boxes (ABC, ACB, BAC, BCA, CAB, CBA) did not significantly influence retention latencies.

3C On the training trial rats were first placed in Box C (Non-Shock box) followed 1min later by footshock training in Box A (Shock box). Forty-eight hour retention latencies in the two previously visited boxes (Box A and Box C) did not differ from each other but were both significantly longer than those in Box B (Novel box). ♦♦♦ , $p < 0.0001$ versus Box A and Box C. N = 13 rats.

3D Rats were trained in Box A (Shock box). Forty-eight hour retention latencies in Box A were significantly longer than those in Box B and C. ♦♦♦, $p < 0.0001$ versus Box A. N = 20 rats.

3E When the interval between training in Box B (Non-Shock box) and Box A (Shock box) was 2min, 48h retention latencies in Box A were significantly longer than those in Box B and Box C (Novel box). ♦♦♦, $p < 0.0001$ versus Box A. N = 15 rats.

3F Yohimbine (0.3mg/kg, sc) administered immediately posttraining enhanced 48h retention latencies for the Shock box (Box A). Retention latencies in Box A (Shock box) were significantly longer than those in Box B (Non-Shock box) and Box C (Novel box). ★ $p < 0.05$, ★★ $p < 0.01$ versus the corresponding control group. N = 12 rats/group.

Testing Accuracy of Inhibitory Avoidance Discrimination Memory

In the second series of experiments, rats were subsequently trained in two inhibitory avoidance apparatuses within a single training session, but footshock was delivered only in one of these two contexts. During the training session, rats were first trained in Box B (Non-Shock box) and 1min later in Box A (Shock box). On the 48h retention test, rats were tested, in a randomized order and without delay, in the Shock and Non-Shock boxes as well as in a Novel box (Box C) they had not seen during the training.

Repeated-measures ANOVA for retention test latencies in these three boxes indicated a significant context effect [$F_{(2,48)} = 52.53$, $p < 0.0001$]. As shown in **Figure 3A**, retention latencies in the Shock box (Box A: 255.8 ± 29.9s) sand Non-Shock box (Box B: 255.1 ± 30.4s) did not differ from each other (paired *t*-test: $t_{29} = 0.63$; $p = 0.53$), but were both significantly longer than those in the novel context (Box C: 13.4 ± 1.5s; paired *t*-tests: Shock box vs. Novel box: $t_{29} = 7.99$; $p < 0.0001$; Non-Shock box vs. Novel box: $t_{29} = 7.84$; ± < 0.0001). Moreover, as shown in **Figure 3B**, retention latencies in the three test contexts were independent of the order of testing [ABC, ACB, BAC, BCA, CAB, CBA: $F_{(5,24)} = 0.26$, $p = 0.93$]. Thus, these findings indicate that with this training procedure rats accurately remembered the two contexts they had visited during the training but lack specific memory of in which training context they had received actual footshock.

To determine whether the difference between retention latencies in Box B and Box C, which were both safe contexts, might have been caused because the contextual modifications made them not equally distinct from Box A, two additional experiments were performed. In the first experiment, Box C now served as Non-Shock context and Box B as Novel context. Rats were trained in Box C

(Non-Shock box) followed 1min later by footshock delivery in Box A (Shockbox). Forty-eight hours later, rats were tested in all three contexts in a randomized manner and without delay. As shown in **Figure 3C**, repeated-measures ANOVA for retention latencies in the three apparatuses revealed a significant context effect [$F_{(2,14)}$ = 37.83, p < 0.0001]. Retention latencies in the Shock box (Box A) and Non-Shock box (Box C) did not differ from each other (paired *t*-test: t_{12} = 0.77; p = 0.45) but were both significantly longer than those in the Novel box (Box B) (paired *t*-tests: Shock box vs. Novel box: t_{12} = 5.95; p < 0.0001; Non-Shock box vs. Novel box: t_{12} = 6.75; p < 0.0001). Thus, these findings indicate that rats had similar retention latencies in the two apparatuses (Shock box and Non-Shock box) they had visited on the training trial, irrespective of whether the Non-Shock box was Box B or C. To further examine whether Box B and C were both sufficiently distinct from Box A, rats were trained in Box A (Shock box) only and tested 48h later in all three contexts in a randomized manner. Repeated-measures ANOVA for retention latencies indicated a significant context effect [$F_{(2,28)}$ = 54.05, p < 0.0001). As shown in **Figure 3D**, retention latencies in Box A (322.1 ± 41.6s) were significantly longer than those in Box B (9.3 ± 0.9s; paired *t*-test: t_{19} = 7.15; p < 0.0001) and Box C (8.9 ± 1.0s; paired *t*-test: t_{19} = 7.14; p < 0.0001). Moreover, retention latencies in Box B and C did not differ from each other (paired *t*-test: t_{19} = 0.84; p = 0.41). These findings indicating that rats readily discriminate the different test contexts, thus, strongly suggest that the similar long retention latencies in the Shock and Non-Shock boxes were caused because rats were unable to remember in which of the two training contexts they had received actual footshock.

To determine whether the difficulty of rats to associate the footshock experience with the correct training context might be due to the short interval between the two training episodes, causing a temporal overlap of the two memory traces, in the next experiment rats were trained in the Non-Shock (Box B) and Shock (Box A) boxes with an increased interval of 2min. As shown in

Figure 3E, repeated-measures ANOVA for 48h retention latencies indicated a significant context effect [$F_{(2,18)} = 46.75$, $p < 0.0001$]. Retention latencies in the Shock box (Box A) were now significantly longer than those in the Non-Shock box (Box B) (paired *t*-test: $t_9 = 5.89$; $p < 0.0001$), indicating discrimination, whereas retention latencies for the Non-Shock and Novel boxes did not differ (paired *t*-test: $t_9 = 0.50$; $p = 0.63$). Thus, the longer interval between the two context exposures at training made it easier for the rats to associate the footshock experience with the correct training context.

Finally, rats were trained on the inhibitory avoidance discrimination task, with a 1min interval between both training episodes, and given an immediate posttraining subcutaneous injection of saline or memory-enhancing dose of yohimbine (0.3mg/kg). Forty-eight hours later, retention was tested by successively testing rats in the three contexts (Box A, B and C), in a randomized fashion. Two-way repeated measures ANOVA indicated a significant yohimbine effect [$F_{(1,66)} = 3.12$, $p = 0.03$] as well as a significant interaction between yohimbine treatment and test context [$F_{(2,66)} = 7.08$, $p = 0.001$]. As shown in **Figure 3F**, saline-treated rats had similar retention latencies in the Shock and Non-Shock boxes (paired *t*-test: $t_{11} = 0.21$; $p = 0.79$), indicating lack of discrimination. Yohimbine treatment significantly increased retention latencies in the Shock box ($p < 0.05$ vs. saline). Moreover, and importantly, retention latencies in the Shock box (Box A) were significantly longer than those in the Non-Shock box (Box B) (paired *t*-test: $t_{11} = 4.99$; $p < 0.01$), indicating discrimination. Yohimbine treatment did not alter retention latencies in the Novel box (Box C) ($p = 0.81$ vs. saline). These findings thus indicate that the posttraining yohimbine administration enhanced rats' specific memory of the association of footshock with the correct training context. Again, we found no evidence that the yohimbine induced generalization across contexts.

DISCUSSION

The present series of experiments was aimed at establishing and validating an inhibitory avoidance discrimination task which allows investigating accuracy of memory. Inhibitory avoidance is a commonly used behavioral task to investigate learning and memory processes in rodents. One of the great assets of this task is that a single footshock stimulation is sufficient to create robust long-term memory, making this task highly suitable to investigate drug effects on memory (Roozendaal and McGaugh, 2011). However, because retention of inhibitory avoidance training is usually tested only in the training context, it is difficult to define exactly what an animal has learned and whether a drug manipulation affected the accuracy of memory. The inhibitory avoidance discrimination task, which is based on training and testing rats in multiple contexts, does allow such more specific conclusions.

The first set of experiments was aimed at investigating whether the short context exposure during inhibitory avoidance training (typically less than 1min) is sufficient to induce accurate memory of the training context. Therefore, rats were trained on the classical inhibitory avoidance task and retention was tested 48h later in either the training apparatus or a novel, contextually modified, apparatus. This procedure of retention testing in the training context as well as a novel, safe context is sometimes used in contextual fear conditioning experiments (Wiltgen and Silva, 2007; Wang *et al.*, 2009). However, a training session in contextual fear conditioning experiments typically involves multiple footshocks and lasts longer than with inhibitory avoidance. Moreover, the training and novel apparatuses in our experiments were very similar and differed only from each other by some tape placed on the walls and floor. Our finding indicating that rats had long retention latencies in the training context whereas they readily entered the dark

compartment of the novel apparatus shows that the brief context exposure during training is sufficient to create robust and accurate memory of the training context. Next, we investigated whether it is possible to test the same rat in the other apparatus as well without altering retention latencies due to the repeated testing. When animals were tested with a 24h delay in the other apparatus, retention latencies in the Shock box were significantly shorter in comparison to retention latencies in that same apparatus on the previous day. Such shorter retention latencies in Box A 24h after rats had first been tested in a novel context (Box B) could be caused by different mnemonic processes such as extinction learning, safety learning or reconsolidation, that might all require memory consolidation (Cammarota *et al.*, 2004; Alberini *et al.*, 2011). However, when rats were tested for retention in both contexts without a delay, retention latencies of rats that were tested in the Shock box during the first or second test session were nearly identical. Thus, these findings indicate that repeated retention testing in both boxes without a delay is a suitable method for assessing accuracy of contextual memory in individual animals. To determine, as proof-of-principle experiment, whether it is possible to assess the effect of a posttraining pharmacological manipulation on memory accuracy, rats were treated with yohimbine after inhibitory avoidance training. We selected yohimbine for this experiment because of extensive evidence indicating that posttraining noradrenergic activation enhances the consolidation of inhibitory avoidance memory (Liang *et al.*, 1986; Introini-Collison *et al.*, 1991; Ferry *et al.*, 1999; McIntyre *et al.*, 2005). Our finding that yohimbine enhanced retention latencies in the Shock box but did not affect retention latencies in the Novel box indicates that the memory enhancement induced by yohimbine is not associated with generalization across contexts and, thus, reduced accuracy.

In the second set of experiments rats were trained on the inhibitory avoidance discrimination task. They were exposed to two inhibitory avoidance apparatuses,

with a 1min delay, but footshock was delivered only in one of the training contexts. Retention was tested 48h later in both apparatuses as well as in a novel apparatus they had not seen before. Our finding that retention latencies in both the Shock box and Non-Shock box were significantly longer than those in a Novel box indicates again that rats had good memory of the two contexts they visited during the training. However, the similar retention latencies in the Shock and Non-Shock boxes further indicates that the presentation of the two training contexts with such a short interval did not allow rats to create accurate memory of the association of footshock with the actual training context. Importantly, the inhibitory avoidance discrimination task incorporates the critical element of contextual discrimination as an episode for the assessment of episodic-like memory in rats. Episodic memory refers to memory for an event that holds spatio-temporal relations (Tulving *et al.*, 1983).

When the interval between training on the two inhibitory avoidance apparatuses was increased to 2min, retention latencies in the Shock box were significantly longer than those in the Non-Shock box and Novel box. Thus, these findings indicate that increasing the interval between the two training episodes facilitates discrimination between both events. Our finding that context discrimination can occur after a single exposure to the context is rather remarkable. Most discriminatory study protocols involving aversive shock require multiple trials, yet a large degree of generalization across different contexts is found (Chess *et al.*, 2009; Czerniawski and Guzowski, 2014). The ability to discriminate most likely depends on the duration of context exposure as well as the level of contextual modification (González *et al.*, 2003; McHugh and Tonegawa, 2007). To test whether posttraining drug manipulation can modulate memory of the association of footshock with the specific training context, yohimbine was administered immediately after training on the inhibitory avoidance discrimination task. In contrast to saline-treated control rats, rats administered yohimbine after

the training had significantly longer retention latencies in the Shock box than in the Non-Shock box. These findings thus strongly suggest that posttraining noradrenergic activation also facilitates the consolidation of memory of episodic-like aspects of the training. As a result, the subsequently formed memory yields a greater degree of accuracy. In agreement with these findings, we recently reported that norepinephrine infused posttraining into the basolateral amygdala enhanced memory precision in an object-in-context recognition task, increasing rats' ability to discriminate in which training context they had seen a particular object (Barsegyan *et al.*, 2014). In contrast, blockade of noradrenergic transmission in the basolateral amygdala with posttraining infusions of propranolol impaired memory on this task, indicating that endogenous noradrenergic activation is involved in regulating the strength and precision of episodic-like memory. These findings are relevant to investigations in humans with respect to whether the emotional impact of an experience not only influences the strength of declarative (episodic) memory, but is also associated with changes in memory accuracy, fidelity and susceptibility to incorporation of miss information (Morgan *et al.*, 2004; Porter *et al.*, 2008; Smeets *et al.*, 2009; Hoscheidt *et al.*, 2014).

New neuroscience technologies increase the need to investigate the exact role of brain regions in memory. *In vivo* neuroimaging, optogenetics and electrophysiological studies as well as molecular studies often show regional differences in brain activity after training that are difficult to interpret with general memory tasks. This is nicely illustrated by findings that drug administration after inhibitory avoidance training into a variety of brain regions, e.g. basolateral amygdala, hippocampus, dorsal striatum, insular cortex, anterior cingulate cortex and prefrontal cortex, induces highly comparable retention enhancement (Roozendaal and McGaugh, 2011).

Thus, although these findings clearly show that these brain regions are all involved

in regulating memory of inhibitory avoidance training, a possible specific role of these brain regions in particular aspects of information processing cannot be discerned. Experiments using context pre-exposure protocols have indicated that inhibitory avoidance can be dissociated into contextual and aversive components. Thus, *N*-Mehtyl-D-aspartic acid (NMDA) receptor blockade in the hippocampus after training does not impair inhibitory avoidance retention in rats pre-exposed to the training context, suggesting that hippocampal NMDA receptors are required for consolidation of a memory for the context, but not for context-shock association, and supporting the view that inhibitory avoidance is based upon an association between context and footshock (Roesler *et al.*, 1998; Roesler *et al.*, 2003).

The findings of the present report are consistent with this view, since they show that inhibitory avoidance is strongly dependent on context discrimination. In fact, subtle changes in the training apparatus resulted in a clear effect on discrimination at testing. Other studies have also investigated a possible unique role of brain regions in memory for the contextual and aversive components of inhibitory avoidance training. In inhibitory avoidance, rats learn that footshock occurs in a specific context. This information can be learned if rats are first exposed to the context and then, on a subsequent day, given a brief footshock in that context (Liang, 2001). With this two-phase inhibitory avoidance training procedure, it was found that the non selective muscarinic cholinergic agonistoxotremorine administered into the hippocampus after context exposure enhanced the subsequent conditioning whereas infusions administered after the footshock training were ineffective (Malin and McGaugh, 2006).

In contrast, oxotremorine infused into the rostral anterior cingulate cortex selectively enhanced memory when administered after the footshock training. Oxotremorine infused into the basolateral amygdala enhanced retention

when administered after either the context or footshock training, indicating a dissociable role of these brain regions in the processing of different aspects of inhibitory avoidance memory. We have shown in the present study that posttraining noradrenergic activation with yohimbine enhances memory of the association of footshock with the correct training context. Since the learning occurs within a single trial, the inhibitory avoidance discrimination task is suitable to study the strength and accuracy of episodic-like and contextual memory in combination with complementary molecular or physiological approaches in order to understand the contribution of different brain regions to different aspects of memory processing.

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Manuscript in preparation

Noradrenergic Activation of the Basolateral Amygdala Facilitates Discrimination on an Inhibitory Avoidance Task Via Downregulation of microRNA-134 in The Dentate Gyrus

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CHAPTER 04

ABSTRACT

Extensive evidence indicates that noradrenergic activation of the basolateral complex of the amygdala (BLA) regulates emotional arousal effects on memory consolidation via interactions with efferent brain regions. To investigate whether BLA activation enhances both the strength and specificity of memory storage processes, male Sprague-Dawley rats were trained on an inhibitory avoidance discrimination task. Rats were subsequently trained in two inhibitory avoidance apparatuses within a single training session, but footshock was delivered only in one of these two contexts. Norepinephrine administered into the BLA, but not central amygdala, immediately after the training enhanced both the strength and accuracy of episodic-like memory of the association of footshock with the correct training context. Norepinephrine administration into the BLA further downregulated plasticity - related microRNA-134 levels in the upper blade of the dentate gyrus 30min after inhibitory avoidance discrimination training and upregulated mRNA levels of its target genes cAMP regulated element-binding (CREB) and brain-derived neurotrophic factor (BDNF). Pharmacological down-re-

gulation of microRNA-134 levels in the hippocampus with infusions of a locked nucleic acid (LNA)-based antisense oligonucleotide facilitated the accuracy of episodic - like memory and discrimination without influencing the strength of memory. In contrast, overexpression of microRNA-134 levels in the hippocampus with a mimic blocked the facilitating effect of norepinephrine administration into the BLA on the accuracy of episodic - like memory without influencing the norepinephrine effect on memory strength. Norepinephrine infusions into the BLA after training on a one-trial inhibitory avoidance task, which does not require context discrimination, did not downregulate microRNA-134 levels in the dentate gyrus and pharmacological inhibition of microRNA-134 in the hippocampus did not affect memory of one-trial inhibitory avoidance training. Our findings indicate that noradrenergic activation of the BLA not only enhances the strength of memory of an emotionally arousing training experience but also facilitates discrimination of episodic - like memories, which requires down-regulation of microRNA-134 and neural plasticity changes within the dentate gyrus of the hippocampus.

/INTRODUCTION

Emotionally arousing experiences tend to be well remembered. Noradrenergic activation of the BLA enhances memory consolidation by modulating synaptic plasticity in efferent brain regions (Roozendaal *et al.*, 2009a). However, it is not clear whether emotionally arousing experiences increase or reduce accuracy of episodic details. Studies in human subjects find opposite results (Heuer and Reisberg, 1990; Hoscheidt *et al,*. 2014; Dunsmoor *et al.*, 2015). Animal studies generally have overlooked whether emotionally arousing experiences are remembered with greater detail or in a generalized manner.

The hippocampus is critical for episodic memory in rats (Steele and Morris, 1999) and in humans (Scolville and Milner, 1956; Tulving, 2002; Henke, 1999). The ability to remember details and to distinguish similar experiences has been ascribed to the dentate gyrus (McHugh *et al.*, 2007; Leutgeb *et al.*, 2007; Yassa and Stark, 2011; Bekinschtain *et al.*, 2012). The dentate gyrus and the BLA are anatomically (Pitkannen *et al.*, 2000) and functionally strongly connected (Ikegaya *et al.*, 1996). Posttraining noradrenergic activation of the BLA enhanced hippocampal plasticity by increase in Arc protein expression in the dorsal hippocampus (McIntyre *et al.*, 2005). Furthermore, under stress connectivity is unidirectional and grows stronger from BLA to hippocampus (Ghosh *et al.*, 2013; Fastenrath *et al.*, 2014). Modulation of hippocampal long-term potentiation by the amygdala seems to be a synaptic mechanism that links emotions with memory (Abe, 2001). However, there is controversy on how emotional memories modulate qualitative aspects of memory. Formation of long-term memory requires new protein synthesis (McGaugh, 2000) which is tightly regulated by microRNAs. MicroRNAs modulate translation of proteins by inhibiting expression for most target genes, by means of reducing steady-state RNA messenger levels of target

genes (Gao *et al.*, 2010). Memory is supported by synaptic plasticity in dendritic spines which is regulated by microRNAs. It has been reported that brain-specific microRNA-134, in cell cultured neurons prevents translation of LIM domain kinase 1 (LIMK1) and cAMP response element binding (CREB) protein which results in actin phosphorylation and gene transcription, respectively (Schraat, 2006; Siegel *et al.*, 2011) whereas brain derived neurotrophic factor (BDNF) relieves the inhibitory effect of microRNA-134 on Limk1 (Christensen and Schraat, 2009). CREB is required for memory consolidation of long term memory (Silva, 2008), as well as Limk1 dependent synaptic plasticity in dendritic spines (Fisher *et al.*, 2004; Rex *et al.*, 2010; Gavin *et al.*, 2012). In addition, brain derived neurotrophic factor (BDNF) might play a critical role underlying the consolidation of similar events (Bekinschtein *et al.*, 2011; Bekinschtein *et al.*, 2013). SIRT1 mediates transcriptional repression via an interaction with a complex containing the sequence specific transcription factor Yin Yang 1 and mice lacking SIRT1 activity have been shown to display impaired synaptic plasticity as well as impaired memory in a fear conditioning task (Gao *et al.*, 2010). In contrast, downregulation of microRNA-134 upon hippocampal injection of a locked nucleic acid (LNA)-based antisense oligonucleotide restored CREB and BDNF levels and rescued plasticity and memory impairments in SIRT1 knockout mice whereas lentivirus mediated overexpression of microRNA-134 in the hippocampusled to aberrant LTP and impaired fear-conditioning memory (Gao *et al.*, 2010). Understanding the regulation exerted by microRNAs in the hippocampus on an emotionally arousing training task might bring a deeper understanding on how emotions modulate accuracy of episodic memory.

In this study we investigate the effect of posttraining norepinephrine infusions on an episodic-like inhibitory avoidance discrimination (IAD) task. IAD task allows investigating accuracy of both contextual and episodic-like aspects of memory. Rats were subsequently trained in two distinctly different inhibitory

avoidance apparatuses with a short delay, but were given footshock in only one of these contexts. On a 48h retention test, the rats were tested in these two training contexts as well as in a novel context. Inhibitory avoidance discrimination task allows investigating whether rats remember the two contexts they visited during the trainingas well as the specific episodic-like memory of the association of footshock with the correct training context. With the aid of posttraining norepinephrine infusions into the BLA we will investigate the role of norepinephrine on modulating accuracy of memory and unravelling a possible mechanism.

MATERIALS AND METHODS

Subjects

Male adult Sprague-Dawley rats (280–320g at the time of surgery) from Charles River Breeding Laboratories (Kisslegg, Germany) were housed individually in a temperature-controlled (22°C) vivarium room and maintained on a 12h/12h light/dark cycle (lights on > 07:00h-19:00h) with *ad libitum* access to food and water. Training and testing were performed during the light phase of the cycle between 10:00h-15:00h. All experimental procedures were in compliance with the European Communities Council Directive on the use of laboratory animals of November 24, 1986 (86/609/EEC) and approved by the Institutional Animal Care and Use Committees of the University of Groningen and Radboud University Nijmegen, The Netherlands.

Cannula Implantation

Rats, adapted to the vivarium for 1 week, were anesthetized with a subcutaneous

injection of ketamine (37.5mg/kg of body weight; Alfasan) and dexmedetomidine (0.25mg/kg; Orion) and received the non-steroidal analgesic carprofen (4mg/kg; Pfizer). Surgery was performed according to a standardized protocol (Fornari *et al.*, 2012a). The rat was positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA), and two stainless steel guide cannulae (23 gauge; Component Supply Co/SKU Solutions, Fort Meade, FL) were implanted bilaterally with the cannula tips 2 mm above the BLA [15mm; coordinates: anteroposterior (AP): 2.8mm posterior to Bregma; mediolateral (ML): +5.0mm lateral to midline; dorsoventral (DV): 6.5mm below skull surface], 2mm above the central amygdala (CeA) (15mm; coordinates: AP: -2.2mm; ML: ±4.1mm; DV: -5.8mm) or 1.5mm above the dorsal hippocampus (11mm; coordinates: AP: -3.6mm; ML: ±1.9 mm; DV: -2.6mm) according to the rat atlas of Paxinos and Watson (2007). The cannulae were affixed to the skull with surgical screws and dental cement. Stylets (11 or 15mm long 00-insect dissection pins) were inserted into each cannula to maintain patency and were removed only for the infusion of drugs. After surgery, rats were administered atipamezole hydrochloride (0.25mg/kg, s.c.; Orion) to reverse anesthesia and were subsequently injected with 3ml of saline to facilitate clearance of the drugs and prevent dehydration. The rats were allowed to recover for a minimum of 7 days before initiation of the training and were handled 3 times for 1min each during this recovery period to accustom them to the infusion procedure.

Inhibitory Avoidance Discrimination Task and Procedures

Rats were subsequently trained in two inhibitory avoidance apparatuses within a single training session, but footshock was delivered only in one of these two contexts. On the retention test, 48h after training, rats were tested in the previously seen Shock and Non-Shock boxes as well as in a Novel Box in a randomized order. This experimental design allows investigating whether rats accurately remember the two apparatuses they had visited

during training and discriminate in which of these two contexts they had received actual footshock. Each apparatus consisted of a trough-shaped alley (91cm long, 15cm deep, 20cm wide at the top, and 6.4cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor (McGaugh *et al.*, 1988). The starting compartment (31cm) was made of opaque white plastic and was well lit; the shock compartment (60cm) was made of two dark, electrifiable metal plates and was not illuminated.

The 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 Non-Shock Box had four vertical white stripes (2cm wide) taped in the dark compartment together with tape placed on the floor closing the gap between the two plates. The Novel box had two white circles (3.5cm diameter) taped on each wall of the dark compartment, and the gap between both plates was closed with tape. All three inhibitory avoidance apparatuses were located next to one other in a sound -and light- attenuated room. For a detailed description of the task, **see Atucha and Roozendaal (in press)**.

For training, rats were initially placed in the light compartment of the Non-Shock box and could explore the entire apparatus for 20s without any footshock delivery. Afterwards, the rats were removed from the apparatus and, after a delay of 1min, placed in the light compartment of the second inhibitory avoidance apparatus (Shock box). After the rat completely entered the dark compartment, the sliding door was closed and a single inescapable footshock (for BLA -and CeA- cannulated rats: 0.6mA; 1s; for hippocampus-cannulated rats: 0.38mA, 1s) was delivered. The rats were removed from the dark compartment 20s after termination of footshock and, after drug treatment, returned to their home cages. Some rats were sacrificed at 15min, 30min or 1h after training and drug

treatment for molecular analysis. Others were left undisturbed in their home cages and tested for retention 48h later. For retention testing, the rats were tested in a randomized order in the previously seen Non-Shock and Shock boxes and, additionally, in a Novel box they had not seen before. For all three boxes, the rats were placed in the starting compartment of the inhibitory avoidance apparatuses and their latency to enter the dark compartment with all four paws (maximum latency of 600s) was measured. Shock was not administered on the retention test trial.

Some rats were trained in the Shock box only and tested 48h later for retention in that apparatus and a Novel box. For training, the rats were placed in the starting compartment of the Shock box, facing away from the door, and were allowed to freely explore the apparatus. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.60mA for 1s) was delivered. Rats were removed from the shock compartment 20s later and, after drug treatment, returned to their home cages. Some rats were sacrificed 30min after training and drug treatment for molecular analysis. Retention was tested 48h after training by placing the rat into the starting compartment of either the Shock box or Novel box and measuring the latency to enter the dark compartment with all four paws (maximum latency of 600s). Immediately after testing in the first context, rats were placed in the other apparatus and their latencies to enter that dark compartment were measured.

Local Drug Treatments

Norepinephrine (Sigma-Aldrich) dissolved in saline or saline control was administered into the BLA (1.0µg in 0.2µl), CeA (1.0µg in 0.2µl) or dorsal hippocampus (2.5µg in 0.5µl) immediately after the training trial. Some rats received intra-BLA infusions of norepinephrine or saline 3h after the training trial. For the molecular

experiments, rats received norepinephrine (1.0µg in 0.2µl) into the BLA in one hemisphere and saline control (0.2µl) was administered into the other hemisphere immediately after the training trial. Some rats received the norepinephrine and saline infusions into the BLA without training. The use of posttraining drug administration provides direct support for the view that the treatment affects memory consolidation processes and that retention performance is, thus, not confounded by possible effects on attentional motivational, or sensory-perceptual mechanism at the time of training or test (McGaugh, 1966).

Cholesterol-tagged LNA-antagomir targeting microRNA-134 (Ant-134; 10 pmol in 0.5µl; Exiqon A/S, Vedbaek, Denmark) or a non-targeting scrambled version of the antagomir (Scr; 10 pmol in 0.5µl; Exiqon A/S, Vedbaek, Denmark) was dissolved in saline and administered into the hippocampus immediately after training on the inhibitory avoidance discrimination or inhibitory avoidance task. Some rats received intra-hippocampus infusions of the antagomir or scrambled control 3h after the training trial.

Overexpression of microRNA-134 was performed by Syn-rno-miT-134-5p, (mimic-134) mature sequence (5'-UGUGACUGGUUGACCAGAGGGG-3'; Qiagen, Hilden, Germany) or a non-target control (NT; Qiagen, Hilden, Germany). First, the mimic or NT was resuspended in 100µl RNAse free water and further diluted 2:3 in Screenfect buffer (Screenfect® A, Eggenstein-Leopoldshafen, Germany). Subsequently, the resuspension was dissolved in 25µl of a mixture of Screenfect buffer and Screenfect reagent (Screenfect® A) in 1:2.73. A final concentration of 0.25 pmol in 0.5µl saline was administered into the hippocampus.

Bilateral infusions of drug or control were given by using 30 gauge injection needles connected to a 10µl Hamilton microsyringe with polyethylene (PE-20) tubing. For

BLA and CeA infusions, the injection needle protruded 2.0mm beyond the cannula tips and a 0.2µl injection volume per hemisphere was infused during 30s by an automated syringe pump (StoeltingCo. Dublin, Ireland). For bilateral hippocampal infusions, the injection needles protruded 1.5mm beyond the tip of the cannula and a 0.5µl injection volume per hemisphere was infused over a period of 75s. The injection needles were retained within the cannulae for an additional 20s after drug infusion to maximize diffusion and to prevent backflow of drug into the cannulae. Norepinephrine was freshly prepared before each experiments whereas stock solutions were prepared for NT, mimic and Ant-134 and kept at -20ºC.

Cannula Placement Verification and Tissue Collection

After completion of behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital and perfused transcardially with a 0.9% saline solution followed by 4% formaldehyde. The brains were removed and stored in 4% formaldehyde. At least 24h before sectioning, brains were placed in a 25% sucrose solution in water for cryoprotection. Coronal sections of 50µm were cut on a cryostat and collected in Tris-buffered saline (TBS) and mounted on gelatin-coated slides, stained with cresyl violet and examined by light microscopy by an observer blind to drug treatment condition.

Determination of placement of injection needle tips in the BLA, CeA and dorsal hippocampus was made according to the atlas plates of Paxinos and Watson (2007). Only animals with needle tips located within the boundaries of the BLA, CeA and/or dorsal hippocampus and without extensive tissue damage at the target areas were included into the behavioral and molecular analyses.

For molecular experiments, rats were deeply anesthetized with an overdose of

sodium pentobarbital at 15min, 30min or 1h after training for microRNA/mRNA measurements. After decapitation, the brains were rapidly removed 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. For verification of cannula placement in the BLA of flash-frozen brains, coronal sections of 50µm were cut on a cryostat and collected on gelatin-coated slides and let to dry. Slides were then fixed in 100% acetone for at least 30min and let to dry before staining with cresyl violet. Determination of the injection needle placement and exclusion of animals was performed as described above.

RNA Isolation and Quantitative RT-PCR

Coronal slices of the dorsal hippocampus at a thickness of 350µm were cut on a cryostat and further dissected using a 0.75mm brain puncher (Stoelting Co). Bilateral punches from the different subfields of the dorsal hippocampus (dentate gyrus upperblade, dentate gyrus lower blade, CA1 and CA3; -2.64 to -3.86mm in range AP) were collected from three consecutive slices to a total of 6 punches. Punches were preserved at -20°C for at least 16h and later at -80°C in RNA later ICE (Ambion® Life Technologies, USA).

Following tissue dissection, RNA later ICE was removed and total RNA was isolated using miRNAeasy® kit. The concentration and purity of total mRNA was assessed using Nanodrop™. For optimized detection and quantification of miRNAs and mRNAs, first DNAse treatment (Thermo Scientific) was carried out as indicated in the manufacturer's protocol. Second, cDNA was synthesized from total RNA using miScript II kit (Qiagen) according to the manufacturer's protocol. Using first-strand cDNA as a template, qPCR reactions were performed for each miRNA using miRNA-specific forward primers of the entire mature miRNA sequence and the Universal qPCR reverse primer. For mRNA qPCR reactions mRNA-specific for-

ward and reverse primers were used. Detection of Creb was performed using the following primers: forward (5'-TCAGCCGGGTACTACCATTC-3') and reverse (5'-TTCAGCAGGCTGTGTAGGAA-3'). Detection of Limk1 was performed using the following primers: forward (5'-CCTCCGAGTGGTTTGTCGA-3') and reverse (5'-CAACACCTCCCCATGGATG-3'). Detection of Bdnf was performed using the following primer: forward (5'-GGTCACAGCGGCAGATAAAAAGAC-3') and reverse (5'-TTCGGCATTGCGAGTTCCAG-3'). Detection of microRNA-124 was performed using the following primer (5'-TAAGGCACGCGGTGAATGCC-3'). Detection of microRNA-132 was performed using the following primer (5'-TAACAGTCTACAGCCATGGTCG-3').Detection of microRNA-134 was performed using the following primer (5'-TGTGACTGGTTGACCAGAGGGG-3'). Detection of microRNA-137 was performed using the following primer (5'-TTATTGCTTAAGAATACGCGTAG-3'). Detection of microRNA-138 was performed using the following primer (5'- AGCTGGTGTTGTGAATCAGGCCG-3'). Samples were normalized to U6 transcript using the following primers: forward (5'-GCTTCGGCAGCACATATACTAAAAT-3') and reverse (5'-CGCTTCACGAATTTGCGTGTCAT-3').

Real-time PCR analysis was performed with miScript SYBR® Green PCR kit (Qiagen) in a Rotorgene quantitative real-time PCR thermo-cycler. All take off Ct values used for analysis were averaged except for those considered outliers being higher or lower than (2 x SD ± mean). Relative expression was calculated using comparative C_T method and normalized to the expression of U6 snRNA and with the ΔΔCt method. For some groups levels of microRNA and mRNA in the hippocampus ipsilaterally to the intra-BLA norepinephrine infusion side of each animal were expressed as the ratio to the levels on the contralateral saline-infused side. For other groups expression levels were compared between animals.

Statistics

Data are presented as mean + SEM. Inhibitory avoidance retention test latencies were analyzed with either two -or three- way ANOVAs with posttraining drug infusions (in either 1 or 2 brain regions) as between-subject variables and latencies of individual animals in the three test environments as within-subject variable. *Post hoc* comparisons used unpaired and paired *t*-tests to determine the source of the detected significances, when appropriate. MicroRNA and mRNA expression levels were analyzed with unpaired or paired *t*-tests. For all comparisons, a probability level of < 0.05 was accepted as statistical significance. The number of rats per group is indicated in the figure legends.

RESULTS

Norepinephrine Administration into the BLA Enhances the Accuracy of Memory of an Inhibitory Avoidance Discrimination Training Experience

We first investigated whether norepinephrine administered bilaterally into the BLA immediately after training on the inhibitory avoidance discrimination task would enhance the accuracy of memory of this training. **Fig. 1A** shows a schematic summarizing the training and test procedures. Rats were first trained in the Non-Shock box, followed 1min later by footshock delivery in the Shock box. Norepinephrine (1.0µg in 0.2µl) or saline control was infused bilaterally into the BLA immediately after the training trial. On a 48h retention test trial, rats were tested in the previously seen Shock and Non-Shock boxes as well as in a Novel box. Longer retention test latencies in the Shock Box than the Non-Shock would indicate that the rats had episodic-like memory of the association of footshock with the correct training context. As shown in **Fig. 1B**, two-way ANOVA for 48h

retention latencies showed a non-significant intra-BLA norepinephrine effect [$F_{(1,46)} = 2.90$; $p = 0.10$], but a significant context effect [$F_{(2,46)} = 20.83$; $p < 0.0001$] as well as a significant interaction between both parameters [$F_{(2,46)} = 11.19$; $p = 0.0002$]. Pair-wise *t*-test comparisons indicated that retention latencies of saline-treated rats in the Shock box did not differ from those in the Non-Shock box ($p = 0.77$), but were both significantly longer than those in the Novel box (both, $p < 0.05$). Hence these findings indicate that although saline-treated control rats remembered the two boxes they visited during the training, they were unable to discriminate in which training context they had received actual footshock.

In contrast, norepinephrine-treated rats had significantly longer retention latencies in the Shock box than in the Non-Shock box ($p < 0.001$) or Novel box ($p < 0.001$). Furthermore, rats administered norepinephrine into the BLA had significantly longer latencies in the Shock box as compared to those of saline-treated rats ($p < 0.001$). This memory enhancement was specific for the Shock box as retention latencies in the Non-Shock box ($p = 0.15$) or Novel box ($p = 0.38$) did not differ between the two groups. Thus, these findings indicate, that rats administered norepinephrine into the BLA, in contrast to saline-treated rats, had accurate and enhanced memory of the association of footshock with the actual training context.

In contrast to immediate posttraining administration of norepinephrine, delayed infusions of norepinephrineinto the BLA given 3h after inhibitory avoidance discrimination training did not affect retention performance. As shown in **Fig. 1C**, two-way ANOVA for retention latencies indicated a significant context effect [$F_{(2,30)} = 0.15$; $p = 0.0009$], but no intra-BLA norepinephrine effect [$F_{(1,15)} = 0.23$; $p = 0.64$] or interaction between both factors [$F_{(2,30)} = 0.15$; $p = 0.85$]. Thus, these findings indicate that the norepinephrine-induced enhancement of memory accuracy was found only when administered during a critical time window shortly

after the training experience and thus likely is dependent on the consolidation of memory. In addition, the norepinephrine effect was specific to the BLA, as immediate posttraining infusions of norepinephrine (1.0µg in 0.2µl) into the adjacent CeA did not influence 48h retention performance [$F_{(1,12)} = 0.02$; $p = 0.89$].

Figure 1 ····· Single Column

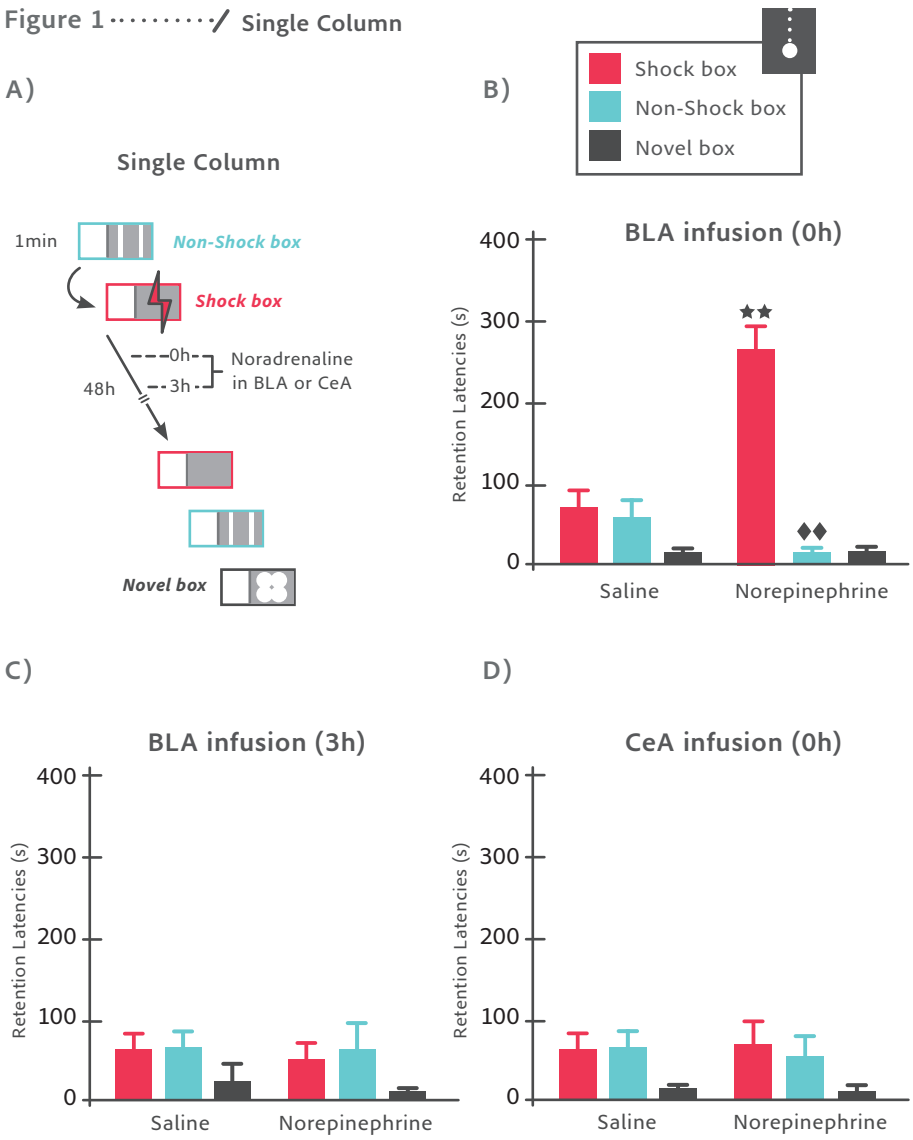


FIGURE 1:

NOREPINEPHRINE ADMINISTRATION INTO THE BLA ENHANCES THE ACCURACY OF MEMORY OF AN INHIBITORY AVOIDANCE DISCRIMINATION TRAINING EXPERIENCE

Inhibitory avoidance retention latencies (mean + SEM) in seconds.

1A. Schematic of the behavioral procedure. Rats were trained in the Non-Shock box (blue) without footshock, followed 1min later by footshock training in the Shock box (red). Norepinephrine (1µg in 0.2µl) or saline was administered bilaterally into the BLA or CeA either immediately posttraining or 3h after the training. On the 48h retention test, rats were tested in the Shock and Non-Shock boxes as well as a Novel box (grey).

1B. Rats administered saline into the BLA immediately after the training had similar retention latencies in the Shock box and Non-Shock box, indicating lack of episodic-like memory. In contrast, norepinephrine-treated rats had significantly longer retention latencies in the Shock box than in the Non-Shock box, indicating accurate episodic-like memory of the association of footshock with the correct training context. Furthermore, the norepinephrine administration increased retention latencies in the Shock box as compared to those of saline-treated rats, indicating memory enhancement. ★★ $p < 0.01$ versus saline treated rats. ♦♦ , $p < 0.01$ versus Shock box within the same group. N = 12-13 rats/group.

1C. Delayed infusions of norepinephrine (1µg in 0.2µl) into the BLA given 3h after training did not increase memory accuracy or retention latencies in comparison to saline-treated rats. N = 8-9 rats/ group.

1D. Posttraining norepinephrine (1µg in 0.2µl) administration into the adjacent CeA did not alter retention latencies. N = 7-9 rats/ group.

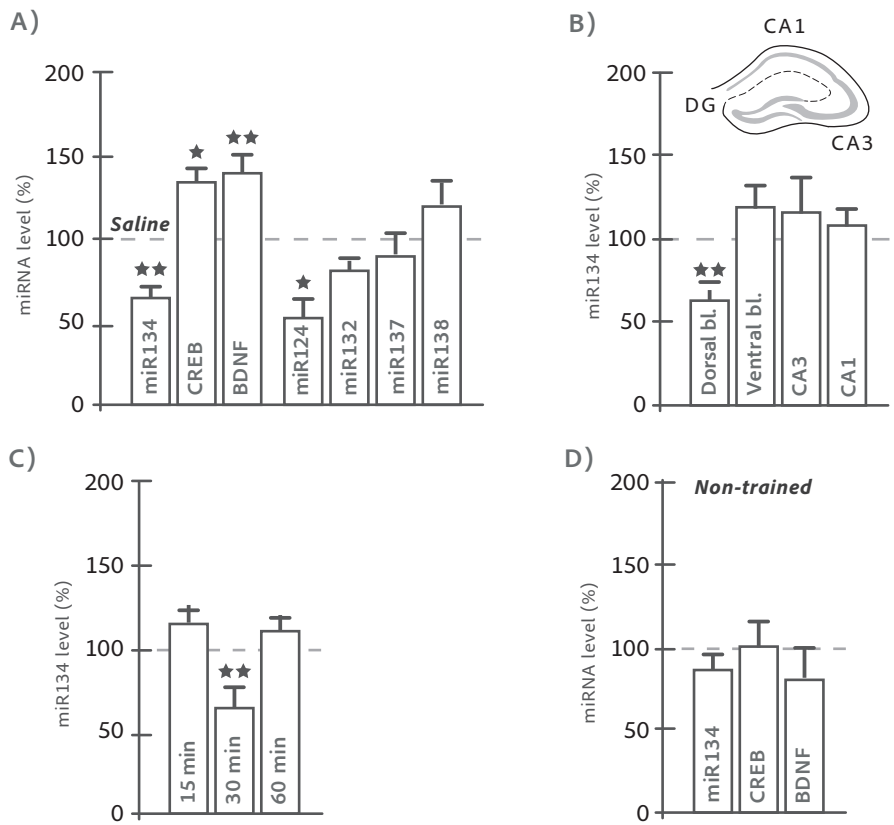
Norepinephrine Administration into the BLA Alters microRNA-134 Levels in the Dentate Gyrus

Accurately discriminating in which of the two training contexts the footshock was delivered is a specific aspect of episodic-like memory (Tulving, 2002). Specifically, the ability to discriminate between similar events has been ascribed to the dentate gyrus (Kesner *et al.*, 2000; Leutgeb and Moser, 2007) and more specifically the dorsal blade of the hippocampus seems to be involved in discrimination and pattern separation (Schmidt *et al.*, 2012). The BLA and dentate gyrus are anatomically (Pitkanen *et al.*, 2000) and functionally strongly connected (Ikegaya *et al.*, 1996; Vouimba *et al.*, 2006). Therefore, in the next series of experiments

we investigated whether norepinephrine infusions into the BLA after inhibitory avoidance discrimination training induces synaptic plasticity changes at the molecular level in the dentate gyrus. Since microRNAs modulate translation processes, we first screened for plasticity and memory-related microRNAs in the dentate gyrus.

To examine the effect of norepinephrine administration into the BLA on microRNA expression during the consolidation period, we trained rats on the inhibitory avoidance discrimination task and administered either norepinephrine (1.0µg in 0.2µl) or saline into the BLA immediately after the training. Rats were sacrificed

Figure 2



30min later and expression levels of different microRNAsand mRNAs were assessed in the dorsal blade of the dentate gyrus. As shown in **Figure 2A**, norepinephrine administration intothe BLA significantly downregulated premature microRNA-134 expression levels ($p < 0.01$ relative to saline) as well as microRNA-134 expression levels ($p < 0.01$) in the dorsal blade of the dentate gyrus. We further found that the norepinephrine administration into the BLA upregulated both Creb mRNA ($p < 0.05$) as well as Bdnf mRNA levelsin the dorsal blade of the dentate gyrus ($p < 0.01$). Creb and Bdnf are both downstream targets of microRNA-134. The norepinephrine administration also downregulated microRNA-124 levels ($p < 0.05$). The norepinephrine administration did not alter levels of plasticity-related microRNA-132 ($p = 0.10$), microRNA-137 ($p = 0.62$) or microRNA-138 ($p = 0.13$).

FIGURE 2:

NOREPINEPHRINE INFUSIONS INTO THE BLA ALTERS MICRORNA-134 LEVELS IN THE DENTATE GYRUS

Expression levels of different microRNAs and mRNAs in the dentate gyrus of rats administered norepinephrine (1µg in 0.2µl) into the BLA in percentage relative to those of saline-treated rats. Rats were trained on the inhibitory avoidance discrimination task (see Fig. 1A) and received posttraining infusions of norepinephrine or saline into the BLA.

2A. Norepinephrine administration into the BLA downregulated expression levels of microRNA-134 and microRNA-124 in the dorsal blade of the dentate gyrus 30min after training and drug administration. In contrast, Creb and Bdnf mRNA levels were upregulated in the dorsal blade of the dentate gyrus 30min after training and norepinephrine administration ★p < 0.05, ★★ p < 0.01, versus saline-treated group. N = 7-8 rats/group.

2B. Norepinephrine (1µg in 0.2µl) administration into the BLA downregulated microRNA-134 levels selectively in the dorsal blade of the dentate gyrus and had no effect in the ventral blade of the dentate gyrus, CA3 or CA1 subfields. ★★ p < 0.01, versus the saline-treated group. N = 6-7 rats/group.

2C. Temporal dynamics of norepinephrine-induced downregulation of microRNA-134 levels in the dorsal blade of the dentate gyrus in comparison to saline treatment. MicroRNA-134 levels were downregulated 30min after training but were unaffected at either 15 or 60min after training and drug treatment. ★★ p < 0.01 versus the saline-treated control. N = 6-7 rats/group.

2D. Norepinephrine infusions into the BLA to non-trained control rats did not alter microRNA-134, Creb or Bdnf expression levels in the dorsal blade of the dentate gyrus 30min after drug administration. N = 6-7 rats/group.

Next, we investigated whether the norepinephrine effect on changing microRNA-134 levels was selective to the dorsal blade of the dentate gyrus or whether similar changes are found in other hippocampal subfields. As shown in **Fig. 2B**, norepinephrine administration into the BLA after inhibitory avoidance discrimination training downregulated microRNA-134 levels in the dorsal blade of the dentate gyrus ($p < 0.01$ relative to saline) but did not alter microRNA-134 expression levels in the ventral blade of the dentate gyrus ($p = 0.087$), CA3 ($p = 0.34$) or CA1 regions ($p = 0.15$).

We further examined the time course of the norepinephrine effect on decreasing microRNA-134 levels in the dentate gyrus. As shown in **Fig. 2C**, microRNA-134 levels in the dorsal blade of the dentate gyrus were significantly lower at 30min ($p < 0.01$ relative to saline) after the training and drug administration, but did not differ at either 15min ($p = 0.35$) or 1h ($p = 0.14$).

Finally, we investigated whether the norepinephrine effect on downregulating microRNA-134 levels in the dentate gyrus is dependent on the training experience. Therefore, in the next experiment norepinephrine (1.0μg in 0.2μl) or saline was administeredbilaterallyinto the BLA of non-trained rats and animals were sacrificed 30min later. As shown in **Fig. 2D**, the norepinephrine administration in non-trained control rats did not significantly alter levels of microRNA-134 ($p = 0.13$), Creb ($p = 0.91$) or Bdnf ($p = 0.29$) mRNA levels in the dorsal blade of the dentate gyrus.

These findings indicate that norepinephrine infusions into the BLA after training on the inhibitory avoidance discrimination task induces a time-dependent downregulation of microRNA-134 levels and an increase in mRNA levels of its downstream targets Creb and Bndf in the dorsal blade of the dentate gyrus.

Pharmacological Manipulation of microRNA-134 Levels in the Dorsal Hippocampus Selectively Alters the Accurary of Memory

In the previous section, we showed that norepinephrine infusions into the BLA downregulated microRNA-134 levels in the dorsal blade of the dentate gyrus. To investigate whether such downregulation of microRNA-134 is involved in regulating the norepinephrine effect on memory accuracy, in the next series of experiments we pharmacologically manipulated microRNA-134 levels in the dorsal hippocampus after training on the inhibitory avoidance discrimination task. In the first experiment, we downregulated microRNA-134 levels an LNA-based microRNA-134 antagomir (10 pmol in 0.5µl) or scrambled control (10 pmol in 0.5µl) administered bilaterally into the hippocampus immediately after inhibitory avoidance discrimination training **(Fig. 3A)**.

As shown in **Fig. 3B**, downregulation of microRNA-134 in the hippocampus after inhibitory avoidance discrimination training selectively enhanced accuracy of episodic-like memory, without influencing the strength of the memory. Two-way ANOVA for 48h retention latencies indicated a significant antagomir treatment effect [$F_{(1,23)} = 5.18$; $p = 0.03$], a significant context effect [$F_{(2,46)} = 22.04$; $p < 0.0001$] as well as a significant interaction between both factors [$F_{(2,46)} = 5.81$; $p = 0.005$]. Pair-wise *t*-test comparisons indicated that retention latencies of rats administered scrambled control infusions in the Shock box did not differ from those in the Non-Shock box ($p = 0.61$), whereas both were significantly longer than in the novel box (both, $p < 0.0001$). The antagomir microRNA-134 infusions into the hippocampus did not entirely mimic the norepinephrine effect in the BLA. While norepinephrine in the BLA enhanced the retention latencies in the Shock box, the antagomir infusions into the hippocampus did not have an effect on retention latencies in the Shock box in com-

parison to those of rats administered the scramble (p = 0.68). Nevertheless, posttraining antagomir infusions into the hippocampus after inhibitory avoidance discrimination training mimicked the effect of posttraining norepinephrine treatment intothe BLA in relation to accuracy of memory. Retention latencies in the Shock box were significantly longer than those in the Non-Shock box (p < 0.0001). As shown in **Fig. 3C**, the antagomir effect on selectively increasing accuracy of memory was not dependent onthe footshock intensity, since with a higher footshock intensity we observe a similar effect on discrimination despite the finding that retention latencies were significantly longer. Two-way ANOVA for 48h retention latencies indicated a significant antagomir treatment effect [$F_{(1,24)}$ = 10.02; p = 0.004], a significant context effect [$F_{(2,48)}$ = 27.21; p < 0.0001] as well as a significant interaction between both factors [$F_{(2,48)}$ = 7.31; p= 0.0002]. Rats administered the scrambled into the hippocampus had similar retention latencies in the Shock box and Non-Shock box (p= 0.74). In contrast, rats administered the antagomir had significantly longer retention latencies in the Shock box than in the Non-Shock box (p < 0.0001). This effect was caused because retention latencies in the Non-Shock box significantly decreased after the antagomir treatment (p < 0.05 relative to scrambled) whereas retention latencies in the Shock box were not altered after antagomir infusion (p = 0.98).

Next, we investigated whether the effect of the antagomir infusion on selectively enhancing accuracy of memory is specific to this drug treatment or that intrahippocampal administration of norepinephrine, another neural plasticity and memory-enhancing drug, would induce similar effects. Rats were trained on the inhibitory avoidance discrimination task and received immediate posttraining infusions of norepinephrine (2.5µg in 0.5µl) or saline into the hippocampus. As shown in **Fig. 3D**, two-way ANOVA for 48h retention latencies indicated, a significant antagomir treatment effect [$F_{(3,49)}$ = 3.58; p = 0.02], a significant context effect [$F_{(2,98)}$ = 44.25; p < 0.0001] as well as a significant interaction between both factors [$F_{(6,98)}$ = 8.98; p < 0.0001]. Posttraining norepinephrine infusions into the hippocampus significantly increased

retention performance but did not facilitate discrimination, but instead increased generalization. Retention latencies for the Shockbox and Non-Shock box did not differ for saline treated (p = 0.94) nor for norepinephrine-treated rats (p = 0.50). This experiment might indicate that norepinephrine and microRNA-134 affect the consolidation of memory of different aspects of information acquired during training on the inhibitory avoidance discrimination task.

Further, in order to investigate whether the effect of norepinephrine infusion into the BLA on downregulation of microRNA-134 levels in the dentate gyrus play a role in regulating the norepinephrine effect on the increased accuracy of memory, we bilaterally overexpressed microRNA-134 in the hippocampus with local infusions of a mimic (0.25 pmol in 0.5µl) or non-target control (0.25 pmol in 0.5µl) and simultaneously infused norepinephrine (1.0µg in 0.2µl) into the BLA immediately after inhibitory avoidance discrimination training. As shown in **Fig 3E**, repeated measures ANOVA indicated BLA treatment effect on retention latencies [$F_{(1,49)}$=79.00; p<0.0001] although the hippocampus treatment did not show a significant effect on retention latencies [$F_{(1,49)}$=2.29; p=0.13], neither the interaction between both factors [$F_{(1,49)}$=0.82; p=0.37]. Nevertheless, context exposure showed a significant effect on retention latencies [$F_{(2,98)}$ = 44.25; p < 0.0001] as well as the interaction between treatment in the BLA and context [$F_{(2,89)}$ = 11.29; p < 0.0001] and the hippocampus and the context [$F_{(2,89)}$ = 9.73; p = 0.00014]. The interaction between treatment in the BLA, treatment in the hippocampus and the context exposure was significant [$F_{(2,89)}$ = 6.92; p = 0.0015]. For rats that received the non-targeting control solution into the hippocampus, the findings were almost identical to those described above, indicating that the norepinephrine infusions into the BLA enhanced both the accuracy of memory as well as memory strength. Pair-wise *t*-test comparisons indicated that rats administered saline infusions into the BLA had similar retention latencies in the Shock box and Non-Shock box (p = 0.97). The norepinephrine infusion into the BLA induced accurate memory as

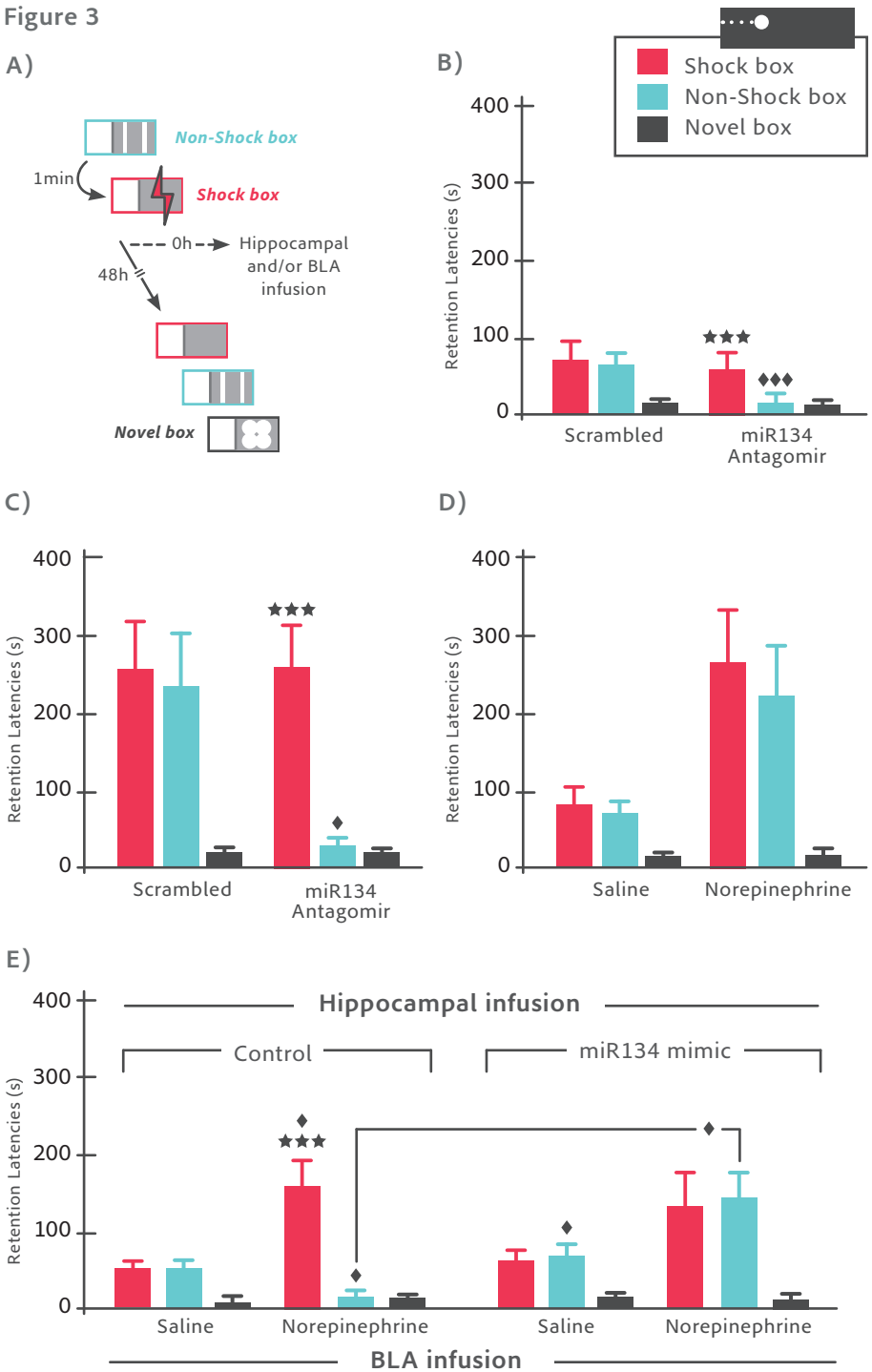
indicated by significantly longer retention latencies in the Shock box compared to the Non-Shock box ($p < 0.0001$). Moreover, it increased the strength of memory as indicated by significantly longer retention latencies in the Shock box than saline-treated control rats ($p < 0.05$). The mimic infusion into the hippocampus did not significantly alter retention latencies in rats administered saline infusions into the BLA. In contrast, overexpression of microRNA-134 in the hippocampus completely blocked the effect of the norepinephrine infusion on inducing discrimination. Retention latencies in the Shock box did not differ significantly from those in the Non-Shok box ($p = 0.84$).

However, the overexpression of microRNA-134 in the hippocampus did not block the memory-enhancing effect of norepinephrine administration into the BLA. Retention latencies in the Non-Shock box were significantly longer than those of rats administered non-target control (0.25 pmol in 0.5μl) into the hippocampus ($p < 0.01$). Thus, these findings indicate that counteracting the effect of norepinephrine infusions into the BLA on the downregulation of microRNA-134 levels with concomitant infusions of a mimic of microRNA-134 into the hippocampus selectively blocked the norepinephrine effect on increasing accuracy of memory without influencing the norepinephrine on strengthening the memory trace. These finding provide strong evidence for the view that downregulation of microRNA-134 plays an important role in facilitating discrimination.

One critical element of the inhibitory avoidance discrimination task is that rats are trained in two highly ambiguous inhibitory avoidance apparatus. Therefore we raised the question whether the norepinephrine effect on downregulating microRNA-134 levels in the dentate gyrus and increasing discrimination is specific to this training protocol. In the next experiment we trained rats in the Shock box only. Norepinephrine (1.0μg in 0.2μl) or saline was infused bilaterally into the BLA immediately after the training. As shown in **Fig. 3F**, repeated measures ANOVA for 48h retention latencies

indicated a significant intra-BLA norepinephrine effect [$F_{(1,20)} = 6.84$; $p = 0.02$] as well as a context exposure effect [$F_{(1,20)} = 22.83$; $p < 0.001$] and a significant interaction between both factor [$F_{(1,20)} = 7.06$; $p = 0.015$]. The norepinephrine administration significantly enhanced retention latencies in the Shock box ($p < 0.0001$ relative to saline) whereas retention latencies in the Novel box did not differ ($p = 0.95$). Despite the fact that the norepinephrine infusions enhanced memory, assessment of microRNA-134 levels in the dorsal blade of the DG, 30min after training in the single box and unilateral drug treatment microRNA-134 expression levels did not differ between norepinephrine -and saline- treated sides within animal ($p = 0.20$). Next, we investigated whether single box inhibitory avoidance training with posttraining administration of norepinephrine into the BLA downregulates microRNA 134 levels in the upper blade of the dentate gyrus. Rats received posttraining unilateral norepinephrine and saline infusions into the BLA and 30min later expression levels were measured into the dorsal blade of the dentate gyrus. As shown in **Fig. 3G**. paired *t*-test comparisons showed that posttraining norepinephrine treatment did not alter miR-134, Creb or Bdnf expression levels significantly ($p = 0.47$; $p = 0.72$; $p = 0.93$, respectively). As shown in **Fig. 3H**, repeated measures ANOVA for 48h retention latencies did not show antagomir treatment effect on retention latencies [$F_{(1,17)} = 0.14$; $p = 0.72$) whereas context exposure effect was significant [$F_{(1,17)} = 32.62$; $p < 0.0001$]. The interaction between both factor was not significant in relation to retention latencies [$F_{(1,17)} = 0.062$; $p = 0.81$]. These findings suggest that downregulation of microRNA-134 in the dentate gyrus is involved in facilitating discrimination.

Figure 3


A)
Non-Shock box
1min
Shock box
0h
Hippocampal and/or BLA infusion
48h
Novel box
B)
Shock box
Non-Shock box
Novel box
Retention Latencies (s)
400
300
200
100
0
Scrambled
miR134 Antagomir
C)
Retention Latencies (s)
Scrambled
miR134 Antagomir
D)
Retention Latencies (s)
Saline
Norepinephrine
E)
Hippocampal infusion
Control
miR134 mimic
Retention Latencies (s)
Saline
Norepinephrine
Saline
Norepinephrine
BLA infusion

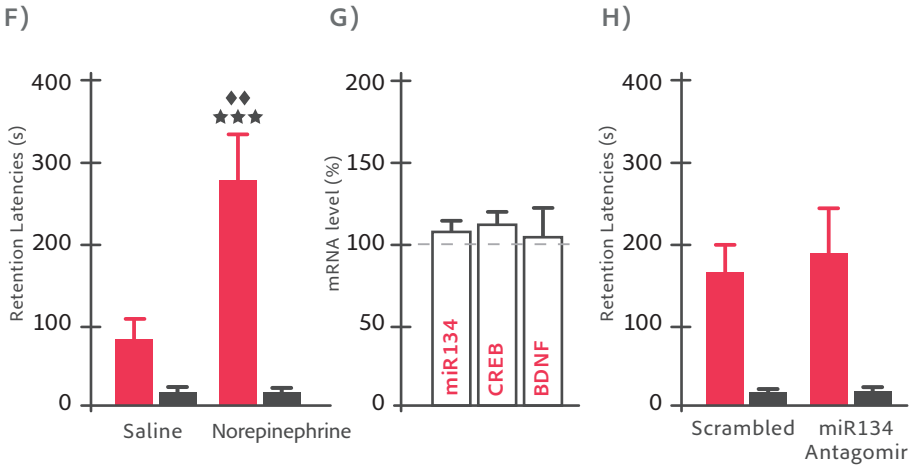


FIGURE 3:

PHARMACOLOGICAL MANIPULATION OF MICRORNA-134 LEVELS IN THE DORSAL HIPPOCAMPUS SELECTIVELY ALTERS THE ACCURACY OF MEMORY

Inhibitory avoidance retention latencies (mean + SEM) in seconds.

3A Schematic of the behavioral procedure. Rats were trained in the Non-Shock box (blue) without footshock, followed 1min later by footshock training in the Shock box (red). Immediately after the training, rats received bilateral drug treatment in the BLA or dorsal hippocampus. On the 48h retention test, rats were tested in the Shock and Non-Shock boxes as well as a Novel box (grey).

3B An antagomir against microRNA-134 (10pmol in 0.5µl) or scrambled control oligonucleotide (10 pmol in 0.5µl) was administered into the hippocampus immediately posttraining. The footshock intensity on the training was 0.35mA in 1s. Forty-eight-hour retention latencies in the Shock box of rats administered the scrambled control did not differ from those in the Non-Shock box, indicating lack of episodic-like memory. In contrast, antagomir-treated rats had significantly longer retention latencies in the Shock box than in the Non-Shock box, indicating accurate episodic-like memory. The antagomir administration did not increase retention latencies in the Shock box as compared to those of scrambled-treated rats. ★★★ $p < 0.01$ versus Non-Shock box within the same group. ♦♦♦, $p < 0.0001$ versus the scrambled control group. N = 11-14 rats/group.

3C Rats received the same infusions of an antagomir against microRNA-134 (10pmol in 0.5µl) or scrambled (10pmol in 0.5µl) into the hippocampus after training with a higher intensity footshock (0.60mA in 1s). Although retention latencies were longer after training with the higher footshock, retention latencies in the Shock box of rats administered the scrambled control did not differ from those in the Non-Shock box. Retention latencies in the Shock box of rats administered the antagomir were significantly longer than those in the Non-shock box but did not differ from those of the scrambled group. ★★★ $p < 0.0001$ versus Non-shock box within the same group. ♦, $p < 0.05$ versus the scrambled control group. N = 6-7 rats/group.

3D Posttraining norepinephrine (2.5µg in 0.5µl) administration into the hippocampus did not enhance discrimination. Retention la-

FIGURE 3:

tencies after norepinephrine administration the effect of concurrent norepinephrine (1µg in 0.2µl) administration into the BLA on memory accuracy but did not alter the memory-enhancing effect. N = 9 rats/group.

3E Rats administered posttraining norepinephrine into the BLA with concomitant non-target (control) infusions into the hippocampus had significantly longer retention latencies in the Shock box than in the Non-Shock box, indicating accurate episodic-like memory. Rats that received mimic microRNA-134 infusions into the hippocampus concomitant with norepinephrine infusions into the BLA showed higher retention latencies for the Non-shock box in comparison to non- target control treated animals with norepinephrine infusions into the BLA. ★★★ $p < 0.0001$ versus Non-Shock within the same group. ♦ , $p < 0.05$ in relation to control condition saline into the BLA and NT into the hippocampus, group. N = 11/15 rats/group.

3F Norepinephrine (1µg in 0.2µl) infusions into the BLA immediately after training on a single box inhibitory avoidance task enhanced retention latencies in the Shock box as compared to those of saline-treated rats. ★★★ $p < 0.0001$, in comparison to Non-shock box within the same group. ♦♦, $p < 0.01$ in comparison to the saline treated rats. N = 9-11 rats/group.

3G Norepinephrine (1µg in 0.2µl) infusions into the BLA immediately after single inhibitory avoidance training, which does not require discrimination, did not downregulate microRNA-134 levels in the dorsal blade of the dentate gyrus 30min after training and drug treatment. N = 8 rats/group.

3H Posttraining antagomir (10pmol in 0.5µl) infusions into the hippocampus after single inhibitory avoidance training also did not affect retention latencies as compared to those administered the scrambled control (10pmol in 0.5µl). N = 9-11 rats/group.

DISCUSSION

It is well known that noradrenergic activation of the BLA enhances the consolidation of memory of an emotionally arousing training experience, but whether the norepinephrine also enhanced memory of the details of the experiences is much less clear. Therefore, in the present study we investigated the effect of norepinephine infusions into the BLA on the facilitation of accurate episodic-like memory of an inhibitory avoidance disrimination task. Our findings indicate that the norepinephrine administration not only enhanced the strength of memory

but also facilitated discrimination of the association of footshock with the correct training context. In addition, norepinephrine infusions into the BLA downregulated microRNA-134 mRNA levels in the dorsal blade of the dentate gyrus 30min after training. This reduction in microRNA-134 levels was associated with an upregulation of mRNA levels its downstream targets Creb and Bndf, two proteins which are critically involved in neural plasticity and memory accuracy. Pharmacological downregulation of microRNA-134 levels in the dentate gyrus with an antagomir oligonucleotide resulted in a better discrimination without affecting the strength of the memory. Conversely, overexpression of microRNA-134 in the hippocampus blocked the intra-BLA norepinephrine effect on the facilitation of discrimination. In addition, posttraining infusions of norepinephrine after training on a single-box inhibitory avoidance task, which is easier and does not require discrimination, did not influence microRNA-134 levels in the dentate gyrus and posttraining downregulation of microRNA-134 in the hippocampus did not affect memory of a single-box inhibitory avoidance training experience. Thus, these findings indicate that noradrenergic activation of the BLA can enhance the accuracy of episodic-like aspects of inhibitory avoidance discrimination training by a neural mechanism that involves downregulation of microRNA-134, and the consequent upregulation of its downstream targets Creb and Bndf in the dorsal blade of the dentate gyrus.

Emotional events tend to be better remembered than neutral experiences. A stressful experience induces the activation of many different hormonal and neurotransmitter systems (Joëls and Baram, 2009; Roozendaal and McGaugh, 2011) and extensive evidence indicates that the BLA is a critical locus for integrating these modulatory influences in order to facilitate the consolidation of these memories (McGaugh, 2000). The activation of the BLA strengthens the storage of different kinds of information through its widespread network of efferent projections to other brain regions, including the hippocampus, caudate nucleus and various cortical areas (McIntyre *et al.*, 2005; Roozendaal *et al*, 2009; Holloway and Erickson, 2012). Many animal studies have shown that the administration of norepinephrine or

β-adrenoceptor agonists into the BLA enhance the consolidation of memory of a wide variety of emotionally arousing experiences (Quirarte *et al.*, 1997; Segev *et al.*, 2012; Barsegyan *et al.*, 2014). Human studies are consistent with this view and have shown that systemic administration of noradrenergic drugs could result in stronger amygdala activation and memory (van Stegeren *et al.*, 2005; Hermans *et al.*, 2011; Hermans *et al.*, 2014) whereas β-adrenoceptor antagonists such as propranolol block the effect of emotional arousal on increased amygdala activation and memory (McGaugh *et al.*, 2004; Strange and Dolan, 2004; van Stegeren, 2008). Although it is now well accepted that remembrance of emotionally arousing experiences is stronger, it is still heavily debated whether the emotional impact of an experience is also associated with changes in memory accuracy, fidelity and susceptibility to incorporation of misinformation (Morgan *et al.*, 2004; Porter *et al.*, 2008; Smeets *et al.*, 2009; Hoscheidt *et al.*, 2014). Findings of human studies show contradictory results. On the one hand, some studies show that emotional arousal can improve the accuracy of memories such that we remember emotionally arousing experiences with greater detail (Segal *et al.*, 2012). Other studies reported that emotional memories tend to be remembered in a more generalized manner (Loftus, 1979; Payne *et al.*, 2002). Although the investigation of memory accuracy became a popular topic in human memory research, it received very little attention in animal studies in part due to the fact that many of the available learning and memory tasks for animals do not allow determining memory accuracy. We very recently developed the inhibitory avoidance discrimination task that allows investigating the neural mechanisms underlying memory accuracy in animal models **(Atucha and Roozendaal in press)**. Our findings clearly show that noradrenergic activation of the BLA after training on the inhibitory avoidance discrimination task not only enhances the strength of the memory but also facilitates discrimination or memory accuracy. The norepinephrine effect was consolidation dependent as delayed infusions given 3h after the training were ineffective. In addition, this effect was specific to the BLA, since posttraining infusions into the adjacent CeA were ineffective. These findings

are consistent with some other recent findings from our laboratory indicating that norepinephrine infused posttraining into the BLA enhanced memory precision in an object-in-context recognition task, increasing rats' ability to discriminate in which training context they had seen a particular object (Barsegyan *et al.*, 2014).

Amygdala activity at encoding can predict episodic memory recall (Cahill *et al.*, 1998; LaBar 1998). A crucial aspect of episodic memories is the ability to discriminate between similar events which has been ascribed to the dentate gyrus (McHugh *et al.*, 2007; Yassa and Stark, 2011; Bekinschtain *et al.*, 2013). Our findings indicate that norepinephrine infused into the BLA after training on the inhibitory avoidance discrimination task induced downregulation of microRNA-134 levels in the dorsal blade of the dentate gyrus.

MicroRNA-134 has two validated downstream targets which are synaptic plasticity associated LIMK1 and transcription factor CREB (Christensen and Schraat, 2009). It has been reported that brain-specific microRNA-134, in cell cultured neurons prevents translation of LIM domain kinase 1 (LIMK1) and cAMP response element binding (CREB) protein which results in actin phosphorylation and gene transcription, respectively (Schraat, 2006; Siegel *et al.*, 2011) whereas brain derived neurotrophic factor (BDNF) relieves the inhibitory effect of micro-RNA 134 on Limk1 (Christensen and Schraat, 2009).

Posttraining microRNA-134, in cell cultured neurons prevents translation of LIMK1 and CREB protein which are crucial for memory consolidation (Schraat, 2006; Siegel *et al.*, 2011). In addition, BDNF relieves the inhibitory effect of micro-RNA 134 on Limk1 (Christensen and Schraat, 2009). Our results showed that posttraining norepinephrine infusions increased Bdnf as well as Creb expression levels in the dentate gyrus 30min after IAD training. Accordingly, a study reported that exression of BDNF was dependent on the complexity of the training (Bekinschtein *et al.*, 2013). To investigate whether this downregulation

of microRNA-134 levels in the dentate gyrus played a causal role in memory accuracy, we pharmacologically downregulated levels of this microRNA in the hippocampus with a specific antagomir antisense oligonucleotide. We found that the antagomir enhanced discrimination but did not have any effect on the strength of memory. On the other hand, overexpression of microRNA-134 blocked the intra-BLA norepinephrine effect on memory accuracy. These findings indicate that downregulation of microRNA-134 levels in the dentate gyrus facilitates discrimination. Previous results on microRNA-134 effects on consolidation described effects on plasticity and/or memory (Bicker *et al.*, 2010, 2014; van Spronsen *et al.*, 2013; Jimenez-Mateos *et al.*, 2015) but not in quality of memory. Our findings suggest that microRNA-134 within the dentate gyrus has a specific role facilitating episodic-like memories rather than enhancing memory in a general manner. MicroRNA-134 regulates two plasticity-related translation of two mRNAs, transcription factor cAMP-response element binding (Creb) mRNA and plasticity-related kinase Limk1 (Christensen and Schraat, 2009). To further support the specificity of microRNA-134 in regulating discrimination, downregulation of microRNA-134 into the hippocampus after classical inhibitory avoidance did not have an effect on retention latencies. Accordingly, BDNF levels were not affected after a low complexity memory task (Bekinschtain *et al.*, 2013).

The ability to discriminate between similar experiences has been postulated to be dependent on pattern separation (Yassa and Stark, 2011). Pattern separation is a computational model based on attractor networks proposed by Marr (Marr, 1971) that explains how similar representations (experiences) are stored in a distinct, non-overlapping fashion (Yassa and Stark, 2011). Pattern separation refers to the conversion of similar patterns of neural activity into distinct representations (Kumaran and McClelland, 2012; Schmidt *et al.*, 2011). In accordance with this interpretation, norepinephrine mediated emotional arousal facilitated discrimination that involved pattern separation (Segal *et al.*, 2012). MicroRNA-134 shows three characteristics that support its role in processing pattern separation-

like discrimination: 1) microRNA-134 facilitation of discrimination depends on consolidation. 2) microRNA-134 actions are restricted to the dorsal blade of the dentate gyrus. 3) microRNA-134 does not affect memory strength but discrimination. MicroRNA-134 has not been localized in the hippocampus but since it has been shown that in cortical areas miR-134 is mainly present in somatostatine positive interneurons (Chai *et al.*, 2013). We could speculate that microRNA-134 is only present in inhibitory neurons in the dentate gyrus. Then, a possible mechanism for microRNA-134 to induce pattern separation-like discrimination would be exerting increased inhibitory regulation. Increased inhibition on neurons that proyect to granular cells in the upper dentate gyrus could modulate activity on excitatory neurons forming a new network that favors memory consolidation of accurate memories (Schmidt *et al.*, 2012). An increased in inhibitory input is associated with early computational theories that explain the hippocampus function on discrimination and pattern separation (Marr, 1971; Schmidt *et al.*, 2012).

In summary, noradrenergic activation of the BLA not only enhances the strength of memory but can also increase accuracy of memory. We have shown that this effect involves downregulation of microRNA-134 levels specifically in the upper blade of the dentate gyrus as well as upregulation of Creb and Bdnf levels. Further experiments designed to investigate the molecular and physiological pathway in which microRNA-134 exerts its role on discrimination can lead to a better understanding on how emotional arousal facilitates discrimination.

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Manuscript in preparation

Noradrenergic Activation of the Basolateral Amygdala Induces Accurate and Hippocampus-Dependent Remote Memories

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CHAPTER 05

Extensive evidence indicates that emotionally arousing experiences are vividly remembered for long periods of time. Posttraining administration of norepinephrine into the basolateral complex of the amygdala (BLA) enhances the consolidation of memory, but the long-term fate of such memories has not been investigated. Studies on systems consolidation have suggested that memories might become hippocampus independent over time and less detailed as a consequence. The present study investigated systems consolidation processes of long-term emotionally arousing memories. Rats were trained on an inhibitory avoidance discrimination task in which they were subsequently trained in two inhibitory avoidance apparatuses, but footshock was given in one apparatus only. Norepinephrine or saline was administered into the BLA immediately after the training. At 2 days after training, both saline -and norepinephrine- infused rats showed accurate episodic-like memory of the association of footshock with the correct training context. At 28 days after training, saline-treated control rats showed

lack of episodic-like memory of the training experience whereas norepinephrine-treated rats remained episodic-like memory. Hippocampal inactivation with the GABAergic receptor agonist muscimol during the retention test at 28 days did not influence retention performance of saline-treated rats whereas the hippocampal inactivation significantly impaired the expression of episodic-like memory of norepinephrine-treated rats. We further found that norepinephrine infusions into the BLA after inhibitory avoidance discrimination training altered DNA methylation processes in the hippocampus. De novo DNA methyl transferase 3a (Dnmt3a) and plasticity-related cAMP regulated-element binding (Creb) mRNA expression levels were both upregulated in the dentate gyrus of rats administered norepinephrine into the BLA after the training. Our findings indicate that noradrenergic activation of the BLA modulates cellular and systems level activity and, as a consequence, enhances not only the strength but also the long-term episodic-like specificity of memory of an emotionally arousing training experience.

/INTRODUCTION

Emotionally arousing experiences are better retained in memory (Schacter *et al.*, 1999; McGaugh, 2000; Joëls *et al.*, 2011). In recent years, neuroscience gained understanding of how memories are formed and enhanced (Roozendaal and McGaugh, 2011; Squire and Wixted, 2011; Stern and Alberini, 2013). Extensive evidence indicates that noradrenergic activation of the basolateral complex of the amygdala (BLA) enhances the consolidation of memory processing of emotionally arousing training experiences (Roozendaal *et al.*, 2009a). In contrast, posttraining infusions of a β-adrenoceptor antagonist into the BLA impair memory of emotionally arousing training (Quirarte *et al.*, 1997; Roozendaal *et al.*, 2006). Noradrenergic activation of the BLA modulates synaptic plasticity and memory storage in efferent brain regions, including the hippocampus and cortical regions (McGaugh *et al.*, 1996; Setlow *et al.*, 2000; Roozendaal *et al.*, 2001; Ikegaya *et al.*, 1996; McIntyre *et al.*, 2005; McReynolds *et al.*, 2010; Holloway-Erickson *et al,.* 2012). Several studies in human subjects are consistent with these findings of animal studies and have shown that emotional events tend to be better remembered for long periods of time (Bradley *et al.*, 1992; Christianson *et al.*, 1992). Nevertheless, little is known about the long-term consequence of emotional memory enhancement.

Memory encodes experiences in episodes, where a certain event is organized in a specific temporal context (Shapiro, 2014). Early studies have shown that episodic memory is hippocampus dependent (Scoville and Milner, 1957). However, extensive evidence from systems consolidation studies have indicated that the hippocampal involvement in the expression of memory might be time limited (Zola-Morgan *et al.*, 1986; Frankland *et al.*, 2004) and that other brain regions such as the anterior cingulate cortex become more relevant when time

passes (Dudai *et al.*, 2004; Frankland *et al.*, 2004; Miller *et al.*, 2010). Such a time-controlled gained independence from the hippocampus is associated with lack of detailed episodic-like memory (Wiltgen and Silva, 2007). These results led to the standard consolidation model (SCM) (Wiltgen and Tanaka, 2013). However, not all studies confirmed SCM, some results indicated that episodic memories remain permanently hippocampus dependent, leading to the multiple trace theory (MTT) (Nadel and Moscovitch, 1997). The BLA has a strong anatomical and functional connection with the hippocampus (Ikegaya *et al.*, 1997; Fastenrath *et al.*, 2014) and it is now well established that BLA activation can enhance the consolidation of episodic-like memory (Medford *et al.*, 2005; Barsegyan *et al.*, 2014). Nevertheless, the involvement of the BLA in modulating systems consolidation processes of emotionally arousing experiences has not been investigated. In the present study we investigated the effects of noradrenergic activation of the BLA on long-term accuracy of episodic-like memory. Rats were trained in two distinctly different inhibitory avoidance apparatuses, but footshock was given only in one of these contexts.

Immediately after training rats received norepinephrine or saline infusions into the BLA and either 2 (recent) or 28 (remote) days later they were tested in both previously visited contexts to examine whether they accurately remembered the association of footshock with the correct training context. To examine whether the expression to short-and/or long-term memory is dependent on the hippocampus, in some rats the hippocampus was temporarily inactivated with the GABAergic receptor agonist muscimol at the time of retention testing.

Some evidence indicates that remote memory might depend on persistent changes in DNA methylation (Miller *et al.*, 2010). DNA methylation has been shown to be critical for the expression of recent (Miller and Sweatt, 2007) and

remote memory (Miller *et al.*, 2010). DNA methylation of 5' cytosine residues is catalyzed by DNA methyl transferases (DNMTs) that can induce long-term transcriptional silencing, thereby regulating synaptic plasticity (Levenson *et al.*, 2006) and possibly maintaining long-term memory (Day and Sweatt, 2010). To investigate whether noradrenergic activation of the BLA alters DNA methylation processes in the hippocampus, norepinephrine or saline was administered into the BLA after training on the inhibitory avoidance discrimination task and 30min later we assessed in the dentate gyrus of the hippocampus expression levels of three Dnmt subtypes: Dnmt1, 3a and 3b and cAMP response-element binding (Creb) mRNA a critical transcription factor for learning and memory (Kandel, 2004). DNMT1 has traditionally been considered a maintenance methyl transferase in DNA replication and has preference for hemimethylated DNA while DNMT3A and DNMT3B induce *de novo* DNA methylation and are importantly involved in new protein synthesis (Siedlecki and Zielenkiewicz, 2006).

MATERIALS AND METHODS

SUBJECTS

Male adult Sprague-Dawley rats (280–320g at the time of surgery) from Charles River Breeding Laboratories (Kisslegg, Germany) were housed individually in a temperature-controlled (22°C) vivarium room and maintained on a 12h/12h light/dark cycle (07:00h-19:00h lights on) with *ad libitum* access to food and water. Training and testing were performed during the light phase of the cycle between 10:00h-15:00h. All experimental procedures were in compliance with the European Communities Council Directive on the use of laboratory animals of November 24, 1986 (86/609/EEC) and approved by the Institutional

Animal Care and Use Committees of the University of Groningen and Radboud University Nijmegen, The Netherlands.

Cannula Implantation

Rats, adapted to the vivarium for 1 week, were anesthetized with a subcutaneous injection of ketamine (37.5mg/kg of body weight; Alfasan) and dexmedetomidine (0.25mg/kg; Orion) and received the non-steroidal analgesic carprofen (4mg/kg; Pfizer). The rats were positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA) and two stainless-steel guide cannulae (15mm; 23 gauge; Component Supply Co/SKU Solutions, Fort Meade, FL) were implanted bilaterally with the cannula tips 2.0mm above the BLA. The coordinates were based on the atlas of Paxinos and Watson (2007): anteroposterior (AP), -2.8mm from Bregma; mediolateral (ML), ±5.0mm from midline; dorsoventral (DV), -6.5mm from skull surface; incisor bar -3.3mm from interaural. Some rats also received bilateral guide cannulae (11mm; 23 gauge) aimed 1.5mm above the dorsal hippocampus: AP: -3.6mm; ML: ±1.9mm; DV: -2.6mm. The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (15 or 11mm long 00 insect dissection pins), inserted into each cannula to maintain patency, were removed only for the drug infusions. After surgery, the rats were administered atipamezole hydrochloride (0.25mg/kg sc; Orion) to reverse anesthesia and were subsequently injected with 3ml of sterile saline to facilitate clearance of drugs and prevent dehydration. The rats were allowed to recover for a minimum of 7 days before initiation of the training and were handled 3 times for 1min each during this recovery period to accustom them to the infusion procedure.

Inhibitory Avoidance Discrimination Task and Procedures

For the inhibitory avoidance discrimination task, rats were subsequently trained in two contextually distinct inhibitory avoidance apparatuses within a single training session, but footshock was delivered only in one of these two contexts. On the retention test, rats were tested in these two previously seen boxes as well as in a novel box. This experimental design allows investigating whether rats accurately remember the two apparatuses they had visited during training and discriminate in which of these two contexts they had received actual footshock. Each apparatus had the same geometry and consisted of a trough-shaped alley (91cm long, 15cm deep, 20cm wide at the top, and 6.4cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor (McGaughetal, 1988).

The starting compartment (31cm) was made of opaque white plastic and was well lit; the shock compartment (60cm) was made of two dark, electrifiable metal plates and was not illuminated. The actual shock apparatus (Shock box) did not have any contextual modifications. The other two apparatuses had some contextual modifications that made them a distinctly different training and/or test context. The safe training context (Non-Shock box) had four vertical white stripes (2cm wide) taped in the dark compartment together with tape placed on the floor closing the gap between the two plates. The Novel box that was only used on the retention test had two white circles (3.5cm diameter) taped on each wall of the dark compartment, and the gap between both plates was closed with tape. All three inhibitory avoidance apparatuses were located next to one other in a sound -and light- attenuated room. For a detailed description of the task, **see Atucha and Roozendaal (2015)**.

For training, rats were initially placed in the light compartment of the Non-Shock box and could explore the entire apparatus for 20s without any footshock delivery. Afterwards, the rats were removed from the apparatus and, after

a delay of 2min, placed in the light compartment of the second inhibitory avoidance apparatus (Shock box). After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.60mA for 1s) was delivered. The rats were removed from the dark compartment 20s after termination of footshock and, after drug treatment, returned to their home cages. Some rats were sacrificed 30min after training for assessment of molecular changes. Others were tested for retention at either 2 or 28 days after training. For retention testing, the rats were tested, in a randomized order and without delay, in the previously seen Non-Shock and Shock boxes and, additionally, in a Novel box they had not seen before. For all three boxes, the rats were placed in starting compartment of the inhibitory avoidance apparatuses and their latency to enter the dark compartment with all four paws (maximum latency of 600s) was measured. Previously we have shown that this repeated retention testing does not affect retention latencies in the different test environments (Atucha and Roozendaal, 2015). Shock was not administered on the retention test trial.

Drug Administration

Norepinephrine (1.0µg; Sigma-Aldrich) was dissolved in saline and administered into the BLA immediately after training on the inhibitory avoidance discrimination task. The use of posttraining drug administration provides direct support for the view that the treatment affects the consolidation of memory processing and that the retention performance is, thus, not confounded by possible effects on attentional, motivational, or sensory-perceptual mechanisms at the time of training or test (McGaugh, 1966).

To control for time specificity, other groups of rats were administered norepinephrine (1.0µg) or saline into the BLA 3h after the training. Bilateral

infusions of drug or an equivalent volume of saline were given via 30 gauge injection needles connected to 10µl Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 2.0mm beyond the cannula tips and a 0.2µl injection volume per hemisphere was infused over a period of 30s by an automated syringe pump (Stoelting Co., Dublin, Ireland). The injection needles were retained within the cannulae for an additional 20s to maximize diffusion and to prevent backflow of drug into the cannulae. The infusion volume was based on previous findings from our laboratory indicating that similar infusions into the adjacent central amygdala do not affect memory consolidation (Roozendaal and McGaugh, 1996, 1997).

The GABAergic receptor agonist muscimol (0.5µg; Sigma-Aldrich) was dissolved in saline and administered into the hippocampus 20-25min before retention testing (Corcoran and Maren, 2001), either 2 or 28 days after training. For hippocampal infusions, the infusion needles protruded 1.5mm beyond the guide cannulas and a 0.5µl-injection volume per hemisphere was infused over a period of 75s by an automated syringe pump. The injection needles were retained within the cannulae for an additional 20s to maximize diffusion and to prevent backflow of drug into the cannulae. All drug solutions were freshly prepared before each experiment.

Cannula Placement Verification and Tissue Collection

For cannula placement verification, rats were deeply anesthetized with an overdose of sodium pentobarbital after completion of behavioral testing and perfused transcardially with a 0.9% saline solution followed by 4% formaldehyde. The brains were removed and stored in 4% formaldehyde. At least 24h before sectioning, brains were placed in a 25% sucrose solution in water for cryoprotection. Coronal sections of 50µm were cut on a cryostat and collected in Tris-buffered saline (TBS) and mounted on gelatin-coated slides,

stained with cresyl violet and examined by light microscopy by an observer blind to drug treat ment condition. Determination of injection needle tip placements in the BLA and dorsal hippocampus wasmade according to the atlas plates of Paxinos and Watson (2007). Only animals with needle tips located within the boundaries of the BLA and/or hippocampus and without extensive tissue damage at the target areas were included into the behavioral analyses.

For molecular experiments, rats were deeply anesthetized with an overdose of sodium pentobarbital 30min after training for Dnmts mRNA measurements. Within 90s after the pentobarbital injection, the rats were sacrificed and the brains rapidly removed 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.

For verification of cannula placement in the BLA on flash-frozen brains, coronal sections of 50µm were cut on a cryostat and collected on gelatin-coated slides and let to dry. Slides were then fixed in 100% acetone for at least 30min and let to dry before staining with cresyl violet. Determination of the injection needle placements and exclusion of animals was performed as described above.

Quantitative Real-time PCR (qRT-PCR)

Coronal slices of the dorsal hippocampus at thickness of 350µm were cut on a cryostat and further dissected using a 0.75mm brain puncher (Stoelting Co). Bilateral punches from the dentate gyrus of the dorsal hippocampus (-2.64 to -3.86mm in range AP) were collected from three consecutives slices to a total of 6 punches. Punches were preserved at -20°C for at least 16h and later at -80°C in RNA later ICE (Ambion® Life Technologies, USA).

Following tissue dissection, RNA later ICE was removed and total RNA was isolated using TRIzol (Life Technologies, USA) according to the manufacturer's protocol. The purity of all isolated RNA samples was determined by agarose gel electrophoresis and UV-spectrophotometric analysis, respectively. Absorbance at 260nm and 280nm was used to assess the purity of RNA. A ratio of 2.0 is accepted as pure for RNA. The mean ± SD of the 260/280nm ratios was 2.0 ± 0.05. Contamination by genomic DNA was removed by treatment of 1µg RNA with 2 U deoxyribonuclease (DNase) (Sigma Aldrich, St. Louis, MO, USA) for 1h at 37°C, followed by DNase inactivation at 65°C for 10min. cDNA was synthesized from 0.5 - 1µg RNA according to the protocol provided with the revert Aid First Strand cDNA Synthesis Kit (Fermentas, Germany) and expression was quantified using the ABI PRISM 7900HT Sequence Detection System in a 384 well configuration. We used 2µl of a 1:20 dilution of the reverse transcription reaction in a quantitative real-time polymerase chain reaction (PCR), using 2 x GoTaq qPCR Master Mix (Promega; A6002) with a 1:50 dilution of CXR and 200nM of each primer. Detection of Dnmt3a was performed using the following primers: forward (5'-ATCGACGCCAAAGAAGTGTC-3') and reverse (5'-GCTATTCTGCCGTGTTCCAG-3'). Detection of Dnmt3b was performed using the following primers: forward (5'-AAAGTCGAAGACGCACAACC-3') and reverse (5'-CTTACCGCAGGACAGACAGC-3'). Detection of Dnmt1 was performed using the following primers: forward (5'-GGAGCAGATCGAGAAGGATG-3') and reverse (5'-CTTGCACTTCCCACACTCAG-3'). Detection of Creb was performed using the following primers: forward (5'-TCAGCCGGGTACTACCATTC-3') and reverse (5'-TTCAGCAGGCTGTGTAGGAA-3'). Samples were normalized to Actin-β transcript using the following primers: forward (5'-CCAACTGGGACGATATGGAG-3') and reverse (5'-AACACAGCCTGGATGGCTAC-3'). We used the following cycling conditions: 10min at 95°C, then 40 cycles of 15s at 95°C, 1min at 60°C, and 15s at 95°C. All reactions were performed in triplicates. All mean Ct values used for analysis were averaged except for those considered outliers being higher or lower than (2 x SD ± mean).

Relative expression was calculated using comparative C_T method and normalized to the expression of Actin-β RNA and with the ΔΔCt method.

Statistics

Behavioral data are expressed as the mean ± SEM. Inhibitory avoidance retention latencies were analyzed with either two -or three- way ANOVAs with latencies of individual animals in the different test contexts as a repeated measure. *Post hoc* comparisons used unpaired and paired *t*-tests to determine the source of the detected significances, when appropriate. Training and retention latencies of individual rats were compared with paired *t*-tests. Dnmt mRNA expression levels were analyzed with unpaired *t*-tests. For all comparisons, a probability of < 0.05 was accepted as statistical significance. The number of rats per group is indicated in the figure legends.

/ RESULTS

Effect of Posttraining Norepinephrine Infusions into the Basolateral Amygdala on Accuracy of Recent and Remote Memories

The aim of these first series of experiments was to investigate whether noradrenergic activation of the BLA immediately after inhibitory avoidance discrimination training influenced both short -and long- term accuracy of episodic-like memory. Therefore, rats were trained in two inhibitory avoidance apparatuses with a delay of 2min, but footshock was delivered in one of these two contexts only. Immediate after the training, rats received bilateral infusions

of a memory-enhancing dose of norepinephrine (1.0μg) or saline into the BLA. Either 2 days (recent) or 28 days (remote) after training, we examined whether rats had accurate episodic-like memory of in which training context they had received actual footshock. Therefore, we assessed retention latencies of each rat in the previously seen Shock and Non-Shock boxes as well as in a Novel box. **Fig. 1A** shows a schematic diagram of the experimental design.

Norepinephrine Administration into the BLA Posttraining Enhances Accuracy of Recent Memory

First, we investigated the effect of posttraining norepinephrine infusion into the BLA on accuracy of episodic-like memory tested 2 days after training. As shown in **Fig. 1B**, two-way ANOVA for retention latencies in the three test apparatuses indicated a significant norepinephrine effect [$F_{(1,38)} = 5.25$; $p = 0.03$], context effect [$F_{(2,38)} = 45.02$; $p < 0.0001$] and a significant interaction between both parameters [$F_{(2,38)} = 6.66$; $p = 0.003$]. Pair wise *t*-test comparisons revealed that saline-treated rats had significantly longer retention latencies in the Shock box than in the Non-Shock box ($p < 0.01$), whereas retention latencies in the Non-Shock box and Novel box did not differ ($p = 0.59$). These longer retention latencies in the Shock box vs the Non-Shock box indicate that saline-treated control rats had accurate episodic-like memory of the association of footshock with the actual training context. Rats administered norepinephrine into the BLA after training also had significantly longer retention latencies in the Shock box than in the Non-Shock box ($p < 0.0001$). Moreover, rats administered norepinephrine into the BLA had significantly longer retention latencies in the Shock box in comparison to those of the saline group ($p < 0.0001$). This memory enhancement was specific for the Shock box as retention latencies in the Non-Shock box or Novel box did not differ between the two groups.

In contrast to immediate posttraining administration of norepinephrine, delayed infusions of norepinephrine given 3h after inhibitory avoidance discrimination training did not affect retention performance. As shown in Fig. 1C, two-way ANOVA for retention latencies indicated a significant context effect [$F_{(2,16)} = 11.45$; $p = 0.0001$], but no norepinephrine effect [$F_{(1,16)} = 0.11$; $p = 0.75$] or interaction between both factors [$F_{(2,16)} = 0.08$; $p = 0.92$]. Thus, these findings indicate that the norepinephrine effect was found only when administered during a critical time window shortly after the training experience and thus likely is dependent on the consolidation of memory.

Figure 1

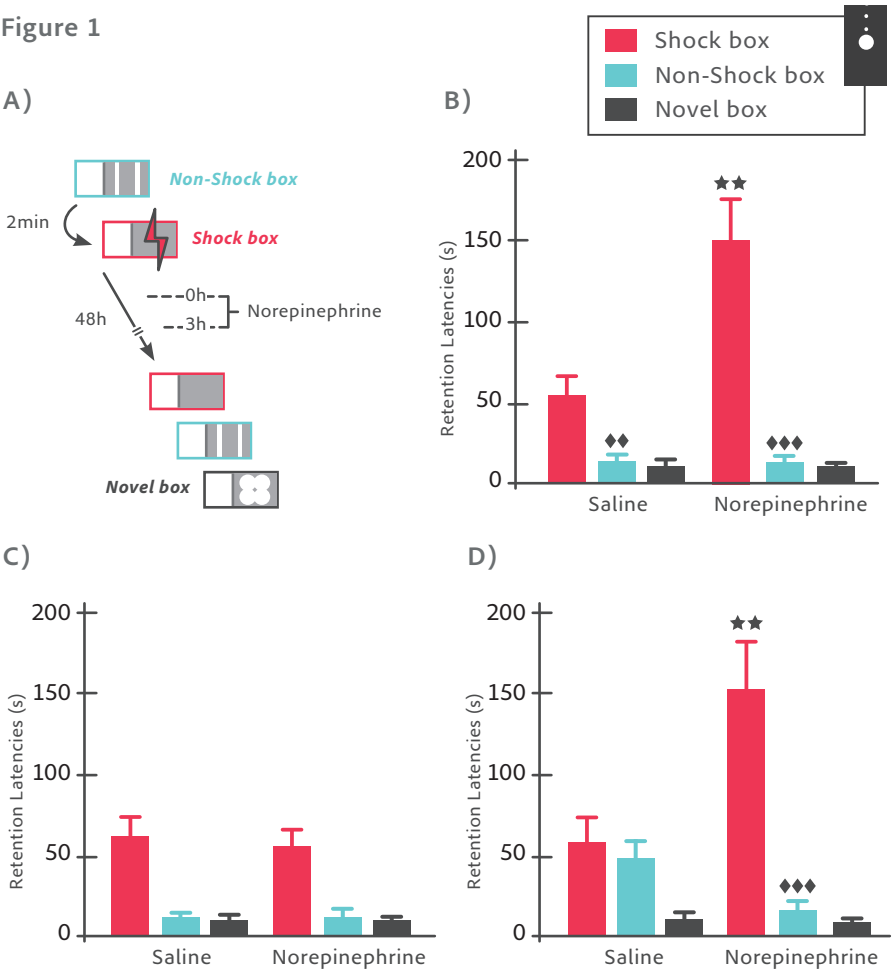


FIGURE 1:

EFFECT OF POSTTRAINING NOREPINEPHRINE INFUSIONS INTO THE BASOLATERAL AMYGDALA ON ACCURACY OF RECENT AND REMOTE MEMORY

Inhibitory avoidance retention latencies (mean + SEM) in seconds.

1A. Schematic of the behavioral procedure. Rats were trained in the Non-Shock box (blue) without footshock, followed 2min later by footshock training in the Shock box (red). Norepinephrine (1µg) or saline was administered into the BLA either immediately posttraining or 3h after the training. On the retention test, given either 2 (recent) or 28 (remote) days later, rats were tested in the Shock and Non-Shock boxes as well as a Novel box (grey).

1B. When tested at 2 days after training, both saline- and norepinephrine-treated rats showed accurate episodic-like memory of the association of footshock with the correct training context. Retention latencies in the Shock box were significantly longer than those in the Non-Shock box. Furthermore, posttraining norepinephrine infusions enhanced retention latencies in the Shock box as compared to those of rats administered saline. ★★p < 0.05, ♦♦ p < 0.01, ♦♦♦ p < 0.0001 versus the corresponding control group. N = 10-12 rats/group.

1C. Delayed infusions of norepinephrine into the BLA given 3h after training did not alter retention latencies in comparison to saline-treated rats. N = 9 rats/group.

1D. When tested at 28 days after training, rats given posttraining saline infusions into the BLA lacked episodic-like memory of the training as indicated by similar retention latencies in the Shock and Non-Shock boxes. In contrast, rats administered norepinephrine into the BLA after training still showed episodic-like memory of the training as indicated by significantly longer retention latencies in the Shock box than in the Non-Shock box. ★★ p < 0.01, ♦♦♦ , p < 0.0001 versus the corresponding control group. N = 12-13 rats/group.

Posttraining Norepinephrine Administration into the BLA Enhances Accuracy of Remote Memory

Second, we investigated, in separate groups of rats, the effect of posttraining norepinephrine infusion into the BLA on accuracy of episodic-like memory tested 28 days after training. As shown in Fig. 1D, two-way ANOVA for retention latencies showed no norepinephrine effect [$F_{(1,46)} = 0.16$; $p = 0.69$], but indicated a significant context effect [$F_{(2,46)} = 18.27$; $p < 0.0001$] as well as a significant interaction effect between both

parameters [$F_{(2,46)} = 8.09$; $p = 0.001$]. Pairwise *t*-test comparisons indicated that retention latencies of saline-treated rats in the Shock box did not differ from their latencies in the Non-Shock box ($p = 0.89$), but were both significantly longer than those in the Novel box (both, $p < 0.05$). Hence, these findings indicate that although saline-treated control rats at this remote time point still remembered the two boxes they visited during the training, they no longer had accurate memory of the association of the footshock with the actual training context. In sharp contrast, rats administered norepinephrine into the BLA after the training experience had significantly longer retention latencies in the Shock box than in the Non-Shock box ($p < 0.0001$) or Novel box ($p < 0.0001$). Furthermore, retention latencies of rats treated with norepinephrine in the Shock box were significantly longer than those of rats administered saline ($p < 0.01$). Thus, these findings indicate that, in contrast to saline-treated rats, rats administered norepinephrine into the BLA immediately after the training had even 28 later accurate and enhanced memory of the association of footshock with the actual training context.

Involvement of the Hippocampus in Mediating Norepinephrine-Induced Memory Accuracy

The findings above indicate that both saline -and norepinephrine- treated rats had accurate episodic-like memory of the training, i.e., the association of the footshock experience with the correct training context, when tested at a recent time point. On the other hand, at the remote time point, the saline-treated rats no longer showed specific memory of the association of footshock with the training context, whereas rats administered norepinephrine into the BLA after the training still showed accurate episodic-like memory. The Standard Consolidation Theory postulates that there is a transition of memory from a hippocampus-dependent to hippocampus-independent form that reflects a

time-dependent process of reorganization (Squire and Bayley, 2007) and is associated with a loss of detailed episodic-like memory (Frankland *et al.*, 2004; Dudai *et al.*, 2004; Wiltgen and Silva, 2007). To examine whether memory of saline-treated rats became hippocampus independent at 28 days after training whereas that of norepinephrine-treated rats remains to depend on the hippocampus, in the following experiments we investigated the involvement of the hippocampus in the expression of the memory at recent and remote time points. Similar as in the experiment above, rats were trained on the inhibitory avoidance discrimination task and given posttraining infusions of norepinephrine or saline into the BLA. Retention was tested at either 2 (recent) or 28 (remote) days after training. At the time of retention testing, the hippocampus was temporarily inactivated with local infusions of the GABAergic receptor agonist muscimol. To examine whether the muscimol effect was reversible, the same animals were tested again on the inhibitory avoidance discrimination task 24h after the muscimol infusion.

Effect of Hippocampal Inactivation at Recent Time Point

First, we investigated the effect of hippocampal inactivation on memory accuracy of saline -and norepinephrine- treated rats tested 2 days after training. As shown in **Fig. 2A**, three-way ANOVA for retention latencies showed a significant norepinephrine effect [$F_{(2,96)}$ = 6.37; p = 0.002] and a significant muscimol effect [$F_{(2,96)}$ = 12.33; p < 0.0001] whereas the interaction between both factors was not significant [$F_{(2,96)}$ = 2.37; p = 0.19]. For rats that received a saline control infusion into the hippocampus, the findings were almost identical to those described above, indicating that both saline and norepinephrine-treated rats showed accurate memory of the association of footshock with the actual training context. Pairwise *t*-test comparisons showed that rats given saline infusions into the BLA posttraining had significantly longer reten-

tion latencies in the Shock box than in the Non-Shock box ($p < 0.05$). Rats administered norepinephrine into the BLA posttraining also had significantly longer retention latencies in the Shock box than in the Non-Shock Box ($p < 0.0001$). Moreover, norepinephrine-treated rats had significantly longer retention latencies in the Shock box than did saline-treated rats ($p < 0.01$).

Hippocampal inactivation during the retention test induced generalization. Retention latencies in the Shock box and Non-Shock box were similar for rats administered either saline ($p = 0.94$) or norepinephrine into the BLA ($p = 0.96$). Interestingly, the muscimol treatment did not significantly alter retention latencies in the Shock box of rats administered either saline ($p = 0.53$) or norepinephrine into the BLA ($p = 0.08$). Rather, muscimol significantly increased retention latencies in the Non-Shock box of rats administered either saline ($p < 0.05$) or norepinephrine ($p < 0.01$). On the one hand, muscimol treatment did not influence retention latencies in the Novel box for saline ($p = 0.92$) or norepinephrine-treated rats ($p = 0.95$). The similar retention latencies in the Shock and Non-Shock boxes indicate that an intact hippocampus is required for the expression of episodic-like memory of the association of footshock with the correct training context.

On the other hand, our findings strongly suggest that the hippocampus does not play a critical role in remembering the aversive experience or discriminating the different training and test contexts *per se*, as rats still showed longer retention latencies in the two training contexts than in the novel test apparatus. Moreover, the muscimol treatment did not block the memory-enhancing effect of posttraining norepinephrine administration into the BLA as retention latencies of norepinephrine-treated rats in the Shock and Non-Shock boxes were significantly longer than those of saline-treated rats (Shock box: $p < 0.02$; Non-Shock box: $p < 0.03$).

Since muscimol was present during the retention test and hippocampal infusions of muscimol could directly influence locomotor behavior (Taira *et al.*, 1993; Farrar *et al.*, 2008), we tested whether hippocampal inactivation with muscimol alone is sufficient to modify retention latencies in the three different test contexts. Therefore, in this control experiment rats were trained on the inhibitory avoidance discrimination task but without footshock delivery. Saline or norepinephrine was infused into the BLA after the training and retention was tested 2 days later. As shown in **Fig. 2B** the muscimol infusion administered into the hippocampus shortly before the test did not significantly alter retention latencies of the rats during retention testing and they readily entered the three different test apparatuses [$F_{(1,10)} = 0.18$, $p = 0.67$].

To investigate whether the muscimol effect on blocking the expression of episodic-like memory was transient, the same rats (shown in **Fig. 2A**) were tested again 24h after the muscimol infusion in the three test environments. As shown in **Fig. 2C**, two-way ANOVA for retention latencies indicates significant norepinephrine [$F_{(1,50)} = 10.19$; $p = 0.004$] and context effects [$F_{(2,50)} = 39.12$; $p < 0.0001$] as well as a significant interaction effect [$F_{(2,50)} = 10.01$; $p = 0.0002$]. Almost indistinguishable from rats that had received saline infusions into the hippocampus, both saline and norepinephrine-treated rats now showed significantly longer latencies in the Shock box relative to the Non-Shock box (both, $p < 0.01$), indicating that the muscimol effect was transient and had worn off 24h after the hippocampal inactivation.

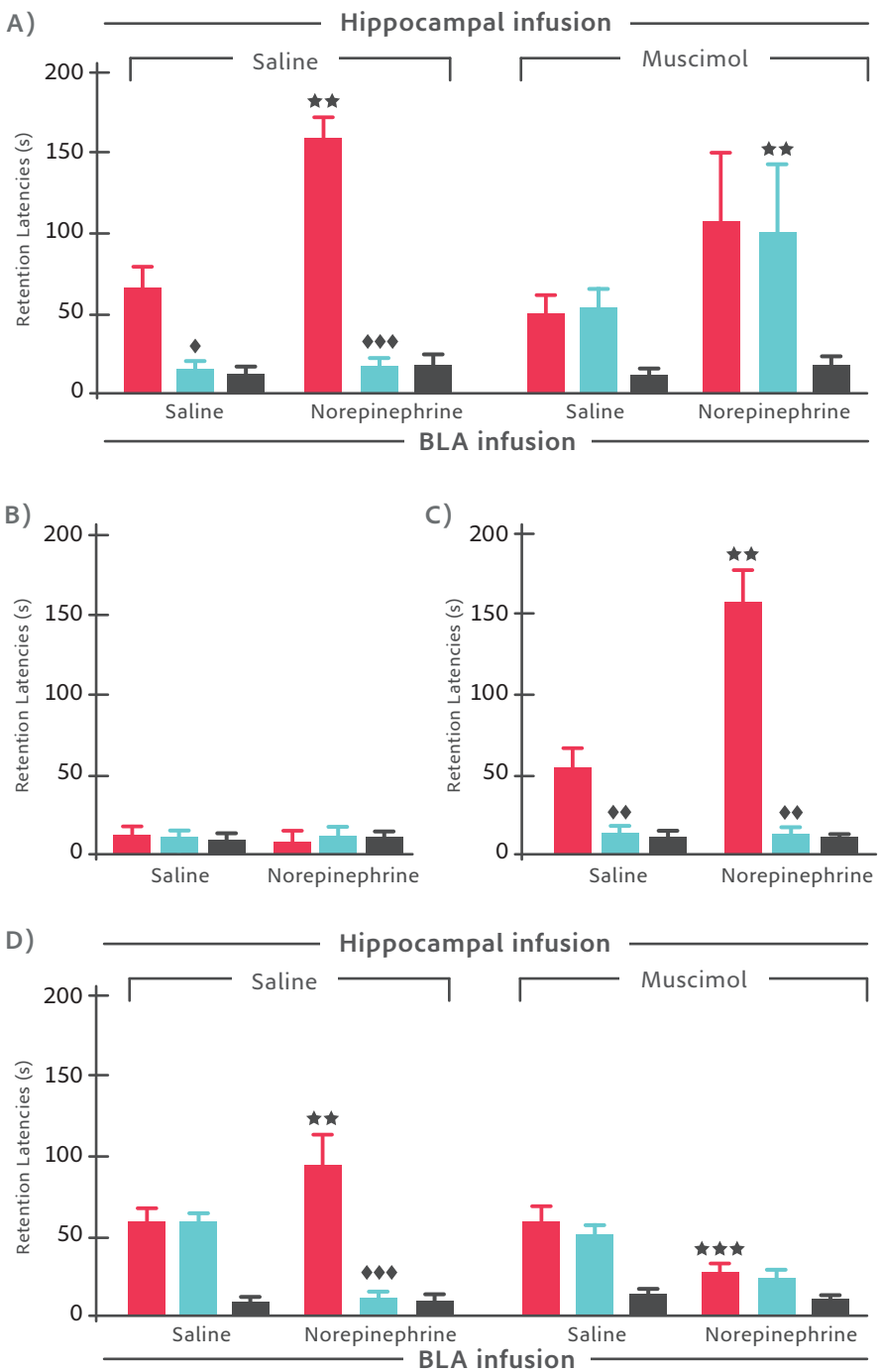
Effect of Hippocampal Inactivation at Remote Time Point

Second, we investigated, in separate groups of rats, the effect of hippocampal inactivation on memory accuracy of saline -and norepinephrine- treated rats

tested 28 days after training. As shown in **Fig. 2D**, three-way repeated measures ANOVA for retention latencies indicated significant norepinephrine [$F_{(2,84)}$ = 14.07; p <0.0001] and muscimol effects [$F_{(2,84)}$ = 11.87; p <0.0001] as well as a significant interaction effect between both treatments [$F_{(2,84)}$ = 14.29; p < 0.0001]. For rats that received a saline control infusion into the hippocampus, the findings were almost identical to those described above, indicating that, at this remote time point, rats administered norepinephrine, but not saline, into the BLA posttraining showed accurate episodic-like memory of the association of footshock with the actual training context. Pairwise *t*-test comparisons indicated that saline-treated rats had similar retention latencies in the Shock box and the Non-Shock box (p < 0.97), but that both were significantly longer than those in the Novel box (p < 0.01). In contrast, norepinephrine-treated rats had significantly longer latencies in the Shock box than in the Non-Shock or Novel boxes (p < 0.0001). Moreover, retention latencies of norepinephrine-treated rats in the Shock box were significantly longer than those of rats given saline infusions into the BLA (p < 0.01).

Hippocampal inactivation with muscimol shortly before retention testing did not alter retention performance of rats given saline infusions into the BLA. Pairwise *t*-test comparisons showed similar retention latencies in the Shock box and Non-Shock box (p = 0.34) whereas retention latencies for the Shock and Non-Shock boxes were significantly longer than for the Novel box (p < 0.0001). Thus, these findings are consistent with the hypothesis that the memory of saline-treated rats at this remote time point is no longer dependent on the hippocampus. In contrast, hippocampal inactivation with muscimol severely impaired retention performance of norepinephrine-administered rats. Retention latencies in the Shock box of norepinephrine-treated rats given muscimol into the hippocampus were significantly shorter than those of rats administered saline into the hippocampus (p < 0.0001). Furthermore, retention

Figure 2


A)
Hippocampal infusion
Saline
Muscimol
Retention Latencies (s)
200
150
100
50
0
Saline
Norepinephrine
Saline
Norepinephrine
BLA infusion
B)
Retention Latencies (s)
Saline
Norepinephrine
C)
Retention Latencies (s)
Saline
Norepinephrine
D)
Hippocampal infusion
Saline
Muscimol
Retention Latencies (s)
Saline
Norepinephrine
Saline
Norepinephrine
BLA infusion

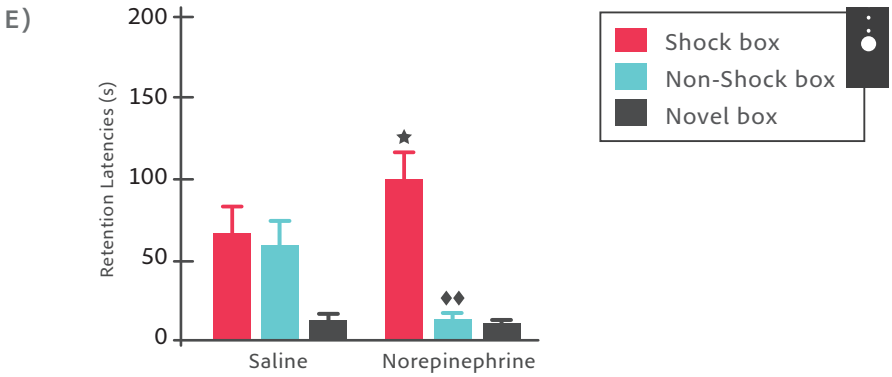


FIGURE 2:

EFFECT OF HIPPOCAMPAL INACTIVATION AT RECENT AND REMOTE TIME POINTS

Inhibitory avoidance retention latencies (mean + SEM) in seconds.

2A. A. Rats were trained in the Non-Shock box, without footshock followed 2min later by footshock training in the Shock box. Immediately after the training, norepinephrine (1µg) or saline was administered bilaterally into the BLA. 20 min before retention testing at 2 days, the hippocampus was inactivated with bilateral infusion of the GABAergic receptor agonist muscimol (0.5µg). Hippocampal inactivation blocked the expression of episodic-like memory. Retention latencies in the Shock box of rats administered either saline or norepinephrine into the BLA did not differ from those in the Non-Shock box. ★★ $p < 0.01$, ♦ $p < 0.05$, ♦♦♦ $p < 0.0001$ versus the corresponding control group. N = 11-14 rats/group.

2B. Muscimol infusions did not alter retention latencies of rats that were exposed to the inhibitory avoidance training box but did not receive footshock. N = 6 rats/group.

2C. Rats that had received muscimol infusions into the hippocampus showed recovered retention performance 24 h later. ★★ $p < 0.01$, ♦♦ $p < 0.01$ versus the corresponding control group. N = 12-14 rats/group.

2D. Rats were trained in the Non-Shock box, without footshock followed 2min later by footshock training in the Shock box. Immediately after the training, norepinephrine (1µg) or saline was administered bilaterally into the BLA. 28 days later, rats were tested in the Shock and Non-Shock boxes as well as a Novel box (grey). 20 min before retention testing, the hippocampus was inactivated with bilateral infusion of the GABAergic receptor agonist muscimol (0.5µg). Hippocampal inactivation did not alter retention latencies of rats administered saline into the BLA. In contrast, hippocampal inactivation significantly impaired retention of norepinephrine-treated rats. Retention latencies in the Shock and Non-Shock boxes did not differ from those in Novel box. ★★ $p < 0.01$, ★★★ $p < 0.0001$, ♦♦♦ $p < 0.0001$ versus the corresponding control group. N = 11-12 rats/group.

2E. Rats that had received muscimol infusions into the hippocampus showed recovered retention performance 24 h later. ★ $p < 0.05$, ♦♦ $p < 0.01$ versus the corresponding control group. N = 11-12 rats/group.

latencies in the Shock box did not significantly differ from those in the Non-Shock box (p = 0.63) or Novel box (p = 0.13) indicating that these rats did not show any retention of the training. These findings indicate that norepinephrine administered into the BLA posttraining maintains the accuracy of episodic-like inhibitory avoidance discrimination training by a process that keeps the memory hippocampus dependent.

To investigate whether the muscimol effect on blocking the expression of episodic -like memory was transient, the same rats were tested again 24h after the muscimol infusion in the three test environments. As shown in **Fig. 2E**, two-way ANOVA indicates a non-significant treatment effect [$F_{(1,42)}$ = 0.39; p = 0.54] but a significant context effect [$F_{(2,42)}$ = 30.71; p < 0.0001] as well as a significant interaction effect [$F_{(2,42)}$ = 10.01; p = 0.0002]. Almost indistinguishable from rats that had received saline infusions into the hippocampus, rats administered saline infusions into the BLA posttraining showed similar retention latencies between the Shock box and Non-Shock box (p = 0.79) whereas norepinephrine-treated rats now again showed significantly longer latencies in the Shock box relative to the Non-Shock box (p < 0.01).

Figure 3

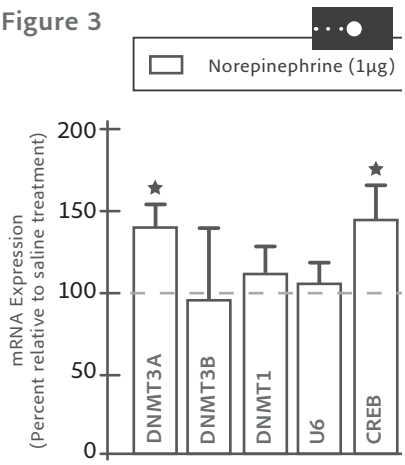


FIGURE 3:

POSTTRAINING NOREPINEPHRINE INFUSIONS INTO THE BLA ENHANCE DNMT3A AND CREB LEVELS IN THE DENTATE GYRUS

3. Rats were trained on the inhibitory avoidance discrimination task and received immediate posttraining infusions of norepinephrine (1µg) or saline into the BLA. Animals were sacrificed30 min after training and drug infusion. Norepinephrine infusion into the BLA upregulated Dnmt3a as well as Creb mRNA levels in the dentate gyrus. Expression levels of Dnmt3b, Dnmt1 and U6 were unaffected. mRNA levels are expressed relative to those of saline-treated rats. ★p < 0.05, ★★p < 0.01 versus the corresponding saline control group. N = 5-7 rats/group.

Posttraining Norepinephrine Infusions into the BLA Enhance Dnmt3a Levels

Next, we examined whether noradrenergic activation of the BLA after inhibitory avoidance discrimination training modulates DNA methyl transferase (DNMT) enzymes in the hippocampus. It has been shown that DNA methylation is critical for memory formation (Miller *et al.*, 2007) as well as for memory maintenance (Miller *et al.*, 2010) and has been established as a dynamic regulator of mnemonic processes in rodents (Day and Sweatt, 2010; Baker-Andresen *et al.*, 2013). Furthermore, DNMT inhibition immediately posttraining affects hippocampal plasticity (Levenson *et al.*, 2006) and impairs memory consolidation process (Miller *et al.*, 2007; Oliveira *et al.*, 2012). Our findings suggest that noradrenergic activation of the BLA maintains memory hippocampus dependent by altering the systems consolidation process. Therefore, in the next experiment we initiated to explore whether posttraining norepinephrine infusions into the BLA modulate dentate gyrus activity at the epigenetic level. We focus here on the dentate gyrus as particularly this subregion of the hippocampus has a strong functional interaction with the BLA during emotionally arousing conditions (Vouimba and Richter-Levin, 2005; Ikegaya *et al.*, 1996; McIntyre *et al.*, 2002). Moreover, we have recent evidence that noradrenergic activation of the BLA enhances accuracy of episodic-like memory of inhibitory avoidance discrimination training by specifically inducing molecular changes in the dentate gyrus subregion of the hippocampus **(Atucha *et al.*, unpublished observation).**

Rats were trained on the inhibitory avoidance discrimination task and immediately afterwards received bilateral infusions of norepinephrine or saline into the BLA. Measurements of Dnmt mRNA levels in the dentate gyrus were assessed 30min after training and drug treatment. The 30min delay-dependent involvement of

DNMT enzymes has been shown in other studies (Miller and Sweatt, 2007; Miller *et al.*, 2008; Han *et al.*, 2010; Oliveira *et al.*, 2012; Mitchnick *et al.*, 2014). We measured mRNA levels of three Dnmt subtypes, Dnmt1, Dnmt3a and Dnmt3b, as well as plasticity-related Creb levels (Yin and Tully, 1996; Zhou *et al.*, 2009) and a ribosomal U6 house keeping gene as control. DNMT3A and DNMT3B enzymes are *de novo* DNA methyl transferases (Siedlecki and Zielenkiewicz, 2006) and have been found to be upregulated in the hippocampus shortly after hippocampus-dependent memory formation. On the other hand, maintenance DNMT enzyme DNMT1, methylates hemimethylated DNA since it is involved in the methylation for the daughter strand following DNA replication during cell division (Bird, 2002). Nevertheless, it has been shown that DNMT1 contributes to object recognition memory in the perirhinal cortex whereas DNMT3A contributes to object location memory in the hippocampus (Mitchnick *et al.*, 2014). As shown in **Fig. 3A**, norepinephrine infusions into the BLA immediately after training selectively augmented Dnmt3a mRNA levels in comparison to the levels of saline-treated rats (t_{11}= 4.12; $p < 0.01$), indicating *de novo* protein synthesis. Dnmt3b and Dnmt1 mRNA levels were not significantly upregulated (t_{11}= 0.72; $p = 0.48$, t_{11}= 0.07; $p = 0.94$, respectively). In addition, plasticity-related Creb mRNA levels in the dentate gyrus were also upregulated in the norepinephrine treatment group (t_{10}= 2.41; $p < 0.05$) whereas U6 control gene mRNA levels were unaltered (t_{11}= 0.40; $p = 0.69$).

DISCUSSION

The aim of this study was to investigate the influence of noradrenergic activation of the BLA immediately after training on an emotionally arousing task on accuracy of recent and remote memory. Our findings indicate that noradrenergic activation of the BLA after inhibitory avoidance discrimination

training enhances not only the strength but also the accuracy of episodic-like memory. Rats that were administered saline into the BLA showed over time hippocampus independent memory, which was associated with lack of episodic memory. In contrast, rats administered norepinephrine into the BLA after training showed episodic-like memory, associated with hippocampus engagement even at remote time points. Overall, our findings suggest that emotional memories remain accurate over time by maintaining hippocampal dependency.

Extensive evidence shows that noradrenergic stimulation of the BLA enhances memory consolidation (Roozendaal *et al.*, 2009a). Our study shows that posttraining noradrenergic activation of the BLA not only enhances the strength but also the accuracy of episodic-like memories over time. Episodic aspects and details are encoded by the hippocampus whereas the cortex over time will extract general knowledge from specific experiences (O'Reilly and Rudy, 2001). Previous reports support a systems consolidation process where the transfer of information from the hippocampus to cortex, leading to hippocampus independent memories over time, is associated with a qualitatively different memory (Wiltgen and Silva, 2007; Frankland *et al.*, 2004). These studies led to the standard consolidation theory where hippocampus independent memory over time is associated with generalization of the training experience associated with lack of contextual details (Wiltgen and Tanaka, 2013).

Other studies, however, showed that in some cases episodic-like and spatial memories remain hippocampus dependent, leading to the multiple trace theory (Nadel and Moscovitch, 1997). The inhibitory avoidance discrimination task allows evaluating memory for contextual details of the boxes as well as the evaluation of memory for the episodic-like aspects of the training. Posttraining infusions of norepinephrine into the BLA enhanced retention for the episodic-

like aspect of memory, and showed to be hippocampus dependent both at recent and remote time points. In contrast, saline administered rats showed accurate memory of the association of footshock with the training context at a recent time point but showed generalization at a remote time point. At this remote time point saline-treated rats generalized at the retention test with saline infusions into the hippocampus as well as with muscimol infusions into the hippocampus. As we used different groups of rats for the recent and remote memory tests, accurate retention performance of norepinephrine-treated rats at this remote time point could not be due to rehearsal effects. We concluded that generalization is associated with lack of hippocampus dependent memory at remote time point whereas accuracy of episodic-like features of the memory during recall is hippocampus dependent.

The hippocampus has been associated with contextual memory retrieval (Hirsh, 1974; Good and Honey 1991; Holland and Bouton, 1999; Wiltgen *et al.*, 2010) and it is required for retrieval of autobiographical remote memories (Steinvorth *et al.*, 2005). Inactivation of the dorsal hippocampus at recall blocks the context-specific expression of a context-association (Holt and Maren, 1999). Accordingly, our findings show that the episodic-like aspect of memory depends on the hippocampus at recent and remote time points whereas the contextual features of the inhibitory avoidance boxes depend on non-hippocampal structures. The degree of hippocampus dependency of a learning experience relies on the specifics of a memory task; object recognition memory as an example depends on cortical structures (perirhinal as well as insular cortex) (Balderas *et al.*, 2008). In addition, it has been shown that damage to the hippocampus does not affect the association between discrete cues (O'Keefe and Nadel, 1978; Squire and Alvarez, 1995). The hippocampus independency in previous systems consolidation-related studies (Wiltgen *et al.*, 2007; Wang *et al.*, 2009) has been interpreted as a loss of detailed memory that resulted

in generalization to other similar environments. In this study, animals did not generalize the details of the boxes since all rats were able to discriminate the novel box from the previously seen boxes whereas generalization resulted in lack of episodic memory.

Noradrenergic enhancement of memory 2 days after training was preserved after hippocampal inactivation by muscimol infusions into the hippocampus; despite the lack of episodic-like memory, the strength of memory was not affected by muscimol infusions. Even in the case of hippocampal inactivation, retention latencies of norepinephrine-treated rats in the Shock box and Non-Shock box were significantly longer than those of saline-treated rats. These findings thus strongly suggest that the memory-strengthening effect of norepinephrine does not critically depend on an intact hippocampus. Noradrenergic activation of the BLA can influence consolidation processes for different cognitive aspects in many different brain regions, such as habitual memory (Wingard *et al.*, 2008), spatial memory (Roozendaal *et al.*, 1996), episodic memory (Barsegyan *et al.*, 2014) and working memory (Roozendaal *et al.*, 2004). Furthermore, norepinephrine administration posttraining induced specific remote memory that appears to be more or less was entirely dependent on an intact hippocampus. Hippocampal inactivation by muscimol completely collapsed retrieval of norepinephrine-treated rats at this remote time point and retention latencies in the Shock and Non-Shock boxes did not differ from those in the Novel box. Furthermore, in contrast to retention testing with an intact hippocampus, when the hippocampus was inactivated retention latencies of norepinephrine-treated rats were significantly shorter than those of saline-treated rats. These findings suggest that remote emotional memories remembered in a detailed manner are hippocampus dependent probably through a multiple trace consolidation process where memory depends entirely on the hippocampus.

Maintenance of memory has been attributed to epigenetics, more specifically to DNMTs (Day and Sweatt, 2010). First, a study showed that methylation is required for consolidation of fear conditioning memory. Training on fear conditioning induced increased expression levels of Dnmt3a and Dnmt3b at 30min (Miller *et al.*, 2007). A follow up study showed that DNA methylation maintains remote memory where methylation of a low affinity calcium enzyme *(calcineurin)* was required for expression of remote memory (Miller *et al.*, 2010). Our study indicates that emotional memories might be modulating consolidation via epigenetic mechanisms. Results showed that noradrenergic activation of the BLA specifically enhanced Dnmt3a mRNA expression in the dentate gyrus 30min after training but not Dnmt3b nor Dnmt1 levels. The dentate gyrus has a critical role of in remembering details (Stark and Yassa, 2011) which is a crucial aspect of episodic memories (Shapiro, 2014). The effects on consolidation of bothDnmt3 enzymes are largely unknown. Dnmt3a has been shown to have more consensus sequences to methylate DNA than Dnmt3a (Hervouet *et al.*, 2009), this might mean that Dnmt3a has a major role in modulating the epigenetic state in the brain. Since DNA methylation has been shown to occur during the early stages of consolidation (Miller *et al.*, 2007) and for at least one gene (*calcineurin*) remained for at least a month in mice after fear conditioning (Miller *et al.*, 2010). DNA methylation measurements of memory-related genes in the hippocampus as well as in the anterior cingulate cortex might indicate the involvement on these regions in remote memory. Although it is not clear whether norepinephrine induced increased levels of dnmt3A shortly after training are involved in longer hippocampus dependent memory.

In summary, the present findings indicate that posttraining noradrenergic activation of the BLA modulates information storage processes in efferent brain regions and as a consequence directs systems consolidation processes of remote

memories. Norepinephrine treatment maintained retrieval of remote memory completely hippocampus dependent whereas saline treated rats showed hippocampus independent memory over time. This noradrenergic stimulation of the BLA immediately after training experience seems to be sufficient to produce long lasting accurate memories and thus, it might reflect the mechanism by which emotional memories are consolidated and remembered.

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Differential Targeting of Brain Stress Circuits With a Selective Glucocorticoid Receptor Modulator

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CHAPTER 06

Glucocorticoid receptor (GR) antagonism may be of considerable therapeutic value in stress-related psychopathology such as depression. However, blockade of all GR-dependent processes in the brain will lead to unnecessary and even counteractive effects, such as elevated endogenous cortisol levels. Selective GR modulators are ligands that can act both as agonist and as antagonist and may be used to separate beneficial from harmful treatment effects. We have discovered that the high-affinity GR ligand C108297 is a selective modulator in the rat brain. We first demonstrate that C108297 induces a unique interaction profile between GR and its downstream effectormolecules, the nuclear receptor coregulators, compared with the full

agonist dexamethasone and the antagonist RU486 (mifepristone). C108297 displays partial agonistic activity for the suppression of hypothalamic corticotropin-releasing hormone (CRH) gene expression and potently enhances GR-dependent memory consolidation of training on an inhibitory avoidance task. In contrast, it lacks agonistic effects on the expression of CRH in the central amygdala and antagonizes GR-mediated reduction in hippocampal neurogenesis after chronic corticosterone exposure. Importantly, the compound does not lead to desinhibition of the hypothalamus-pituitary-adrenal axis. Thus, C108297 represents a class of ligands that has the potential to more selectively abrogate pathogenic GR-dependent processes in the brain, while retaining beneficial aspects of GR signaling.

INTRODUCTION

Adrenal glucocorticoid hormones are essential for adaptation to stressors, but prolonged or excessive exposure to glucocorticoids has been consistently implicated in the development of stress-related psychopathologies, such as depression (Kloet *et al.*, 2005). Antagonism of their most abundant receptor type, the glucocorticoid receptor (GR), can be beneficial in stress-related psychiatric disease [e.g., to abrogate psychotic and depressive features in patients with Cushing's syndrome (Nieman *et al.*, 1985) and in patients suffering from psychotic major depression (De Battista *et al.*, 2006)]. The GR is widely distributed in the brain (Rosenfeld *et al.*, 1988), where it affects many different processes including learning and memory (Roozendaal and McGaugh, 2011, Joëls *et al.*, 2006), adult neurogenesis (Fitzsimons *et al.*, 2012), and neuroendocrine negative feedback regulation (Watts, 2005). Although GR antagonism of articular processes may be of therapeutic benefit, blocking other GR-mediated effects may actually counteract the potential therapeutic efficacy. For example, GR antagonists interfere with glucocorticoid negative feedback and lead to increased cortisol levels (Spiga *et al.*, 2007, Ratka *et al.*, 1989), which inadvertently activate mineralocorticoid receptors (MRs) to which corticosteroids bind in the brain and diminish the efficacy of antagonism at relevant sites.

The GR is a nuclear receptor (NR) that affects gene transcription through a number of transcriptional mechanisms. For several NRs, 'selective receptor modulators' exist. These can act as an agonist, as well as an antagonist depending on the tissue or gene targets, with the estrogen-receptor ligand tamoxifen as a well-known example (Johnson and O'Malley, 2012). Selective GR modulators (SGRMs) may be used to separate beneficial from unwanted glucocorticoid effects. Anti-inflammatory SGRMs with diminished side effects have been pursued, based on the distinction between GR effects that depend on direct DNA binding and those that take place via protein-protein interactions between the GR and other transcription factors (De Bosscher *et al.*, 2003). Selective receptor modulation

may also be based on specificity of ligand-induced interactions between the GR and its major downstream effector molecules, the NR coregulators (Coghlan *et al.*, 2003).

Many receptor-corregulator interactions depend on the GR's ligand-binding domain (GR-LBD) and on specific corregulator amino acid motifs that contain an LXXLL sequence, known as 'nuclear receptor-boxes' (NR-boxes). These interactions are governed by the conformation that is induced by a particular ligand and may be screened for in vitro (Koppen *et al.*, 2009). The importance of individual corregulators for brain GR function is largely unknown, but an exception is steroid coactivator (SRC)-1 [or nuclear receptor coactivator 1 (NCoA1)]. SRC-1 is necessary for GR-mediated negative gene regulation in the hypothalamus-pituitary-adrenal HPA axis (Winnay *et al.*, 2006; Lachize *et al.*, 2009) and for the induction of corticotropin releasing hormone (CRH) gene expression in the central nucleus of the amygdala (CeA) (Lachize *et al.*, 2009). Its two splice variants SRC-1A and -1E seem to exert opposite effects on CRH expression (van der Laan *et al.*, 2008). Selective activation of GR interactions with SRC-1A, brought about via an SRC-1A-specific NR-box, would be expected to separate GR-mediated effects on CRH expression in the hypothalamus and amygdala.

Here, we show proof of principle for selective GR modulation in the brain with relevance for stress regulation, cognition, and psychopathology. We show that a previously described selective high-affinity GR ligand induces a unique corregulator interaction profile that distinguishes between the two splice variants of SRC-1. C108297 (or compound 47 from ref. Clark *et al.*, 2008) has a Ki of 0.9nM for GR and > 10μM for progesterone receptor (PR), MR, and androgen receptor (Clark *et al.*, 2008). It shows GR antagonism in relation to GR-dependent CRH mRNA regulation in the amygdala and corticosterone-induced reduction in hippocampal neurogenesis. The agonistic effects of C108297 include enhanced memory consolidation of emotionally arousing training and a suppression of hypothalamic CRH expression. The compound does not lead to net inhibition of glucocorticoid negative feedback, as indicated by unaltered circulating corticosterone levels.

MATERIALS AND METHODS

Peptide Interaction Profiling

Interactions between the GR-LBD and co-regulator NR-boxes were determined using a MARCoNI assay with 55 immobilized peptides, each representing a co-regulator derived NR-box (PamChip nº 88011; Pamgene International) (Koppen *et al.*, 2009). Each array was incubated with a reaction mixture of 1nM GST-tagged GR-LBD, ALEX A488-conjugated GST antibody, and buffer F (PV4689, A-11131, and PV4547; Invitrogen) and 1μM DEX, RU486, C108297, or solvent (2% DMSO in water). Incubation was performed at 20°C in a PamStation96 (Pamgene International). GR binding to each peptide on the array, reflected by fluorescent signal, was quantified by analysis of .tiff images using BioNavigator software (Pamgene International).

Two-Hybrid Studies

To generate fusions to the DNA-binding protein Gal4, partial co-regulator cDNAs were cloned into the pCMV-BD vector (Stratagene): SRC-1 residues 621-1020, SRC-1A residues 1021-1441, and NCoR1 residues 1962-2440 (Perissi *et al.*, 2010). COS-1 cells were transfected using Lipofectamine2000 (Invitrogen) with a combination of a Gal4 co-regulator fusion plasmid, the pGR-VP16 transactivator plasmid, and the pFR-Luc reporter gene (Stratagene). Twenty-four hours after transfection, the medium was replaced with medium containing 0.1% DMSO, DEX, RU486, or C108297 (all 1μM). The next day, the medium was replaced with 0.1ml Hank's balanced salt solution plus 0.1ml of Steadylight (Perkin-Elmer), and luminescence was counted on a Topcount instrument (Perkin Elmer).

Animal experiments

Animal experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC), certificates and licenses were granted under the Animals (Scientific Procedures) Act 1986 by the United Kingdom Home Office, or experiments were approved by the Local Committees for Animal Health, Ethics, and Research of the Dutch universities involved. Male rats were used, housed in temperature-controlled facilities on a 12h day/night schedule with food and water available ad libitum. Modes of administration and duration of drug treatment differed in accordance with the standards used in the different in vivo paradigms

Binding to Brain GR

Group-housed Sprague-Dawley rats were orally dosed with corticosterone (3mg/kg) or C108297 (20mg/kg and 100mg/kg) dissolved in 10% DMSO/ 90% methylcellulose (0.5% wt/vol). After 3h, the rats were killed and half-brains were snap-frozen in liquid nitrogen. Tissue was homogenized in buffer using a Bead Ruptor at 4°C for 15min. Free GR ligands were cleared by incubation of homogenates with dextran-coated charcoal (Sigma; C-6197). Receptor binding was determined by incubating homogenate with 2.5nM [3H]DEX (Amersham; TRK645) at 4°C for 18h. Nonspecific binding was determined by addition of 20µM unlabeled DEX. Unbound ligand was removed using dextran-coated charcoal. [3H]DEX activity was quantified as counts per minute on a Perkin-Elmer Topcount, using a Packard Optiplate and MicroScint40.

Hippocampal Gene Expression

From the other halves of the brains 200µm thick coronal sections were mounted on glass slides (Gerhard Menzel). Hippocampal CA1 tissue punches were taken with a Harris Uni-Core hollow needle (Electron Microscopy Sciences; 1.2mm internal diameter), starting around 2.56mm posterior to Bregma (Paxinos and Watson, 1998). RNA isolation, cDNA synthesis, and quantitative PCR have been described elsewhere (Datson *et al.*, 2009). Validated hippocampal GR target genes were selected from microarray analysis (Rat Genome 230 2.0 Arrays; Affymetrix) (Datson *et al.*, 2011). Tubulin β2a was used to normalize expression (Pfaffl, 2001).

Subchronic Treatment: Agonism in Relation to CRH and the HPA Axis

Group housed Wistar rats (200–220g; Harlan) underwent ADX in the morning as described (Sarabdjitsingh *et al.*, 2009). One week later, animals were treated twice daily (s.c., 1ml/kg) with vehicle (polyethylene glycol-300), C108297 (20mg/kg), or DEX (0.5mg/ kg) (Karssen *et al.*, 2005). On day 5, 3 hours after the morning injection, half of the animals underwent 30min of restraint stress. A tail-cut sample was collected 15min after the onset of restraint. Animals were killed by decapitation either under basal conditions or at 30min after onset of the restraint. CRH and c-fos mRNA and CRH hnRNA were quantified by in situ hybridization on whole PVN and CeA as described previously (Karssen *et al.*, 2005). Corticosterone and ACTH were measured by radioimmunoassay (MP Biomedicals).

Subchronic Treatment: Antagonism in Relation to CRH and the HPA Axis

Procedures were as described above but in intact rats, this time using RU486 (40mg/kg) as a reference drug. Tail cuts that were performed at 08:00 hours and 20:00 hours on

day 4 for basal plasma corticosterone levels. To determine acute stress responses in 'naïve rats', we subjected rats to an acute 0.4mA footshock in an inhibitory avoidance shock box (Claessens *et al.*, 2012), with or without a single pretreatment with the doses of RU486 and C108297 that were used in the subchronic setting.

Neurogenesis

Grouphoused Wistar rats (200g) were habituated to the animal facility for 10 days. Corticosterone (Sigma; C-2505; 40mg/kg) or vehicle (arachis oil) was injected (s.c.) daily at 09:00 hours on 21d. Animals received C108297 (50 mg/kg) or vehicle [0.1% ethanol in coffee cream; Campina] by gavage on the final 4 days of corticosterone treatment at 09:00h and 16:00h. Animals were killed 1 day after the last treatment. All animals received 5-bromo-2-deoxyuridine (BrdU) (200mg/kg, i.p.) on day 1, 3 hours after the first corticosterone injection. Tissue processing for immunostainings was performed as described (Hu *et al.*, 2012). Data on vehicle-treated groups were also reported elsewhere (Hu *et al.*, 2012).

Inhibitory Avoidance Behavior

One-trial inhibitory avoidance training and retention was performed as described (Fornari *et al.*, 2012b), using single-housed Wistar rats (300–350g; Charles River) and a footshock intensity of 0.5mA for 1s. RU486 (40mg/kg) or vehicle (polyethylene glycol) was administered (s.c.) 1h before the training session. C108297 (20mg/kg) or corticosterone (1mg/kg) was dissolved in DMSO and administered (100µL, s.c.) immediately after the training trial, so that treatment did not interfere with memory acquisition. Retention was tested 48h later. A longer latency to enter the former shock compartment with all four paws (maximum latency of 600s) was interpreted as better memory.

Statistical Analysis

Data were analyzed using Graphpad Prism using (as appropriate) one or two-way ANOVA, followed by Tukey's Bonferroni *post hoc test*, respectively, and Kruskal-Wallis for data that deviated from a normal distribution.

/ RESULTS

C108297 Displays Selective Modulator Activity in Vitro

To explore possible selective modulator activity of C108297 based on the GR-co-regulator interactions, we used a MARCoNI peptide array (Koppen *et al.*, 2009) to determine interactions between (recombinant) GR-LBD and co-regulator NR boxes **(Fig. 1A)**. Reference drugs were the full agonist dexamethasone (DEX) and the prototypical antagonist RU486 at saturating doses. Without ligand, GR displayed only weak interactions with co-regulator motifs. DEX induced significant interactions between GR-LBD and 28 motifs from co-activator proteins. RU486 induced modest interactions with motifs from two co-repressor proteins, nuclear receptor co-repressors (NCORs) 1 and 2 (Schulz *et al.*, 2002). C108297 induced interactions with a subset of the motifs that were recruited after DEX treatment, suggesting selective modulator activity. C108297 did not induce interactions with NCoR motifs. For quantitative analysis, see **Fig. 2**. The partial recruitment of co-regulator motifs of C108297-bound GR suggests that the compound combines agonistic and antagonistic effects (dependent on the gene-specific co-regulator use by GR).

C108297 Reaches the Brain

We tested whether C108297 can reach the brain to affect GR-dependent processes. Three hours after oral treatment of rats, C108297 (20mg/kg) led to 35 ± 15% occupancy of brain GR binding determined ex vivo in one hemisphere, compared with the negative control. This level of

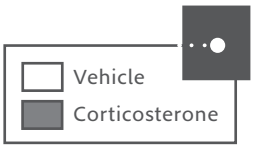

Figure 1

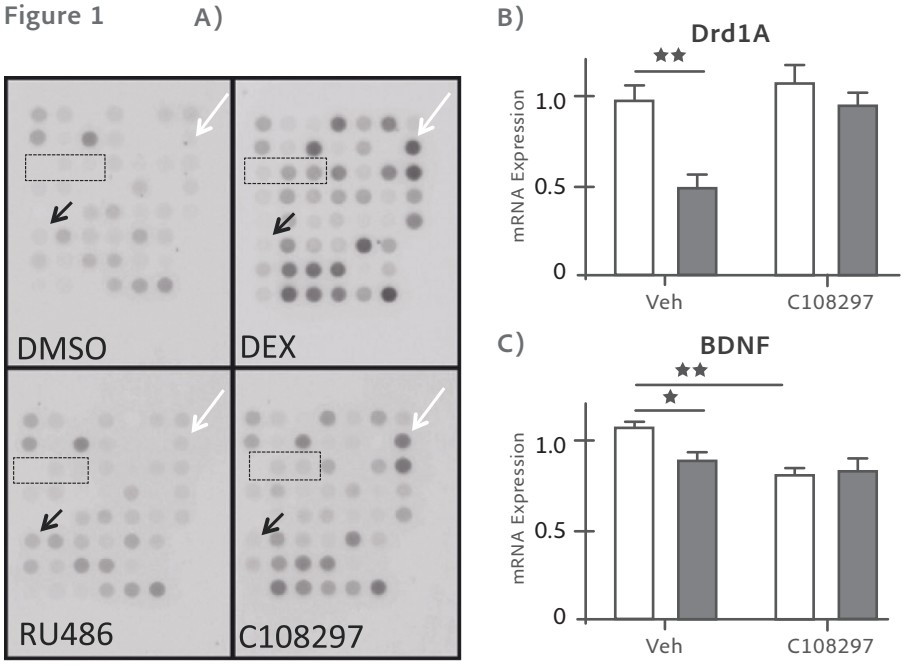

FIGURE 1:

C108297 BEHAVES LIKE A SELECTIVE MODULATOR IN VITRO AND IN VIVO

1A. Ligand-induced interactions between the GR-LBD and coregulator motifs. DEX induced many interactions compared with DMSO. RU486 induced modest interactions with corepressor motifs (black arrow: NCoR1). C108297 showed an intermediate profile. GR-LBD interactions ith the central motifs from SRC-1 were much weaker or absent (boxed), but others were retained (white rrow indicates SRC-1 motif IV).

1B. Hippocampal Drd1a mRNA was regulated by corticosterone after vehicle but not C108298 treatment.

1C. BDNF mRNA was down-regulated by both corticosterone and C108297. Asterisks indicate significant differences from the control group (★p < 0.05; ★★p < 0.01).

occupancy did not differ from that observed for the positive control of 3mg/kg corticosterone [well above the ED50 of 0.6mg/kg (Reul and de Kloet, 1985)], which resulted in 44±15% GR occupancy. This degree of occupancy is considered effective for many corticosterone effects via GR (e.g., ref. Schaaf *et al.*, 1998), and the dose of 20mg/kg C108297 was used in all other in vivo experiments described below, with the exception of the work on neurogenesis that was initiated earlier.

C108297 Displays Gene-Specific Agonism and Antagonism GR Target Genes in VIVO

To confirm gene-specific antagonism of C108297, we tested mRNA regulation of two previously characterized hippocampal GR target genes (Datson *et al.*, 2011). Rats were treated with 3mg/kg corticosterone with or without pretreatment with C1082987 (20mg/kg) or with C108297 alone. For Drd1a mRNA (coding for the dopamine 1A receptor), two-way ANOVA showed main effects of corticosterone ($p < 0.01$) and C108297 ($p < 0.05$) but no interaction (but endogenous corticosterone was present). Drd1a mRNA was significantly lower after corticosterone (3mg/kg) treatment but not after (pre)treatment with C108297 (20mg/kg) **(Fig. 1B)**. For BDNF regulation, two-way ANOVA showed main effects of corticosterone, C108297 (both $p < 0.05$) and an interaction ($p < 0.001$). C108297 by itself down-regulated BDNF mRNA levels and did not prevent the corticosterone effect **(Fig. 1C)**.

C108297 Distinguishes Between SRC-1 Splice Variants

Out of many potential co-regulators of GR, SRC-1 is among the few that have been linked to regulation of specific GR target genes (Winnay *et al.*, 2006; Lachize *et al.*, 2009). Its splice variants SRC-1A and -1E may mediate different effects in relation to stress adaptation (van der Laan *et al.*, 2008). Because C108297 seemed to differentiate

between the SRC-1 splice variants, we focused on these for further analysis. Quantitative analysis of the MARCoNI data showed that C108297 differentiates between the three NR boxes that are common to the two SRC-1 splice variants and NR box IV, which that is unique to SRC-1A (Kalkhoven *et al.*, 1998) **(Fig. 2A)**. Two-way ANOVA indicated highly significant differences between ligands and motifs and a strong interaction between the two ($p < 0.001$ for main effects and the interaction). DEX was able to induce strong GR interactions with all four SRC-1 motifs, but C108297 induced substantial agonist-like binding only for the SRC-1A specific NR-box **(Fig. 2B)**, confirming potentially selective recruitment of this splice variant by the GR-C108297 complex.

We validated the ligand-directed differential recruitment of SRC-1 splice variants using larger protein fragments in a two-hybrid system in mammalian COS-1 cells **(Fig. 2C)**. Two-way ANOVA showed significant effects of drug and protein fragment and an interaction ($p < 0.001$ for all effects). Both DEX and C108297 induced a strong GR-LBD interaction with a 420-aa fragment containing the SRC-1A specific NR-box IV. DEX, but not C108297, induced interactions with the SRC-1 domain containing the three central NR-boxes. A fragment from the co-repressor NCoR1 was recruited by GR-LBD only after incubation with the antagonist RU486. Thus, the ligand selective interactions of GR also occurred with large protein fragments in cell line context.

C108297 Has Selective Partial Agonist Activity in the Brain of Adrenalectomized Rats

The selective modulator type interactions of GR with SRC-1 variants led to the hypothesis that C108297 in vivo acts as an agonist for GR-mediated regulation of the Crh gene in the core of the HPA axis but not in the CeA (van der Laan *et al.*, 2008; Meijer *et al.*, 2000). To test agonism, we used adrenalectomized rats in a 5 days treatment paradigm in which half of the animals underwent a single

Figure 2

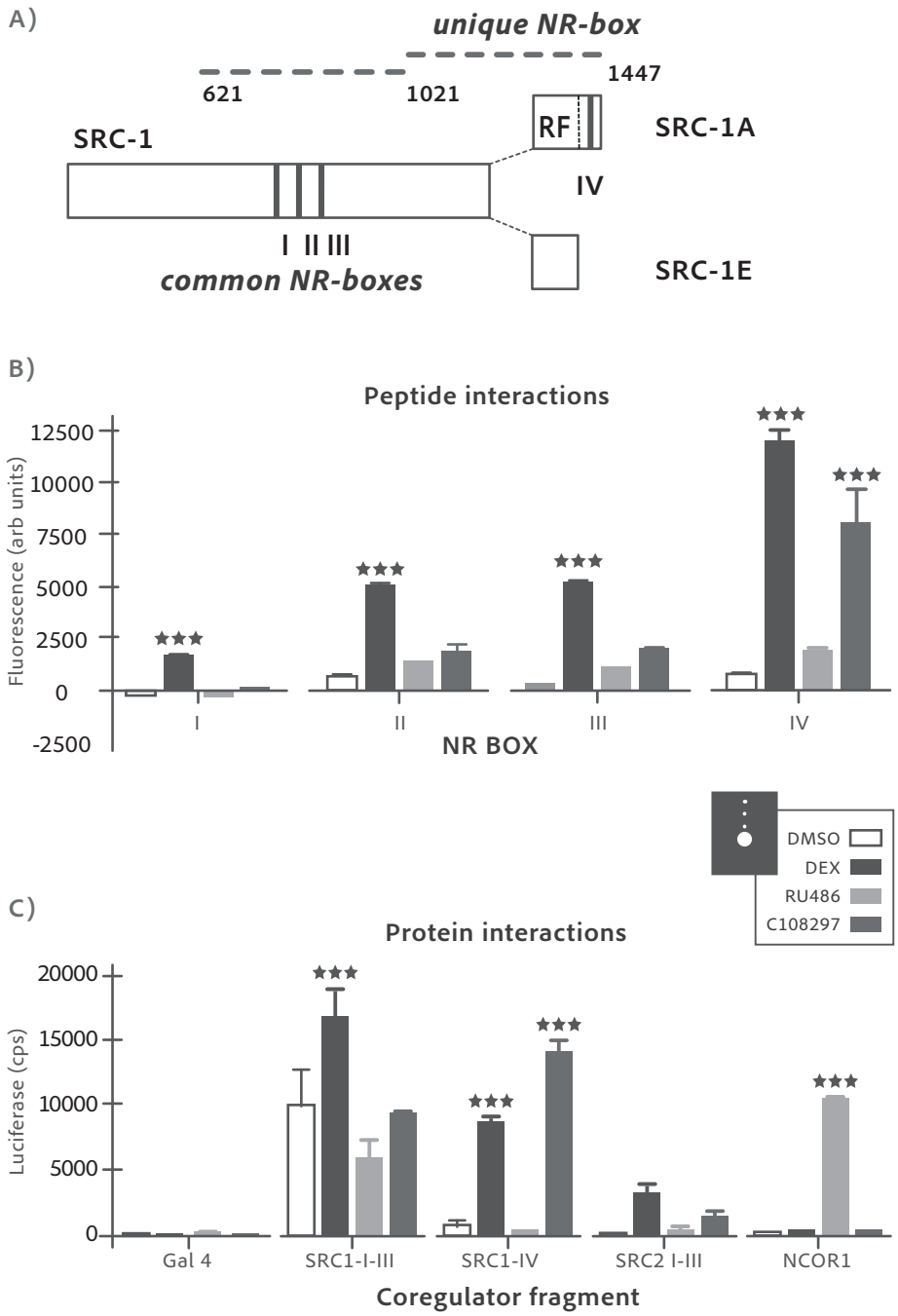
A)
unique NR-box
621
1021
1447
RF
SRC-1A
SRC-1
IV
I II III
common NR-boxes
SRC-1E
B)
Peptide interactions
Fluorescence (arb units)
12500
10000
7500
5000
2500
0
-2500
I
II
III
IV
NR BOX
DMSO
DEX
RU486
C108297
C)
Protein interactions
Luciferase (cps)
20000
15000
10000
5000
0
Gal 4
SRC1-I-III
SRC1-IV
SRC2 I-III
NCOR1
Coregulator fragment

FIGURE 2:

SCR-1 SPLICE VARIANT 1E IS SELECTIVELY RECRUITED BY GR-C108297

2A. Protein structure of SRC-1 harboring three NR central boxes (roman numerals). SRC-1A harbors a repressor function (RF) and the additional NR-box IV. Protein fragments marked by dotted lines refer to C.

2B MARCoNI quantification showed that unlike DEX, C108297 induced interactions only between GR and NR-box IV.

2C. In a two-hybrid assay only DEX induced interaction with the SRC-1 fragment common to both splice variants. The SRC-1A-specific protein fragment was also recruited by GR-C108297. A fragment of corepressor NCoR1 only interacted after incubation with RU486. Asterisks indicate significant difference from the control condition ($p < 0.001$).

restraint stress on day 5, 30min before they were euthanized. This paradigm allows measurement of a number of both basal and stress-induced HPA axis variables (Karssen *et al.*, 2005). It is well established that CRH expression in the hypothalamic paraventricular nucleus (PVN) and CeA both respond to treatment with our control agonist DEX, but in an opposite direction (Makino *et al.*, 1995).

CRH mRNA in both brain regions responded to drug but not to acute stress (two-way ANOVA: drug effect of PVN, $p < 0.001$; CeA, $p = 0.011$; stress effect not significant). In the PVN **(Fig. 3A)**, CRH mRNA was strongly suppressed by DEX. C108297 also showed modest agonism that reached significance in the stressed animals. CRH mRNA in the CeA **(Fig. 3B)** was increased after DEX treatment in non stressed animals but unaffected by C108297. In the stressed rats, the differences between the treatment groups failed to reach significance. A more substantial agonist effect of C108297 was observed for stress-induced CRH heteronuclear (hn) RNA in the PVN. This response was equally strongly suppressed by DEX and C108297 (**Fig. 3C**; one-way ANOVA: $p < 0.001$). In the CeA, the levels of CRH hnRNA were below detection, even after prolonged exposure of the films. Thus, C108297 showed (partial) agonism in the PVN but not in CeA.

Figure 3

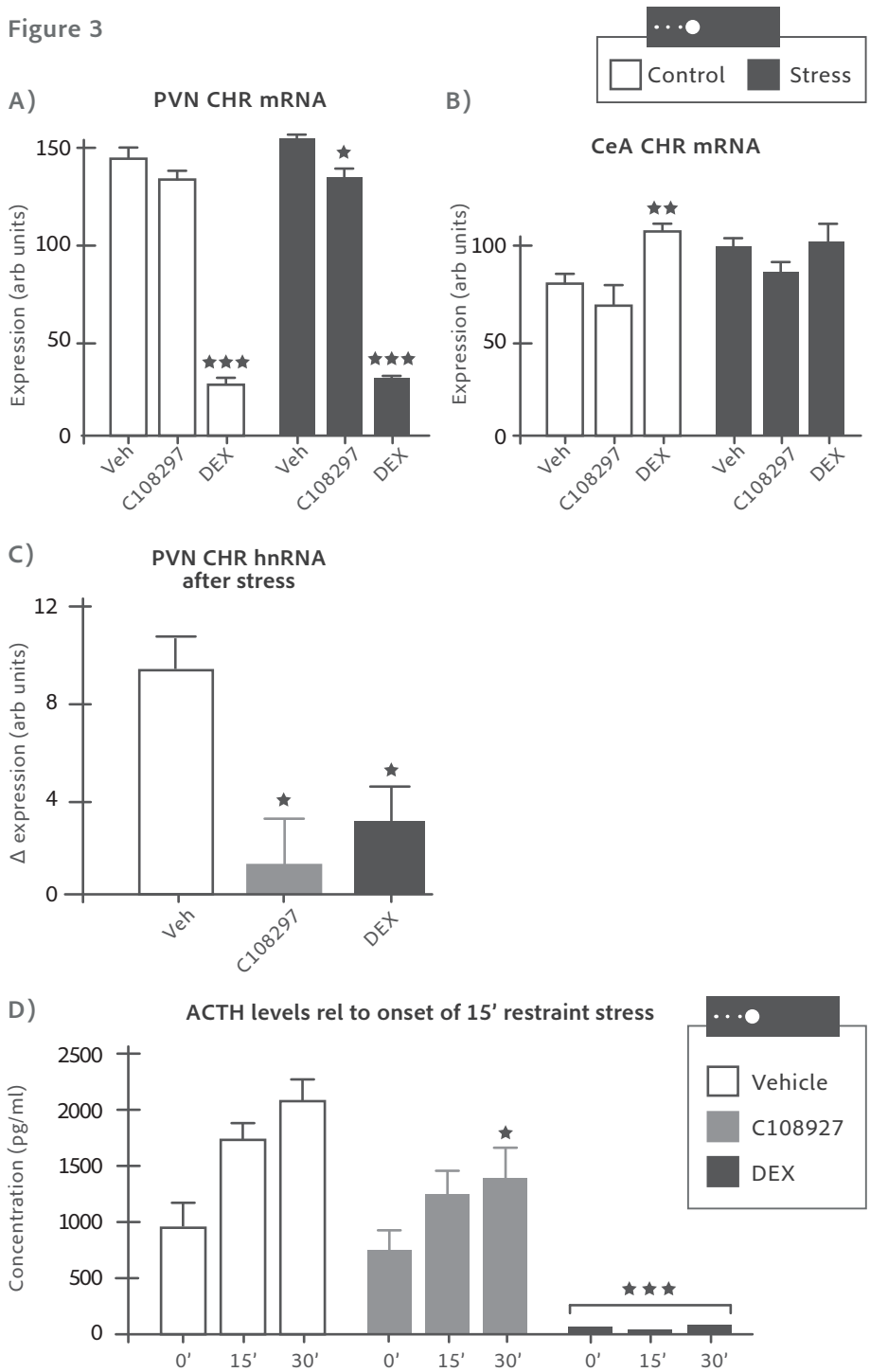
Control
Stress
A)
PVN CHR mRNA
Expression (arb units)
150
100
50
0
Veh
C108297
DEX
Veh
C108297
DEX
B)
CeA CHR mRNA
Expression (arb units)
100
50
0
Veh
C108297
DEX
Veh
C108297
DEX
C)
PVN CHR hnRNA
after stress
Δ expression (arb units)
12
8
4
0
Veh
C108297
DEX
D)
ACTH levels rel to onset of 15' restraint stress
Concentration (pg/ml)
2500
2000
1500
1000
500
0
0'
15'
30'
0'
15'
30'
0'
15'
30'
Vehicle
C108927
DEX

FIGURE 3:

SELECTIVE GR MODULATION IN THE STRESS SYSTEM: C108297- AGONISM IN ADX RATS AFTER SUBCHRONIC TREATMENT COMPARED WITH THE PROTOTYPIC AGONIST DEX

3A. In the PVN, where SRC-1A is expressed at high levels, DEX led to strong down-regulation of CRH mRNA ($p < 0.001$). C108297 had a modest agonist effect that reached significance in the stressed group ($p < 0.05$).

3B. In the CeA, DEX up-regulated CRH mRNA in nonstressed rats ($p < 0.05$), but C108297 was without effect.

3C. The acute response of the Crh gene in response to restraint stress was strongly attenuated both by pretreatment with DEX and C108297.

3D. DEX led to a complete blockade of the HPA axis ($p < 0.001$), whereas C108297 leads to a very weak attenuation of the adrenocortical stress response ($p < 0.05$).

Figure 4

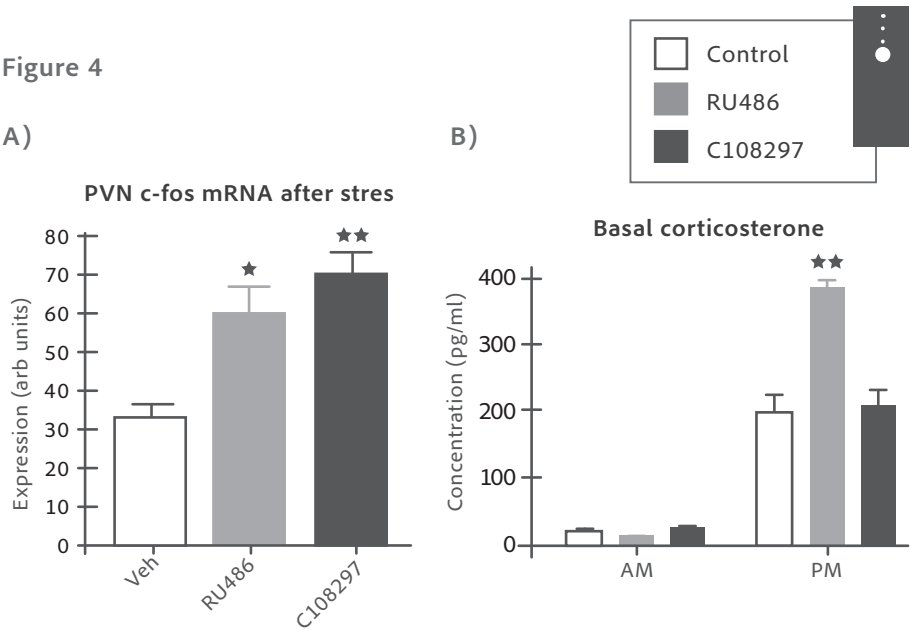

FIGURE 4:

SELECTIVE GR MODULATION IN THE STRESS SYSTEM: ANTAGONISM IN ADRENALLY INTACT RATS AFTER SUBCHRONIC TREATMENT COMPARED WITH THE PROTOTYPIC ANTAGONIST RU486.

4A. The acute c-fos response to stress in the PVN was enhanced both by pretreatment with RU486 and C108297.

4B. RU486 treatment led to increased circadian peak levels of plasma corticosterone. C108297 does not have this effect. ★ $p < 0.05$; ★★ $p < 0.01$.

To assess other (ant)agonist-like effects of C108297 on HPA axis activity, we determined basal and acute restraint stress-induced adrenocorticotropin (ACTH) secretion after 5 days of treatment [**Fig. 3D**; two-way ANOVA effects of time after on set of stress: drug-pretreatment ($p < 0.001$) and an interaction ($p < 0.01$)]. DEX led to a complete suppression of basal and stress-induced ACTH release. Sub-chronic C108297 treatment did not affect basal ACTH levels in these adrenalectomized (ADX) animals but led to a modest suppression of stress-induced ACTH release, possibly indicating a weak agonistic effect.

C108297 Has Selective Antagonist Activity in Adrenally Intact Rats

To determine neuroendocrine antagonistic effects against endogenous corticosterone we compared 5 days treatment of C108297 (20mg/kg) with RU486 (40mg/kg) in adrenally intact rats, followed by restraint stress on day 5 in half of the animals. The stressor strongly induced expression of both CRH hnRNA in the PVN (two-way ANOVA: $p < 0.001$) and led to a modest increase in CRH mRNA two-way ANOVA: $p < 0.05$), but these parameters were not affected by drug treatment (not shown), consistent with a lack of GR involvement in the immediate curtailing of the transcriptional CRH response in acute stress situations (Aguilera *et al.*, 2007). There was no effect of sub-chronic drug treatment or the stressor on amygdala CRH mRNA. The only central measure that responded to subchronic drug treatment in intact rats was the c-fos response to restraint stress in the PVN (one-way ANOVA: $p < 0.001$). Both RU486 and C108927 treatment led to elevated c-fos mRNA expression 30min after the onset of stress **(Fig. 4A)**.

With regard to stress-induced activation of the HPA axis, the two compounds also led to similar changes, indicative of antagonism by C108297. At 15min after the onset of the restraint stress, corticosterone levels were about 25% lower in both the RU486 (301 ± 69ng/ml) (Wulsin *et al.*, 2010) and C108297 (273 ± 52ng/ml) treatment groups

Figure 5

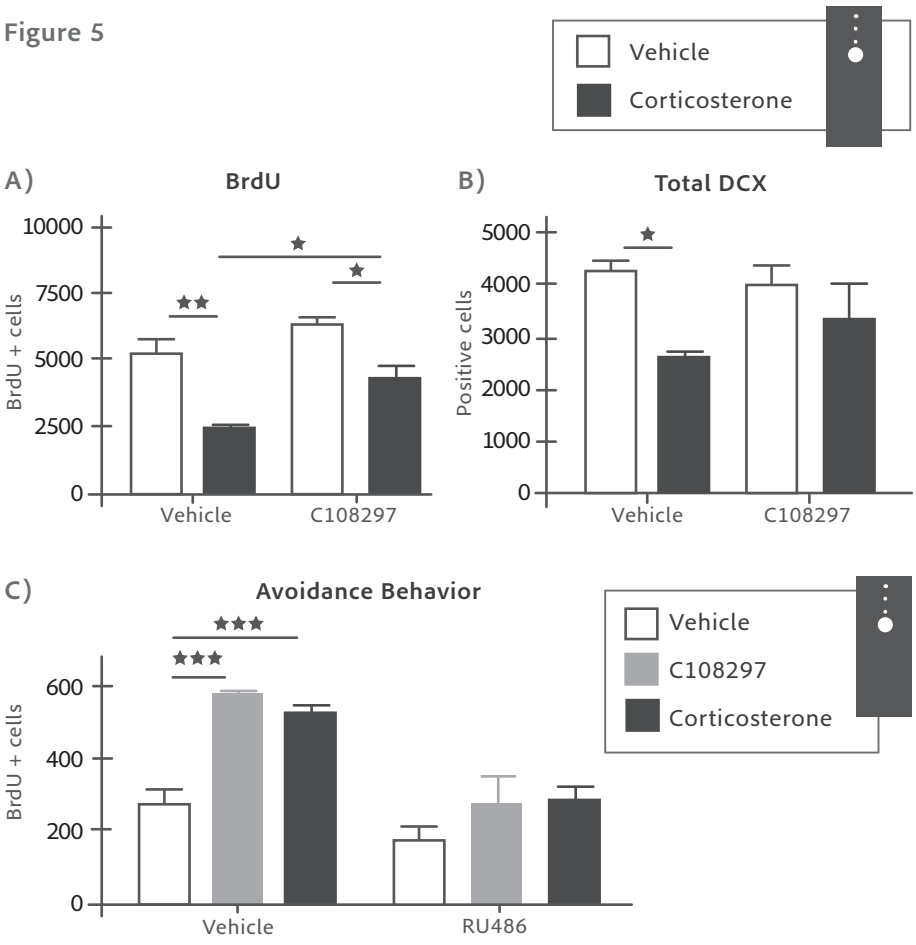

FIGURE 5:

C108297 ACTS AS GR ANTAGONIST IN NEUROGENESIS AND AS AGONIST IN MEMORY RETENTION

5A. Chronic corticosterone suppressed the number of BrdU-positive cell, and 4 d of C108297 treatment increased this number. BrdU scores were significantly higher in animals that received C108297 in combination with chronic corticosterone, compared with corticosterone-treated animals that did not receive C108297.

5B. Total DCX-positive cells were significantly fewer after 3 wk of corticosterone treatment but not in animals that also received C108297.

5C. Acute posttraining C108297 (20 mg/kg) or corticosterone (1 mg/kg) led to long 48h retention test latencies in the inhibitory avoidance task, and these effects were blocked by pretreatment with RU486. Significant differences: ★ $p < 0.05$; ★★ $p < 0.01$; ★★★ $p < 0.001$.

compared with controls (409 ± 36ng/ml). In contrast, RU486 increased the amplitude of the basal diurnal corticosterone rhythm by increasing evening corticosterone levels without affecting morning levels, as described (Spiga *et al.*, 2007), but C108297 did not have this antagonistic effect (**Fig. 4B**; $p < 0.001$ for drug, time, and interaction effects).

Agonism and Antagonism on Neurogenesis and Behavior

To further evaluate the efficacy of C108297 in animal models with relevance for psychopathology, we evaluated the effect of C108297 in two paradigms: corticosterone induced suppression of neurogenesis and memory consolidation of inhibitory avoidance training.

C108297 was tested for reversal of GR-dependent reduction in adult neurogenesis after 3 weeks of treatment with a high dose of corticosterone (40mg·kg-1·d-1). RU486 was shown previously to fully normalize the reduction in neurogenesis induced by corticosterone or chronic stress (Hu *et al.*, 2012). In a comparable design, C108297(50mg/kg) was administered during the last 4 days of corticosterone treatment. Two-way ANOVA indicated that the number of cells that stained for BrdU (a marker for newborn cell survival) was affected by chronic corticosterone treatment ($p = 0.008$) and by C108297 treatment ($p < 0.001$), but there was no significant interaction. *Post hoc* analysis revealed that the difference between C108297 and vehicle groups only reached significance in animals treated chronically with corticosterone **(Fig. 5A)**. The number of double-cortin (DCX) positive cells in the dentate gyrus, indicative of neuronal differentiation of newborn cells, was affected by chronic corticosterone treatment ($p = 0.002$) but not by C108297 ($p > 0.4$), although there was a trend toward an interaction ($p = 0.089$). *Post hoc* analysis indicated a significantly lower number of DCX positive cells after chronic corticosterone treatment only in the group treated with the

vehicle for C108297 **(Fig. 5B)**. Thus, C108297 partially counteracted the effects of chronic corticosterone treatment.

To determine whether C108297 affected memory consolidation, rats were trained on an aversively motivated single-trial inhibitory avoidance task, which is known to be potentiated by GR activation (Fornari *et al.*, 2012b). A corticosterone (1mg/kg) treatment was included as a positive control. Retention test latencies, as assessed 48h after training, indicated a significant drug treatment effect (Kruskal-Wallis test: $p < 0.001$; **Fig. 5C**). Rats treated with either corticosterone or C108297 had significantly longer retention latencies than vehicle-treated rats ($p < 0.001$). This effect could be blocked by RU486 pretreatment. These findings indicate that C108297 has substantial GR agonism in this paradigm.

/ DISCUSSION

High levels of circulating glucocorticoids as a consequence of acute or chronic stress are known risk factors in the development of psychopathologies, either as predisposing factors or during precipitation of disease. GR antagonists have therapeutic potential (Wulsin *et al.*, 2010; Bachmann *et al.*, 2003) but given the ubiquitous expression of the GR, they have many undesired side effects (Sapolsky *et al.*, 2000). Disinhibition of the HPA axis is a side effect that actually counteracts the goal of any such treatment (i.e., blockade of GR signaling). SGRM compounds that combine antagonistic and agonistic GR properties may lead to a better-targeted interference with stress-related brain processes.

Based on the C108297 induced interactions between GR and its co-regulators, we hypothesized and confirmed that this compound is a selective GR modulator,

with relevance for the brain. Interestingly, clear antagonist effects on the brain were accompanied by lack of negative-feedback inhibition of the HPA axis, which in itself suggests the possibility of antagonizing a number of GR effects without affecting systemic basal glucocorticoid levels and the associated change in activity of, for example, MR-dependent processes (Joëls *et al.*, 2008). C108297 is expected to have selective modulator effects also in peripheral tissues that we did not examine here (Asagami *et al.*, 2011). We did not determine binding to MRs and PRs or specific MR/PR readouts here, but previous studies showed 0% displacement from MR and 26% from PR at 10μM C108297, i.e., over a 1000 fold selectivity for GR (Clark *et al.*, 2008). In peripheral tissues, we cannot exclude some binding to PR with the 20mg/kg dose C108297, but under non saturating conditions for brain GR, activation of other steroid receptors is unlikely. Selective targeting to the brain may constitute a particularly efficacious way to interfere with a number of central GR-dependent processes, with very few side effects.

In the MARCoNI assay the overall strength of the GR bound to C108297 interactions with co-regulator motifs is somewhat lower than for GR bound to DEX, suggesting that C108297 is a partial agonist. Some of the antagonist effects that we observed after a single dose in vivo may indeed reflect partial agonism relative to circulating corticosterone. However, because some of the co-regulator interactions become zero, whereas others still reach substantial levels, the molecular profile is that of a selective modulator. It is unclear at this point whether theGRfollows a two-state agonist conformation, with C108297 leading to a similar, but less stable, conformation to DEX (Raaijmakers *et al.*, 2009), or whether C108297 leads to a unique conformation of the GR-LBD. C108297 clearly differs from the well-known (but nonselective) antagonist RU486, because it lacks the capacity to induce interactions with domains from corepressors NCoR1 and -2, and the associated intrinsic (repressive) activity that may come from those interactions (Schulz *et al.*, 2002).

Reversal of glucocorticoid-induced effects was observed for expression of the *Drd1a* gene in the hippocampus. This effect may be of relevance for reversal of negative effects of glucocorticoids on cognition (Ortiz *et al.*, 2010). Given chronically, C108297 also antagonized the effects of corticosterone on adult neurogenesis. Here, C108297 seemed to be less potent than RU486 (Sapolsky *et al.*, 2000), perhaps because of a lack of interactions between GR and the classical co-repressors. Notwithstanding, reversal of decreased neurogenesis may be relevant for antidepressive effects (Sahay and Hen, 2007). In relation to regulation of brain CRH, the compound seems to have beneficial effects in the context of stress-related psychopathology, as was predicted by its interactions with the coregulator SRC-1 splice variants (Lachize *et al.*, 2009; van der Laan *et al.*, 2008). The compound lacked efficacy for the potentially anxiogenic induction of CRH via GR (Kolber *et al.*, 2008) even in ADX rats. It showed a mild degree of agonism on basal CRH expression in the PVN, and pretreatment had a substantial suppressive (agonistic) effect on stress-induced CRH transcription (van der Laan *et al.*, 2009). Moreover, there was a clear lack of antagonism by C108297 on basal regulation of the HPA axis, which is an important advantage over complete antagonists such as RU486 when trying to interfere with central consequences of hypercorticism (Spiga *et al.*, 2007).

C108297 does not cause an overall dampening of brain stress responses. Like RU486, it enhanced stress-induced neuronal activity in the PVN, indicating either changed responsiveness of the parvocellular neurons or changed activity of neuronal afferents to the PVN. The apparent agonism on BDNF expression (Schaaf *et al.*, 1998) also shows that some consequences of stress may be mimicked by the compound. The GR-dependent increased consolidation of inhibitory avoidance memory also is in line with well-known stress effects and can be either adaptive or maladaptive (Joëls *et al.*, 2006; Kaouane *et al.*, 2012).

Our data emphasize the multiple levels of GR-mediated control over the HPA axis. For example, RU486, as well as C108297, led to an increased c-fos response to stress in the PVN but to an attenuated stress-induced ACTH release. This dissociation has been observed by others after direct and acute manipulation of the PVN (Evanson *et al.*, 2010). The extent to which CRH and c-fos respond to stressors in 'naïve' rats is in general highly dependent on multiple factors, including the type of stressor and time after stress (Helfferich and Palkovits, 2003; Figueiredo *et al.*, 2003).

A small part of the selective GR modulation in vivo may be explained by differential recruitment of SRC-1A and -1E, and the role of numerous GR co-regulator interactions in mediating the many effects of GR activation on brain will be subjected to further research. SGRMs such as C108297 and their molecular interaction profiles, combined with knowledge of the regional distribution of co-regulators in the brain, can, in the future, assist in dissecting the molecular signaling pathways underlying stress related disorders. In fact, although our analysis was necessarily not comprehensive [e.g., in relation to non genomic GR signaling (Karst *et al.*, 2010)], C108297 itself may have a beneficial profile compared with a situation of hypercortisolism.

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A Novel Mixed Glucocorticoid/ Mineralocorticoid Selective Modulator With Dominant Antagonism in the male Rat Brain

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CHAPTER 07

Adrenal glucocorticoid hormones are potent modulators of brain function in the context of acute and chronic stress. Both mineralocorticoid and glucocorticoid receptors (MRs and GRs) may mediate these effects. We studied the effects of a novel ligand, C118335, on the rat brain. C118335 has high affinity for GRs and modest affinity for MRs. In vitro profiling of receptor-coregulator interactions suggested that the compound is a selective modulator for GRs that may have both agonistic and antagonistic effects. Its molecular profile on MRs was highly similar to the full antagonist eplerenone. The compound showed predominantly antagonistic effects on hippocampal mRNA regulation of known glucocorticoid target genes.

Systemic administration of C118335 also blocked corticosterone-induced enhancement on memory consolidation in an inhibitory avoidance task. The intrinsic effect of C118335 was a strong and dose - dependent reduction of memory consolidation that -surprisingly- reflected involvement of MRs, and not GRs, in this type of memory consolidation. Finally, we didn't find disinhibition of the hypothalamus - pituitary - adrenal axis after treatment with C118335. Mixed GR/MR ligands like C118335 may be of great use to unravel mechanisms of glucocorticoid signalling, and are potential prototypes of drugs to treat the harmful effects of chronic overexposure to glucocorticoids.

INTRODUCTION

The effects of adrenal glucocorticoid hormones on the brain are of crucial importance for adaptation to stress, in part through their effects on learning and memory. These steroids act via mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs), two members of the nuclear receptor family that can act as transcription factors. The transcriptional effects of the high-affinity MR have been associated with responsiveness to stressful stimuli, while GR effects have been linked to later adaptive processes such as negative feedback and consolidation of newly formed memories. More rapid non-genomic effects that are mediated by MRs and GRs may support immediate stress responses (de Kloet *et al.*, 2005; Sarabdjitsingh *et al.*, 2014).

Excessive or prolonged glucocorticoid exposure increases vulnerability to psychopathologies like major depressive disorder. Full GR antagonists have been pursued as potential therapeutic targets in research (Bachmann *et al.*, 2003; Spiga *et al.*, 2007; Wulsin *et al.*, 2010) and clinical settings (DeBattista *et al.*, 2006), but have so far not fulfilled their promise in large-scale clinical trials. Even if antagonizing brain GR in some situations is clearly beneficial (van der Lely *et al.*, 1991; Nieman *et al.*, 1985), pure GR antagonists, like RU486, may not be the ideal drugs to combat stress-related psychopathology. First, pathogenicity may be caused by either excessive stimulation of MR/GR target genes (Meijer, 2006; Polman *et al.*, 2013), but also by a loss of receptor responsiveness (Touma *et al.*, 2011). Second, full antagonism will lead to loss of negative feedback in the hypothalamus-pituitary-adrenal (HPA) axis, resulting in increased glucocorticoid levels that will counteract GR antagonism (Spiga *et al.*, 2007, Ratka *et al.*, 1989). Third, decreasing GR signaling will lead to a shift of cortisol action towards MR-dependent effects, which may or may not be beneficial in particular settings (Joëls *et al.*, 2008).

Selective modulator type of ligands allow manipulation of GR-mediated effects in the brain stress system in a more specific manner (Zalachoras *et al.*, 2013a, Solomon *et al.*, 2014). Such compounds combine agonism and antagonism in a tissue or gene-dependent manner, based on the large range of signaling partners of the ligand-activated receptor. One mechanism, known for GR as 'dissociated signaling', is based on less efficient DNA binding of the receptor but intact protein-protein interactions with other, non-receptor, transcription factors (De Bosscher *et al.*, 2008). Another basis for selective modulation is differential recruitment of nuclear receptor coregulators by MR or GR after binding of ligands (Coghlan *et al.*, 2003). The coregulators form a large and diverse group of transcriptionally active proteins, which mediate the transcriptional effects of nuclear receptors. The several hundreds of identified coregulators display tissue and cell type-specific expression patterns, and gene- and receptor-specific interactions (Lachize *et al.*, 2009; O'Malley, 2007; Zalachoras *et al.*, 2013b).

Here, we investigated the effects on the brain of the novel ligand C118335 that has a unique receptor pharmacology: low nanomolar affinity for GR, modest affinity for MR, and no binding to progesterone and androgen receptors (Hunt *et al.*, 2012). A coregulator-interaction assay suggested that the compound is a selective modulator for GR, and a predominant antagonist at the MR. Transcriptional effects on individual glucocorticoid target genes suggest that combined GR and MR antagonism is dominant in hippocampus and striatum, while there was modest agonism on negative feedback in the HPA axis. Effects of C118335 in an inhibitory avoidance task, revealed a contribution of MR in memory consolidation.

/ MATERIALS AND METHODS

Peptide Interaction Profiling

Interactions between the MR and GR ligand binding domain (LBD) and coregulator nuclear receptor (NR) boxes were determined on a MARCoNI assay. The method has been previously described, in detail, elsewhere (Koppen *et al.*, 2009; Desmet *et al.*, 2014). Briefly, each array was incubated with a reaction mixture of 1 nM GST-tagged MR-LBD or GR-LBD, ALEXA488-conjugated GST- antibody, and buffer F (PV4689, A-11131, and PV4547; Invitrogen, Bleiswijk, The Netherlands) and vehicle (2% DMSO in water) and the receptor ligands at the indicated concentration (GR agonist dexamethasone [DEX], the GR antagonist RU486, the mixed MR/GR agonist cortisol, the MR antagonist eplerenone or, the Selective GR Modulator C108297, or C118335). Incubation was performed at 20°C in a PamStation96 (Pamgene International, Den Bosch, The Netherlands). GR binding to each peptide on the array, reflected by fluorescent signal, was quantified by analysis of .tiff images using BioNavigator software (Pamgene International).

All animal experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and experiments were approved by the Local Committees for Animal Health, Ethics, and Research of the Dutch universities involved.

Gene Expression Studies

Male Sprague-Dawley rats (10-14 weeks old) were used (Charles River, Kisslegg, Germany). The rats were group housed with food and water available *ad libitum* under a 12h/12h light/dark regime (lights on: 07:00h - 19:00h). 5-7 rats per group were injected subcutaneously with vehicle (90% PEG, 10% DMSO), C118335 (100 mg/kg), followed 30min later by an injection of corticosterone (3mg/kg) or vehicle.

Three hours after the second injection animals were sacrificed by an intraperitoneal injection of overdose Euthasol (ASTfarma, Oudewater, The Netherlands) followed by decapitation. Their brains were harvested and snap frozen in isopentane on dry ice and subsequently stored at -80°C. Trunk blood was also collected in EDTA-coated tubes, centrifuged at 13000 rpm for 15min and plasma collected and stored at -20°C until further processing.

Radioimmunoassay

Plasma corticosterone levels were determined with radioimmunoassays using ^{125}I RIA kits (MP Biochemicals, Santa Ana, CA, USA) as per the manufacturer's instructions.

Punching: 200µm thick brain sections were taken on a Leica 3050 cryostat (Rijswijk, The Netherlands) and mounted on uncoated glass slides (Menzel-Gläser, Braunschweig, Germany). Subsequently, tissue was punched out from the caudate putamen and the CA1-CA2 region of the dorsal hippocampus using appropriate Harris Unicore punching needles (Tedpella, Redding, CA, USA).

RNA Isolation, cDNA Synthesis and qPCR

The samples were homogenized on a TissueLyser II (Retsch Qiagen, Haan, Germany), centrifuged and 200µl of chloroform (Merck KGaA, Darmstadt, Germany) was added to each sample. After centrifugation at 11000rpm for 15min, the aqueous phase (top phase) was taken and 5µl of 5mg/ml linear acrylamide (Ambion, Austin, TX, USA), as a carrier, and 500µl isopropyl alcohol (Merck KGaA, Darmstadt, Germany) were added, followed by centrifugation at 11000rpm for 10min and removal of the supernatant. The RNA pellet was washed twice with 75% ethanol (Merck KGaA, Darmstadt, Germany), air-dried and dissolved in 10µl demineralized H_2O. The purity and concentration of the RNA samples were measured on the Nanodrop 1000 (Isogen

Life Science, De Meern, The Netherlands). The integrity of the samples was measured on Standardsens chips on a Bio-Rad experion system (Hercules, CA, USA).

For cDNA synthesis, RNA samples were pretreated with DNase (Promega, Madison, WI, USA) to remove potential genomic DNA contamination according to the manufacturer's specifications. For the incubation a MyCyclertm Thermal Cycler (Bio-Rad) was used. Subsequently, cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad). Four µl 5x iScript reaction mix, 1µl iScript reverse transcriptase (RT) and 5µl nuclease free water were added to each DNase pretreated sample. A control sample without RT treatment was also included in which the 1µl RT was replaced by 1µl nuclease free water. The samples were placed in a MyCyclertm Termal Cycler and incubated for 5min at 25°C, 30min at 42°C and 5min at 85°C (Touma *et al.*, 2011).

Quantitative polymerase chain reaction (qPCR) was performed to measure gene expression in the different brain regions. The efficiency of the used primers was first measured for each gene in each region. To perform the qPCR, the FC FastStartDNA Masterplus SYBR Green I (Roche Applied Science, Basel, Switzerland) kit was used. 2.5µl per cDNA sample was added to a mix of 2µl 5x Sybr green mix, 0.5µl 10 µM of both the forward and reverse primers **(table 1)** and 4.5µl DEPC H_2O to a total volume of 10µl. For the reactions 20µl LightCycler Capillaries (Roche) were used placed in a LightCycler Sample Carousel 2.0 (Roche). The carousel was centrifuged on a LC Carousel Centrifuge 2.0 (Roche), subsequently placed in a LightCycler 2.0 (Roche) to perform qPCR. All samples were measured in duplicate. The samples were incubated for 10min at 95°C, followed by 45 replication cycles (10s denaturation at 95°C, 10s annealing at 60°C and 10s elongation at 72°C) and finally a melting curve was made (65°C to 95°C, 0.1°C/s).

Inhibitory Avoidance Experiment

Male Sprague-Dawley rats (10-14 weeks old) from Charles River Breeding Laboratories (Kisslegg, Germany) were housed individually in a temperature-controlled (22ºC) vivarium room and maintained on a 12h/12h light/dark cycle (lights on: 07:00h/19:00h) with *ad libitum* access to food and water. Rats were handled 3 times for 1min each prior to training. Training and testing were performed during the light phase of the cycle, between 10:00h-15:00h. Rats were trained and tested in an inhibitory avoidance apparatus consisting of a trough-shaped alley (91cm long, 15cm deep, 20cm wide at the top, and 6.4cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor (McGaugh *et al.*, 1988). The starting compartment (31cm) was made of opaque white plastic and well lit; the shock compartment (60cm) was made of two electrifiable metal plates and was not illuminated. Training and testing were conducted in a sound -and light- attenuated room.

For training, the rats were placed in the starting compartment, facing away from the door, and were allowed to enter the dark (shock) compartment. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.38mA, 1s) was delivered. The rats were removed from the shock compartment 15s after termination of footshock and, after drug treatment, returned to their home cages. On the 48h retention test, as on the training session, the latency to re-enter the shock compartment with all four paws (maximum latency of 600s) was recorded and used as a measure of retention. Shock was not administered on the retention test trial.

For the first experiment, a single subcutaneous systemic injection of C118355 (80mg/kg) or vehicle (100% DMSO) was administered 1h prior to training in combination with an immediate posttraining administration of corticosterone (1mg/kg) or vehicle. Corticosterone was dissolved in 100% polyethylene glycol. For the second experiment, C118335 (50 or 80mg/kg), the MR antagonist spironolactone

(40mg/kg) or the GR antagonist RU486 (40mg/kg) was dissolved in 100% DMSO and administered subcutaneously, in a volume of 2ml/kg, immediately after the inhibitory avoidance training trial.

Statistical Analysis

To analyze effects of C118335 on corticosterone secretion a *t-test* between the vehicle and the C118335-treated group was used in non-corticosterone-treated rats. In order to determine successful corticosterone treatment, a two-way ANOVA was used. For the analysis of the Ct values from the qPCR the mathematical model from Pfaffl (Bachmann *et al.*, 2003) was used. Tubulin and Actin-β were used as reference (housekeeping) genes. The geometric mean of these two genes was used as the reference value. The Grubbs' outlier test was conducted and outliers were excluded from the analysis. The values were analyzed by two-way ANOVA, with glucocorticoid treatment and antagonist treatment as factors, followed by Tukey's *post-hoc test*.

Inhibitory avoidance training and retention latencies were analyzed by one-way or two-way ANOVA, followed by *post-hoc* comparison tests, when appropriate. Paired *t-tests* were used to compare training and retention latencies of each animal and determine whether learning had occurred. A probability level < 0.05 was considered statistically significant for all analyses.

RESULTS

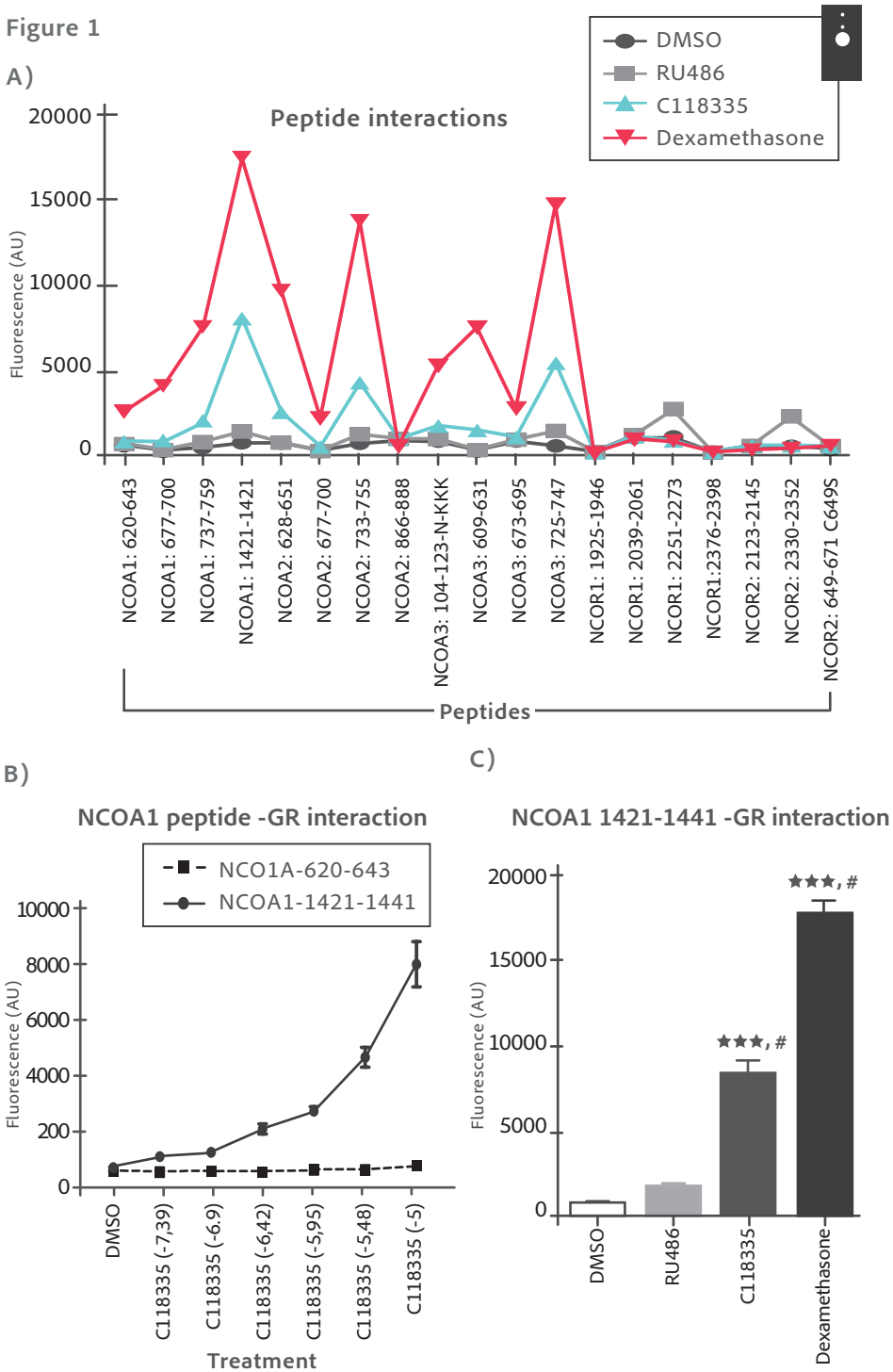
C118335-induced coregulator interactions are selective modulator-like for GR and antagonist-like for MR.

In the MARCoNI assay, C118335 induced a pattern of GR-coregulator interactions that was intermediate to dexamethasone and RU486. The number of interactions observed after C118335 binding was much lower than with dexamethasone **(Figure 1A)**. However, it selectively recruited a number of NR boxes, such as the SRC-1 NR-box IV in a dose-dependent manner **(Figures 1B, 1C)**, but to a lesser extent than dexamethasone (Figure 1C). On the other hand, C118335 did not induce interactions between the GR-LBD and SRC-1 NR-box I, unlike dexamethasone **(Figure 1D)**. In contrast to RU486, it did not lead to recruitment of corepressor motif NCOR1 2251-2273 **(Figure 1E)**. This suggests that the compound will act as an antagonist on most processes that depend on the coregulators represented at the array, but may show substantial partial agonism for others.

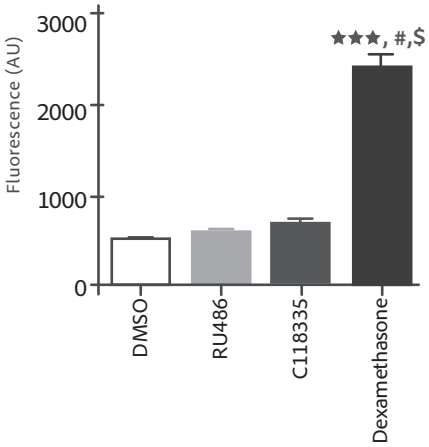
When bound to MR-LBD, C118335 showed a pattern that was fully identical to eplerenone, the most selective MR antagonist available. However, eplerenone and C118335-occupied MR was able to induce interactions with most of the coregulator motifs that are also induced by the full agonist cortisol, and based on this array both C118335 and eplerenone would be predicted to act as partial agonists, rather than full agonists. The data suggest that -with the caveat of its ten-fold lower affinity for MR- C118335 will act identical to eplerenone in transcriptional regulation.

C118335 acted as an antagonist on the canonical GR target genes FKBP5 and SGK-1 in the CA1-CA2 region of the hippocampus and striatum. Treatment with corticosterone in the absence of other ligands resulted in a strong upregulation of FKBP5 mRNA levels in the CA1-CA2 region of the hippocampus. Treatment with C118335 prevented the corticosterone-induced FKBP5 upregulation **(Figure 2A)**. Similarly, C118335 resulted in decreased expression of SGK-1 regardless of glucocorticoid treatment in these adrenally intact animals **(Figure 2B)**. Corticosterone nor C118335 affected Fkbp5 mRNA in the striatum **(Figure 2C)**.

Figure 1


A)
Peptide interactions
DMSO
RU486
C118335
Dexamethasone
Fluorescence (AU)
20000
15000
10000
5000
0
NCOA1: 620-643
NCOA1: 677-700
NCOA1: 737-759
NCOA1: 1421-1421
NCOA2: 628-651
NCOA2: 677-700
NCOA2: 733-755
NCOA2: 866-888
NCOA3: 104-123-N-KKK
NCOA3: 609-631
NCOA3: 673-695
NCOA3: 725-747
NCOR1: 1925-1946
NCOR1: 2039-2061
NCOR1: 2251-2273
NCOR1:2376-2398
NCOR2: 2123-2145
NCOR2: 2330-2352
NCOR2: 649-671 C649S
Peptides
B)
NCOA1 peptide -GR interaction
NCO1A-620-643
NCOA1-1421-1441
Fluorescence (AU)
10000
8000
6000
4000
200
0
DMSO
C118335 (-7,39)
C118335 (-6.9)
C118335 (-6,42)
C118335 (-5,95)
C118335 (-5,48)
C118335 (-5)
Treatment
C)
NCOA1 1421-1441 -GR interaction
Fluorescence (AU)
20000
15000
10000
5000
0
★★★, #
★★★, #
DMSO
RU486
C118335
Dexamethasone

D)



E)

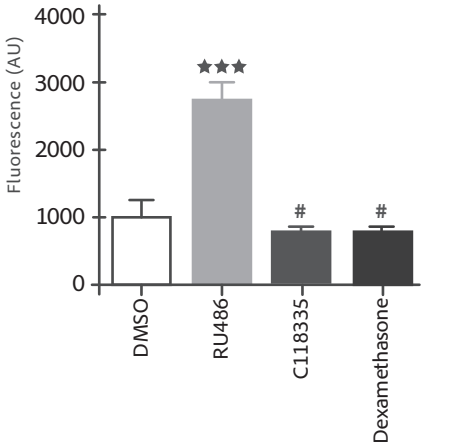


FIGURE 1:

C118335 did not induce as many GR-LBD – coregulator peptide interactions as dexamethasone. However, it induced partial recruitment of the SRC-1a specific NR box-IV (NCOA1-1421-1441).

1A. Overview of the ligand-induced interactions between GR-LBD and coregulator motifs after treatment with DMSO, the classical antagonist RU486, the novel GR ligand C118335 and dexamethasone.

1B. C118335 induced GR-LBD – SRC-1 NR-box IV interactions in a dose-dependent manner, while it did not induce considerable GR-LBD- SRC-1 NR-box I (NCOA1-620-643) interactions at any concentration.

1C. C118335 induced significantly stronger interactions between GR-LBD and SRC-1 NR-box IV than DMSO, though not as strong as the dexamethasone induced interactions one-way ANOVA, $p < 0.0001$, $F_{(3,15)} = 168.6$, tukey's post hoc test:★★★, $p < 0.001$ compared to DMSO group; #, $p < 0.001$ compared to RU486 group.

1D. C118335 did not induce SRC-1 NR-box I –GR-LBD interaction, unlike dexamethasone: One-way ANOVA: $p < 0.0001$, $F_{(3,15)} = 227.6$, tukey's post-hoc test:★★★, $p < 0.001$ compared to DMSO group, #, $p < 0.001$ compared to RU486 group, $, $p < 0,001$ compared to C118335 group.

1E. C118335 did not induce interactions with the corepressor motif NCOR1-2251-2273 : One-way ANOVA : $p < 0.0001$, $F_{(3,15)} = 16.89$, tukey's post hoc test:★★★, $p< 0.001$ compared to DMSO group; #, $p <0.001$ compared to RU486 group.

Figure 2

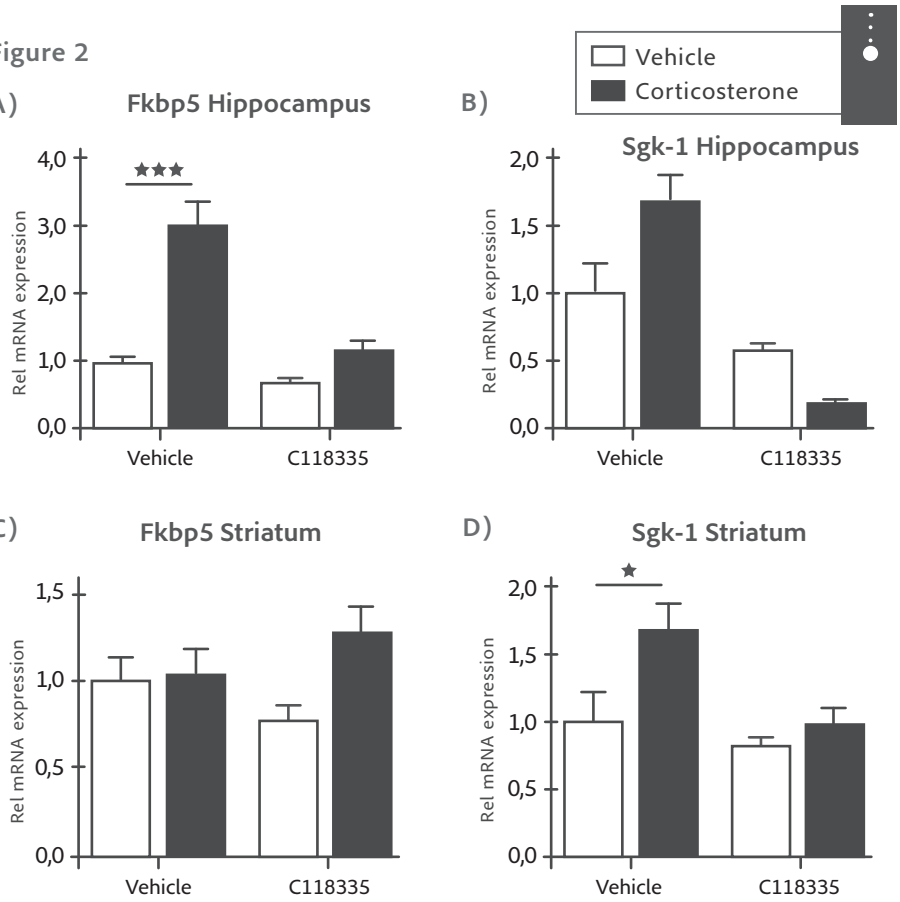


FIGURE 2:

FKBP5 and SGK-1 expression in the hippocampus and dorsal striatum

2A C118335 blocked the corticosterone-induced upregulation of FKBP5 in the hippocampus. There was a significant Glucocorticoid treatment effect [$F_{(1,20)} = 16.56$, $p < 0.001$], a significant Compound treatment effect [$F_{(1,20)} = 16.23$, $p < 0.001$] and a significant Glucocorticoid X Compound interaction [$F_{(1,20)} = 7.301$, $p < 0.05$].

2B C118335 downregulated SGK-1 expression in the hippocampus regardless of glucocorticoid treatment. A compound effect was observed [$F_{(1,20)} = 25.71$, $p < 0.001$] and a marginally non-significant interaction effect [$F_{(1,20)} = 4.127$, $p < 0.06$]. Bonferroni post-hoc test, ★★★, $p < 0.001$, n = 5-6 per group.

2C No Compound effect was found on FKBP5 expression in the striatum [$F_{(1,21)} = 0.003$, $p > 0.95$]. However, a trend towards a Glucocorticoid treatment effect was found [$F_{(1,21)} = 3.274$, $p = 0.084$].

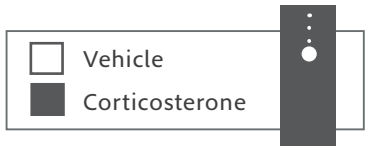
2D C118335 treatment prevented the corticosterone-induced upregulation of SGK-1. There was a significant Glucocorticoid treatment effect [$F_{(1,20)} = 8.197$, $p < 0.01$] and a significant Compound effect [$F_{(1,20)} = 8.295$, $p < 0.01$].

However, SGK-1 expression was upregulated in the striatum after treatment with corticosterone, an effect that was blocked by pretreatment with C118335 **(Figure 2D)**.

Trunk blood corticosterone levels: To test intrinsic effect of C118335 (100 mg/kg) on HPA axis activity, we compared plasma corticosterone levels in animals that were not treated with corticosterone. C118335 treatment induced significantly lower corticosterone plasma levels than in vehicle controls **(Figure 3A)**. As expected corticosterone-treated animals had higher corticosterone plasma levels than the respective vehicle-treated groups. Of relevance for the interpretation of the gene expression data, vehicle-treated animals also had relatively high levels of plasma corticosterone **(Figure 3B)**.

C118335 blocks corticosterone-induced memory enhancement of inhibitory avoidance training, and impairs memory consolidation: Given that we found predominant antagonism of C118335 on two commonly used glucocorticoid target genes in the brain, we investigated whether C118335 is able to block the memory-enhancing effect of posttraining corticosterone on consolidation of inhibitory avoidance training. C118335 (80mg/kg) or vehicle was given 1h before the training trial, followed by corticosterone (1mg/kg) or vehicle immediately after inhibitory avoidance training. Two-way ANOVA for entrance latencies on the training trial indicated no significant C118335 effect [$F_{(1,34)} = 0.32$; $p = 0.58$], posttraining corticosterone effect [$F_{(1,34)} = 1.49$, $p = 0.23$] or interaction between both factors [$F_{(1,34)} = 1.71$; $p = 0.20$]. As shown in **Figure 4A**, two-way ANOVA for 48h retention latencies indicated significant C118335 [$F_{(1,34)} = 14.77$, $p = 0.0005$], corticosterone effects [$F_{(1,34)} = 10.41$; $p = 0.003$] as well as a significant interaction between both parameters [$F_{(1,34)} = 10.75$; $p = 0.002$]. *Post-hoc* comparisons indicated that corticosterone induced significant retention enhancement ($p < 0.01$). Pretreatment with C118355 1h before training completely blocked the corticosterone effect ($p < 0.01$) and retention latencies of rats treated with

Figure 3



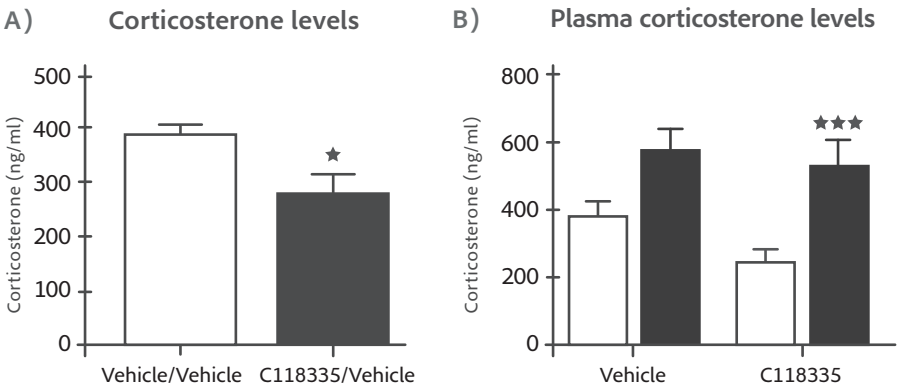


FIGURE 3:

3A. Endogenous corticosterone levels after treatment with vehicle or C118335. Rats treated with C118335 had lower plasma corticosterone levels compared to vehicle [two-tailed t-test: $t_{(10)} = 2.346$, $p = 0.04$].

3B. Two-way ANOVA reveled a Glucocorticoid treatment effect, where treatment with corticosterone increased circulating corticosterone plasma levels [$F_{(1,21)} = 14.71$, $p = 0.001$]. N = 5-6 per group. Bonferroni post-hoc test: ★★★, $p < 0.001$. No Compound effect was found in this analysis.

Figure 4

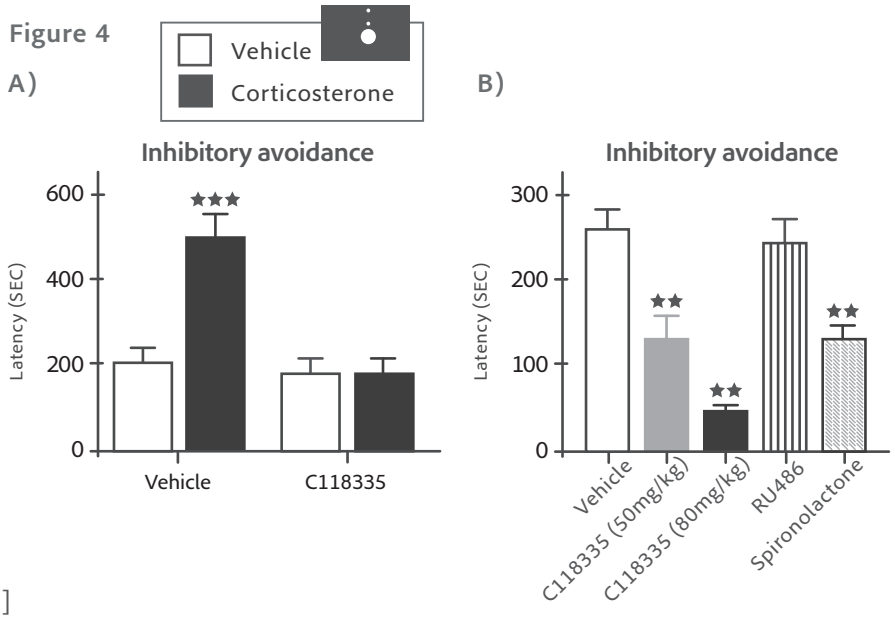


FIGURE 4:

4A. **C118335 (80 mg/kg) blocked corticosterone-induced memory enhancement of memory consolidation of an inhibitory avoidance training. Pretraining C118335 (80 mg/kg) did not affect retention latencies. Post hoc comparison, ★★★p < 0.001, in relation to corresponding control group, n = 8-10 per group.**

4B. **Posttraining C118335 (50 mg/kg) as well as (80mg/kg) showed an antagonist effect in an inhibitory avoidance test. Posttraining RU486 injections did not affect retention latencies whereas posttraining spironolactone decreased retention latencies of the inhibitory avoidance training. Post hoc comparisons, ★★p < 0.01, in relation to corresponding control group, n = 10-13 per group.**

Table 1: Primer sequences used for qPCR analysis and the expected product sizes

	Forward Primer (5' 3')	Reverse Primer (5' 3')	Product length (bp)
Actb	TGACCCTAAGGCCAACCGTG	ACACAGCCTGGATGGCTACC	90
BDNF	GGTCACAGCGGCAGATAAAAAGAC	TTCGGCATTGCGAGTTCCAG	188
FKBPs	CAGAGCAGGATGCCAAGGAA	TCCCATGGTCTGACTCTCG	95
SGK1	AGAGGCTGGGTGCCAAGGAT	CACTGGGCCCGCTCACATTT	129
Tubb2a	GAGGAGGGCGAGGATGAGGCTT	GACAGAGGCAAACTGAGCACCAT	121

corticosterone together with C118335 did not differ from those of vehicle-treated control rats (p = 0.97).

To investigate whether C118335 might have intrinsic effects on memory consolidation, C118335 (50 or 80mg/kg) or vehicle were administered subcutaneously immediately after inhibitory avoidance training. To investigate whether the C118335 effect resembled GR or MR antagonism, other groups of rats received posttraining subcutaneous injection of either the GR antagonist RU486 (40mg/kg) or the MR antagonist spironolactone (40mg/kg). One-way ANOVA for entrance latencies on

the training trial, before drug treatment, indicated no group effect [$F_{(4,50)} = 1.55$; $p = 0.20$]. Forty-eight-hour retention latencies of rats treated with vehicle after the training trial were significantly longer than their entrance latencies on the training (paired *t*-test: $t_{13} = -8.07$; $p < 0.0001$), indicating that they exhibited robust retention of the training experience. As shown in **Figure 4B**, one-way ANOVA for 48h retention latencies revealed a significant group effect [$F_{(4,50)} = 8.35$; $p < 0.0001$]. *Post-hoc* comparison tests indicated that both doses of C118335 (50 and 80mg/kg) induced significant retention impairment (both, $p < 0.01$). Posttraining administration of the GR antagonist had no effect ($p = 0.32$) whereas the MR antagonist impaired retention latencies ($p < 0.01$). Thus, these findings indicate that the C118335 effect on memory consolidation resembled that of MR, and not GR, antagonism.

DISCUSSION

The molecular interaction profile that the mixed GR/MR ligand C118335 induces at the receptor predicts selective GR modulation and antagonism for MR. Functional studies in the brain suggest a predominant action as antagonist at both receptor types. The effects of high levels of corticosterone on validated GR-dependent transcriptional targets and behavior were blocked. C118335 pretreatment blocked the memory-enhancing effect of corticosterone on inhibitory avoidance memory, an effect that likely involves antagonism of the GR. Surprisingly, posttraining systemic administration of the compound had strong intrinsic memory-impairing effects that apparently were mediated via MRs. However, no antagonism was observed at the level of disinhibition of the HPA axis, suggesting indeed tissue-specific agonism that can be expected from a selective modulator type of drug.

Manipulation of MR and GR signaling is of importance for basic research and of

therapeutic interest. The most widely used GR antagonist RU486 (mifepristone) can abolish psychosis in Cushing's disease (van der Lely *et al*., 1991; Nieman *et al*., 1985), and has been considered as a drug against stress-related psychopathology, in particular psychotic major depression (DeBattista *et al*., 2006). However, it also has affinity for the androgen receptor (AR) and progesterone receptor (PR), which has led to the development of more selective compounds. However, full GR antagonism may be less efficacious than selective receptor modulation, which combines agonism and antagonism (Zalachoras *et al*., 2013). Part of the reason why full GR antagonism may not be an optimal strategy to block the consequences of hypercortisolism is disinhibition of the HPA axis, which may counteract GR blockade in the brain, may affect MR function or MR/GR balance in a ill controlled manner.

The effects of C118335 that we have observed here, suggest that in brain antagonism at the GR is dominant, as it antagonized the effects of high doses of administered corticosterone, at mRNA targets that are expected to be GR targets at these levels of hormone (Webster *et al*., 2014; Scharf *et al*., 2011), in particular given the relatively high basal corticosterone levels observed in our study (Reul and de Kloet, 1985). Also blockade of the effect of corticosterone on memory consolidation in the inhibitory avoidance test is consistent with GR antagonism (Roozendaal and McGaugh, 1996). The MARCoNI array predicts that also GR agonism can occur at transcriptional targets that depend on the coactivator motifs that are recruited by C118335-occupied GR. The suppressed corticosterone levels in C118335-treated rats suggest that suppression of the HPA axis is one process where the compound exerts agonism (Ratka *et al*., 1989). The MARCoNI data also show that on GR the two selective modulators C118335 and C108297 (Zalachoras *et al*., 201) have a substantial overlap in the downstream coregulators that they recruit, but that there are around 5-15 co-regulator interactions that are unique to each modulator (depending on the criterion of statistical significance, or effect

size). Differential effects of the two selective modulators on any process may be related to a subset of those uniquely recruited coregulators.

C118335 has a high affinity for GR with a Ki of 8 nM in binding assays, and a functional Ki of 24 nM in luciferase assays. MR affinity is about 8 fold lower, as determined in luciferase assays on synthetic promoters (Hunt *et al*., 2012). Sgk-1 is an MR target in kidney (Chen *et al*., 1999), but in brain has been mostly associated with GR (Anacker *et al*., 2013). The suppression of mRNA levels of hippocampal Sgk-1 mRNA below basal levels may reflect antagonism of MR. Alternatively, Sgk-1 mRNA regulation via GR has different sensitivity in both brain regions. The effects of posttraining C118335 treatment on memory consolidation of inhibitory avoidance training were identical to those of the MR antagonist spironolactone, and much stronger than those of the GR antagonist RU486. We conclude that at the present dose, C118335 acts as a predominant GR antagonist in brain, and agonist at the HPA axis and as an antagonist on brain MRs.

The memory-perturbing effect of MR antagonism in inhibitory avoidance is in itself a surprising finding. MRs have been associated with memory formation, mainly so in transgenic models (Lai *et al*., 2007; Ferguson and Sapolsky, 2007). In behavioral tasks, MRs have been classically associated with response selection whereas GRs are important for memory consolidation (de Kloet et al., 1999; Oitzl *et al*., 2001). The blockade of corticosterone-induced increase in latency by pretreatment with C118335 in the task is in accordance with this notion (Zalachoras *et al*., 2013a). However, while pretreatment in itself did not affect performance in the task, immediate posttraining administration of C118335 impaired memory consolidation, similar to spironolactone and in contrast to RU486. Earlier studies in mice found no effect of posttraining treatment with spironolactone looking at context and cue dependent conditioning (Zhou *et al*., 24). It is conceivable that in the passive avoidance type of memory formation non-genomic MR activation plays

a role in the early stages of memory consolidation, and C118335 may antagonize this activity. We have no mechanistic prediction for the binding of C118335 to membrane associated MRs, which has a slightly different pharmacological profile from the genomic MR (Karst *et al.*, 2005). However, in the MARCoNI assay, the intrinsic activity of C118335 is identical to that of eplerenone, and the most direct assumption is that C118335 acts as an antagonist of the membrane-associated MR. Nevertheless, the effect may also be indirect, as the downregulation of Sgk-1 mRNA that we observed (which can be rapidly translated into protein changes) (Chen *et al.*, 1999), may have had functional consequences for GR functioning (Anacker *et al.*, 2013).

In conclusion, we offer here a proof-of-principle for the efficacy of a novel selective GR modulator/ MR antagonist, with predominant antagonistic effects on brain cortisol signaling. While its full spectrum of agonistic and antagonistic effects at GR, and its potency as an MR antagonist remain to be determined, C118335 or similar compounds may have therapeutic potential in the treatment of hypercortisolemia-induced psychopathology.

Acknowledgements

We would like to thank Melvin Albers for assistance with the measurements.

Conflict of interest

HH and is employed by Corcept Therapeutics, and made C118335 available. Corcept financed part of the costs of the experiments. RH is employed by Pamgene Int, who made MARCoNI arrays available for this study.

Summary And General Discussion

- **Emotional memory consolidation**
- **Importance of the memory task**
- **Memory consolidation of accurate memories**
- **Maladaptive situation and solutions**

CHAPTER 08

Emotional Memory Consolidation

Not all experiences are equally well remembered, significant and emotional life events are typically remembered better and with greater detail. Extensive evidence indicates that stress hormones released as a result of an emotionally arousing experience modulate the consolidation of memory processing (Roozendaal *et al.*, 2009). Stress hormones such as glucocorticoids enhance the memory consolidation process of emotionally arousing experiences (de Kloet *et al.*, 1999; Sandi and Pinelo-Nava, 2007; de Kloet, 2000; Roozendaal *et al.*, 2000; McGaugh and Roozendaal, 2002; Roozendaal, 2002; de Quervain *et al.*, 2009; Roozendaal *et al.*, 2006; Schwabe *et al.*, 2012).

Stressful situations require an efficient behaviour which involves to coordinate the activity of large scale brain networks (Mesulam *et al.*, 2009). Upon a stressful situation, the coordination of brain activity might be orchestrated by stress-induced neuromodulators that shift the network balance toward the salience network (Seley *et al.*, 1936; Hermans *et al.*, 2014). The salience network investigated in functional magnetic-resonance imaging (fMRI) studies includes cortical (fronto-insula, dorsal anterior cingulate, inferotemporal and temporoparietal regions) and subcortical (amygdala, thalamus, hypothalamus and midbrain) regions and it highly depends on the noradrenergic system (Hermans *et al.*, 2011). Human neuroimaging studies indicate enhanced insular cortex (IC) activity during encoding of emotionally arousing information as well as affection (Buchel *et al.*, 1998; Marschner *et al.*, 2008; King *et al.*, 2009; Hermans *et al.*, 2011). In order to study memory enhancement in laboratory conditions with rodents, we used an emotionally arousing training task and pharmacological manipulation of the noradrenergic system or glucocorticoid receptor immediately after the learning event. We used the inhibitory avoidance task and a modified version, the inhibitory avoidance discrimination task to study the consolidation process of

emotional memories. Inhibitory avoidance training induces a very long-lasting memory, which enables to study both memory consolidation as well as systems consolidation processes.

Although human neuroimaging studies include the IC as being part of the salience network (van Marle *et al.*, 2010), animal studies have mainly focused on stress-induced effects on interconnected brain regions such as the basolateral complex of the amygdala (BLA), prefrontal cortex and hippocampus (Revest *et al.*, 2005; Sandi and Pinelo-Nava, 2007; Roozendaal *et al.*, 1997b). Animal studies largely ignored the IC as a stress-sensitive brain region. Learning and memory studies in rodents investigating the role of the IC focused on the involvement of this brain region in taste memory consolidation (Miranda *et al.*, 2008a; Stehberg *et al.*, 2011) and of touch and odor (Augustine, 1996). The study presented in **Chapter 2** of this thesis showed that the IC is implicated in regulating glucocorticoid effects on the consolidation of an emotional learning experience. I investigated whether the IC had a specific contribution mediated by glucocorticoids to inhibitory avoidance memory in relation to the footshock and context components of the training. The two-phase inhibitory avoidance procedure, originally developed by Liang (2001), consists of training rats on the inhibitory avoidance task for two consecutive days.

On the first day, the rat can explore the inhibitory avoidance apparatus and on the second day the rat is placed directly into the dark compartment and receives a brief footshock. Posttraining glucocorticoid receptor agonist infusions into the IC enhanced the consolidation of both components (i.e., memory of the context and the foostchock) of a modified inhibitory avoidance procedure. A single-trial inhibitory avoidance training experience increased the number of phosphoERK1/2-positive neurons in the anterior IC 30min after the training, whereas, strikingly, systemic posttraining administration of a memory-enhancing dose corticosterone reduced the number of phosphoERK1/2-positive neurons.

It has been shown that increased phosphoERK1/2 immunoreactivity in the IC reflects an increased neuronal activity. We interpreted this finding as a better signal-to-noise ratio based on conclusions from neuroimaging studies (Pruessner *et al.*, 2008; van Marle *et al.*, 2010). Several studies in rodents and human subjects highlight the involvement of the IC in many complex cognitive functions, like emotion, perceptual functions, sensory-motor integration and body awareness. Since the IC is implicated in many cognitive and affective processes, it might function as a hub of integration between different stimuli and brain regions.

The IC seems to show a complex relation between performance and activity. Experiments such as dose-response curves upon emotional arousal conditions focused on understanding the molecular activity within the IC will bring a deeper understanding on the functional meaning of this region. Furthermore, inactivation studies to understand the functional connectivity of the IC within other key brain regions of the salience network might bring a better understanding on the role of this brain region in modulating memory of arousing experiences.

Importance of the Memory Task

New neuroscience technologies increase the need to investigate memory processes in a more detailed manner. Nevertheless, often behavioural tasks fail to show such specificity. In general, memory tasks are broad and demand many cognitive aspects. As an example, drug microinfusions after inhibitory avoidance training into a number of brain areas modulate memory consolidation in a time and dose dependent manner (Roozendaal and McGaugh, 2011). Although these findings show that these brain regions are all involved in regulating memory of inhibitory avoidance training, this task does not allow making further conclusions concerning a possible differential involvement of these brain regions

in subserving memory of specific types of information acquired during the training. In **Chapter 3**, we developed a new version of the classical inhibitory avoidance task, the inhibitory avoidance discrimination task. In this task rats are trained in two contextually modified inhibitory avoidance apparatuses with 1min delay in between, which induces poor episodic-like memory. Thus, this task in combination with drug manipulations that modulate memory, allows studying the mechanisms underlying episodic-like aspects of memory.

The major feature of this task is that it discerns between memory for contextual details of the boxes and the memory for the episodic-like aspect. The task consists of the presentation of two similar, yet distinctly different, inhibitory avoidance boxes where footshock is only delivered in one of the boxes. Retention is tested later in both apparatuses as well as in a novel apparatus that rats had not seen before. My findings show that when the interval beween the two training episodes was short (1min), retention latencies in both apparatuses were similar; rats showed similar strength (retention latencies) for both previously visited boxes. Thus, rats could not distinguish the box associated to footshock from the other box presented during the training. Nevertheless, rats distinguished the Novel box. Thus, rats showed specific memory for the details of the boxes but lacked episodic-like memory (association between footshock and the specific context). In contrast, when the interval between the two training episodes was longer (2min), rats showed episodic-like memory of the training event on a 48h retention test.

Rats exposed to the inhibitory avoidance discrimination task, with the short 1min interval between episodes, showed lack of episodic-like memory on a 48h retention test which allows the investigation of drugs that might enhance episodic-like memory. Accordingly, noradrenergic activation with a systemic administration of the noradrenergic stimulant yohimbine immediately after

training on the inhibitory avoidance discrimination task facilitated the strength as well as the episodic-like aspect of memory of the training experience. Since the learning occurs within a single trial, the inhibitory avoidance discrimination task is suitable to study the strength and accuracy of episodic-like and contextual memory in combination with complementary approaches. This task can be used with molecular or physiological approaches in order to understand the contribution of different brain regions to different aspects of memory processing. Furthermore, these findings are relevant to investigations in humans with respect to which aspects of memory are affected by the emotional impact of an experience such as misinformation, accuracy or generalization (Morgan *et al.*, 2004; Porter *et al.*, 2008; Smeets *et al.*, 2009; Hoscheidt *et al.*, 2014).

Memory Consolidation of Accurate Memories

Noradrenergic activation of the BLA and accuracy of memory

A great deal is known about memory modulation of single training events (Roozendaal and McGaugh, 2011). Yet, little is known about memory modulation mechanisms of two consecutive experiences that allows studying accuracy of memory. By utilizing the inhibitory avoidance discrimination task, in **Chapter 4** I investigated the role of noradrenergic activation of the BLA in facilitating accuracy of an episodic-like memory.

Administration of norepinephrine into the BLA (but not adjacent central amygdala) immediately (but not 3h) after training on the inhibitory avoidance discrimination task enhanced specifically memory for the apparatus in which footshock was delivered. Norepinephrine treatment specifically decreased plasticity related microRNA-134 (Schratt *et al.*, 2006) levels in the dorsal blade of the dentate gyrus (but not in ventral blade of the dentate gyrus, CA3, or CA1) 30min after training.

In addition, pharmacological posttraining downregulation of microRNA-134 levels with hippocampal infusions of a locked nucleic acid (LNA)-based antisense oligonucleotide facilitated the episodic-like memory or discrimination without influencing the strength of memory. Thus, microRNA-134 seems to be specifically involved in consolidation of accurate memories.

The inhibitory avoidance discrimination training procedure by itself induces generalized memory (i.e., no discrimination). However, noradrenergic activation of the BLA or antagomir against microRNA-134 infusions into the hippocampus facilitated discrimination between the boxes presented during the training. Since downregulation of microRNA-134 enhances plasticity (Christensen and Schratt, 2009), one possible interpretation would be that enhanced plasticity is required in the dorsal blade of the dentate gyrus in order to accurately consolidate episodic-like memories. Opposite to this view, we found that a memory-enhancing dose of norepinephrine infused posttraining into the hippocampus did not enhance accuracy of memory but rather increased general memory strength. Next, we provided evidence for the view that the effect on discrimination induced by noradrenergic activation of the BLA is mediated by downregulation of microRNA-134 in the hippocampus. Overexpression of microRNA-134 by infusions of mimic microRNA-134 into the hippocampus blocked the effect of norepinephrine administration into the BLA on discrimination, but it left the memory-enhancing effect of norepinephrine intact. MicroRNA-134 has been shown to have two specific targets which are plasticity-related Creb mRNA and Limk1 mRNA (Christensen and Schratt, 2009).

These results suggest that modulation of microRNA-134 levels only occur in relation to memory aspects supporting accuracy of episodic-like memory. Similar findings were described in relation to brain-derived neurotrophic factor (BDNF) in relation to the ability to discriminate. BDNF protein synthesis in the dentate gyrus

is required in order to show discrimination in a highly ambiguous behavioral task whereas BDNF levels were not altered after training on a less demanding task (Bekinschtein *et al.*, 2013).

Extensive evidence indicates that noradrenergic modulation of the BLA enhances memory performance. As expected, posttraining noradrenergic activation of the BLA enhanced memory of the classical inhibitory avoidance task (where only one box associated with footshock was presented during training). Surprisingly, posttraining downregulation of microRNA-134 in the hippocampus did not affect retention performance on the classical inhibitory avoidance task. In accordance with this finding, norepinephrine infusions into the BLA after classical inhibitory avoidance training did not affect microRNA-134 levels within the dorsal blade of the dentate gyrus. One study reported that microRNA-134 blockade did not influence object recognition memory or object location memory (Jimenez-Mateos *et al.*, 2015). MicroRNA-134 expression is controlled by the histone deacetylase sirtuin 1 (SIRT1) (Gao *et al.*, 2010). SIRT1 mediates transcriptional repression via an interaction with a complex containing the sequence-specific transcription factor Yin Yang 1. Mice lacking SIRT1 activity have been shown to display impaired synaptic plasticity as well as impaired memory in a fear conditioning task (Gao *et al.*, 2010). In contrast, downregulation of microRNA-134 upon hippocampal injection of a locked nucleic acid (LNA)-based antisense oligonucleotide restored CREB and BDNF levels and rescued plasticity and memory impairments in SIRT1 knockout mice. In addition, lentivirus-mediated overexpression of microRNA-134 in the hippocampus led to aberrant long-term potentiation and impaired fear conditioning memory (Gao *et al.*, 2010).

Thus, it seems that the downregulation of microRNA-134 in the dorsal blade of the dentate gyrus only occurs in specific conditions that underlie accuracy of memory consolidation. This finding has not been previously reported and it offers the

opportunity to study the molecular and network substrates of discrimination and generalization processes.

How does microRNA-134 enhance accuracy of memory consolidation?

In **Chapter 4**, we described opposite effects for posttraining downregulation of miR-134 and posttraining norepinephrine infusions into the hippocampus. Downregulation of microRNA-134 facilitated discrimination whereas norepinephrine infusions induced generalization. Catecholamines (dopamine, norepinephrine and epinephrine) are known to enhance signal-to-noise ratio (Heinz and Smolka, 2006). Noradrenergic activation in the hippocampus seems to be required for plasticity to occur since increased spiking activity induced by amphetamine was blocked by antagonism of adrenergic receptors (Segal *et al.*, 1991). In addition, it has been shown that norepinephrine-driven phosphorylation of GluR1 facilitates synaptic delivery of GluR1-containing AMPA receptors that results in a lower threshold for long-term potentiation. Noradrenergic activation in the hippocampus might increase excitability of a large population of excitatory neurons. Nevertheless, discrimination might require a more specific activation pattern, since norepinephrine infusions into the hippocampus after inhibitory avoidance discrimination training did not facilitate discrimination.

The ability to discriminate between similar experiences is a crucial feature of episodic memory and has been ascribed to the dentate gyrus (Yassa and Stark). Experimental evidence points at the dentate gyrus as a critical mediator for discrimination (Kesner *et al.*, 2000; Leutgeb *et al.*, 2007). Our findings show that posttraining noradrenergic activation of the BLA specifically downregulated microRNA-134 levels in the dorsal blade of the dentate gyrus whereas CA1, CA3 and the lower blade of the dentate gyrus were unaffected. Therefore, it seems likely that downregulation of the microRNA-134 into the hippocampus

had a specific effect. Antagomir infusion directed against microRNA-134 binds specifically to microRNA-134 (Jimenez-Mateos *et al.*, 2015). Therefore, it will only exert its effects in the presence of microRNA-134. Due to the difficulty of working with small and easily degradable microRNAs, we lack data of microRNA-134 distribution in the hippocampus. Based on findings on cortex indicating that microRNA-134 is mainly present in somatostatin-positive inhibitory neurons (Chai *et al.*, 2013), we could speculate the same might be true for the hippocampus. Assuming that microRNA-134 is mainly present in inhibitory neurons within the dentate gyrus, downregulation of microRNA-134 would increase plasticity of inhibitory neurons rendering a higher regulation on the network. If microRNA-134 would only be expressed in inhibitory neurons, blockade of microRNA-134 in the hippocampus would selectively affect inhibitory neurons and as a consequence it would increase the level of inhibitory input to the network. Interestingly, such an increased inhibitory input within the dentate gyrus would be consistent with early computational theories that explain hippocampal function in discrimination and pattern separation (Marr, 1971; Schmidt *et al.*, 2012).

Noradrenergic activation of the BLA and systems consolidation of remote memories

Everyday experiences tend to be easily forgotten but emotionally arousing events tend to be remembered in a detailed manner for long periods of time. As a general consensus, newly acquired episodic memories depend on the hippocampus to be retrieved (Squire and Bayley, 2007). However, there is no consensus on whether the initial episodic memory remains hippocampus dependent or not. Experimental studies and reports from human patients as well as findings of experiments in animals show conflicting results on the involvement of the hippocampus in remote memories. This controversy has led to different systems consolidation theories. Moreover, it has not been studied whether systems consolidation processes for strong emotionally arousing experiences might be different from emotionally neutral experiences. In **Chapter 5**, we studied the

effect of noradrenergic activation of the BLA on strength and accuracy of remote memories. Rats were trained on the inhibitory avoidance discrimination task, this time with a 2min delay, and immediately after the training, rats received norepinephrine or saline infusions into the BLA. Retention was tested either 2 days (recent) or 28 days (remote) later. Rats were tested in both previously visited contexts and in and additional novel box.

The standard consolidation model (SCM) claims that the hippocampus plays a time-limited role in consolidating new memories into long-term memories (Frankland *et al.*, 2004; Squire and Wixted, 2011). In contrast, the multiple trace theory (MTT) proposes that episodic memories continue to depend on the hippocampus for long periods of time (Nadel and Moscovitch, 1997). We investigated the effect of noradrenergic activation of the BLA on the hippocampal involvement at the time of memory retrieval. Hippocampal inactivation for saline-treated rats into the BLA resulted in generalization 2 days after training and had no effect 28 days after training. As predicted by SCM, saline-treated rats showed a time-limited engagement of the hippocampus leading over time to generalization, (Kim and Fanselow, 1992; Anagnostaras *et al.*, 1999; Frankland *et al.*, 2004; Wiltgen and Silva, 2007; Wiltgen and Tanaka, 2013). Surprisingly, hippocampus inactivation for norepinephrine-treated rats at such remote time point resulted in severe memory impairment.

Initial consolidation of episodic memories depends on the hippocampus (Moscovitch *et al.*, 2005). In accordance, both groups showed hippocampus-dependent recent episodic-like memory. Nevertheless, a month after training saline-treated rats showed hippocampus independent memory and lack of episodic memory. Saline-treated rats followed a standard consolidation model (SCM) with time-limited role of the hippocampus. However, a month later, norepinephrine-treated rats showed episodic-like memory that was still

hippocampus dependent. Norepinephrine-treated rats followed multiple trace type of consolidation since episodic memories continued to depend on the hippocampus. Posttraining infusions of norepinephrine into the BLA altered the systems consolidation process rendering entirely hippocampus dependent accurate long-term memory. Our data suggests that emotional memories remain accurate over time by shifting the systems consolidation process from standard consolidation to multiple trace model. It seems that norepinephrine engages the hippocampus more predominantly and as a consequence remains dominant in comparison to other brain regions. On the other hand other studies support the idea that initially memory might depend on hippocampal as well as non-hippocampal memory systems (Nadel and Moscovitch, 1997) which might compete during the learning (Maren and Fanselow, 1997).

Noradrenergic activation of the BLA and maintenance of memory

Noradrenergic activation of the BLA enhances hippocampal activity (Ikegaya *et al.*, 1997; Vouimba *et al.*, 2006). Accordingly, we found that posttraining administration of norepinephrine into the BLA enhanced plasticity within the dentate gyrus 30min after training. Norepinephrine-treated rats showed increased c-AMP response element (Creb) mRNA levels as well as DNA methyl transfererase 3A (Dnmt3A) mRNA levels. The transcription factor CREB is critical for memory consolidation (Silva, 2008) and Dnmts might be responsible for memory maintenance (Day and Sweatt, 2010). Therefore, alterations in Dnmt3A enzyme levels can alter the transcription levels of many genes. This subtle epigenetic change could be key in altering systems consolidation process. An experiment to block specifically Dnmt3A immediately after training would show the role of this enzyme in altering molecular dynamics and later network dynamics. Unfortunately, available drugs to inactivate Dnmts are general and affect most of these enzymes rather than specific ones.

Although emotional enhancement of episodic memory is generally an adaptive phenomenon, under certain conditions it can also contribute to neuropsychiatric disorders and cognitive decline (Yassa and Stark, 2011; Leal *et al.*, 2014). Episodic memory deficits are clear symptons of Alzherimer's disease (DeCarli *et al.*, 2004). Reports of patients show specific patterns of changes in CA3/DG regions of the hippocampus (Yassa *et al.*, 2010). Therefore, understanding the process of generalization or lack of discrimination would provide important clues to our understanding of neuromodulatory mechanisms that fail to function normally, leading to the development of such psychiatric conditions.

Maladaptive Situation and Solutions

Stress-related neurocognitive problems have a major medical and economic impact on society. Glucocorticoid-related drugs are used commonly as treatment for stress related disorders. Unfortunately, glucocorticoids are ubiquitous and show pleiotropic effects (Chen and Qiu, 1999) which leads to many undesirable side effects (Sapolsky *et al.*, 2000; Judd *et al.*, 2014). Stress-induced high levels of glucocorticoids are known to be a risk factor for development of psychopathologies. There is an increasing need for more specific tools and treatments that can target specifically glucocorticoid receptors without undesirable side effects. In **Chapters 6** and **7** we present two novel selective glucocorticoid receptor modulators that have clinical potential.

Exposure to stress induces adrenal glucocorticoid hormonal release which is essential for the adaptation to stressors. As shown in **Chapter 2** glucocorticoids enhance memory of an emotionally arousing training experience in a time and dose dependent manner. In addition, glucocorticoids regulate adult neurogenesis (Fitzsimons *et al.*, 2012) and neuroendocrine negative feedback regulation (Watts *et al.*, 2005). Nevertheless, increased levels of glucocorticoids and excessive

exposure to them is associated with psychopathologies (de Kloet *et al.*, 2005). Antagomism of glucocorticoid receptor (GR) is used as treatment for psychosis and depression (Nieman *et al.*, 1985; DeBattista *et al.*, 2006) but current drugs such as RU 486 that is a GR antagonist but also binds to progesterone receptors. On the other hand full GR antagonism would induce disinhibition of the HPA axis that results in increased glucocorticoid levels. More selective drugs are needed to specifically palliate undesired glucocorticoid effects but at the same time preserving their positive functions. Selective glucocorticoid receptor modulators (SGRM) affect specifically nuclear receptors such as GR. The compounds described in **Chapters 6** and **7** are SGRM that show specific interaction profiles and show potential as therapeutic drugs.

As shown in **Chapter 6**, the C108297 compound is a novel SGRM with a specific GR-coregulator interaction profile. This compound showed a partial agonistic activity and showed a unique interaction profile between GR and its downstream effector molecules (nuclear receptor coregulators). For example, specifically SRC-1a nuclear receptor was preferentially recruited while other GR-coregulator interactions were blocked. C108297 showed a partial agonistic and antagonistic activity in the brain. On the one hand C108297 supressed hypothalamic CRH gene expression and antagonized GR mediated reduction of neurogenesis in the hippocampus but it did not lead to disinhibition of the HPA axis. On the other hand posttraining systemic injections of C108297 enhanced memory consolidation of an emotionally arousing task in a GR dependent manner.

In **Chapter 7**, we described the effect of C118355, a novel SGRM with a specific interaction profile that induces selective GR modulation but antagonism for mineralocorticoid receptor (MR). C118355 did not disinhibit the HPA axis but C118355 antagonized corticosterone-induced gene expression in the brain as well as on memory effects. Posttraining systemic administration of C118355

impaired memory consolidation of an emotionally arousing task in a dose dependent manner. In addition, it blocked corticosterone induced enhancement on memory consolidation in the same task. Surprisingly, pretreatment of C118355 did not affect memory consolidation, posttraining systemic injections showed MR dependent memory consolidation impairment similar to spironolactone. Since the interaction profile of C118355 was similar to eplerenone we could conclude that this novel compound might also act as an antagonist of the membrane-associated MR. Although further experiments are needed some of these effects could be indirectly GR dependent.

The experiments described in this thesis support the involvement of the insular cortex as a part of the salience network and it also supports the role of the BLA on modulating memory consolidation. Noradrenergic activation of the BLA alters the consolidation process and induces long lasting accurate memory by maintaining memory entirely hippocampus dependent over time.

Appendix

- References
- Nederlandse samenvatting
- Donders Graduate School for Cognitive Neuroscience Series
- Acknowledgements
- Publication List
- Curriculum Vitae

CHAPTER 09

>References

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Nederlandse Samenvatting

Het onderzoek in dit proefschrift is tweeledig, in deel I worden enkele studies beschreven die als doel hebben de effecten van stress en consolidatie van emotioneel geheugen beter te begrijpen. In deel II worden de effecten van nieuwe medicijnen bestudeerd, die aangrijpen op de glucocorticoide receptor (GR), één van de receptoren in de hersenen waar corticosteron op aangrijpt. Deze effecten worden beschreven op genetisch, moleculair en cognitief niveau.

Deel I:

Consolidatie van emotioneel geheugen

Niet al onze herinneringen zijn even precies of even sterk en er zijn verschillende factoren die dit kunnen beïnvloeden. Een belangrijke factor is stress, en dit proefschrift behandelt de effecten van stress op de consolidatie van informatie (oftewel het opslaan van herinneringen). De hormonen die vrijkomen tijdens een stressvolle ervaring kunnen deze opslag beïnvloeden. Daardoor worden sommige ervaringen beter (en soms slechter) onthouden. Vanuit een medisch en therapeutisch perspectief is er veel interesse om geheugenmodulatie door stress beter te begrijpen. Bijvoorbeeld in de context van posttraumatische stressstoornis (PTSS), waar traumatische herinneringen de kwaliteit van leven in patiënten sterk beïnvloeden. Om de mechanismen van geheugenvorming onder stress beter te begrijpen, zijn in deze studies proefdieren (ratten) gebruikt.

Experimenten uit **hoofdstuk 2** van dit proefschrift tonen aan dat de insulare cortex (IC) betrokken is bij stressmodulatie van geheugenconsolidatie. Dit is getest door dieren een emotionele leerervaring te laten ondergaan in een taak waarin dieren moeten leren vermijdingsgedrag te onderdrukken ('inhibitory avoidance'; IA).

Dit wordt gedaan door dieren in een box te zetten met een licht en een donker (afgeschermd) deel. De dieren worden aan het begin in het lichte deel gezet en omdat ratten van nature de voorkeur geven aan donkere ruimtes, zullen ze naar het donkere deel lopen. In dit deel krijgt de rat een milde, elektrische voet-schok. Als de rat later opnieuw in de box wordt geplaatst, zal het zijn natuurlijke gedrag (lopen naar het donkere compartiment) onderdrukken, om een voetschok te voorkomen. Het aantal seconden dat een rat in het lichte compartiment blijft, wordt als maat voor geheugen gebruikt; hoe langer, hoe beter het dier zich de aversieve ervaring herinnert. Toediening van het stresshormoon corticosteron na de leerervaring versterkt de herinnering van de box, en in **hoofdstuk 2** werd gevonden dat daarbij de activiteit in de IC afneemt. In een variant op de IA taak werden de dieren blootgesteld aan het apparaat op dag 1, waarna ze op dag 2 in hetzelfde apparaat een schokje kregen. Hierdoor werd de herinnering van de context en de schok los van elkaar gevormd. De IC lijkt betrokken te zijn bij geheugenvorming van beide aspecten, wat gemoduleerd werd door de aanwezigheid van stresshormonen. De IC cortex zou daarom als 'hub' kunnen functioneren, waarin cognitieve en emotionele ervaringen worden geïntegreerd.

Ontwikkeling van een geheugentaak

Er is veel bekend over hoe geheugenvorming beïnvloedt kan worden door externe factoren, zoals stress, tijdens een enkele, simpele leerervaring. We weten echter minder over de effecten van blootstelling aan opeenvolgende ervaringen, en hoe meerdere herinneringen elkaar beïnvloeden. Het begrijpen hiervan kan ons iets leren over de kwaliteit van geheugen. In hoofdstuk 3 worden experimenten beschreven waarin een nieuwe geheugentaak is ontwikkeld om geheugenkwaliteit of nauwkeurigheid te meten. Deze taak is een variant op de IA taak, waarin dieren moeten leren om vermijdingsgedrag te onderdrukken. In deze variant (de IA-discriminatie taak, of IAD) worden de dieren, kort na elkaar

blootgesteld aan twee verschillende boxen met een licht/donker compartiment (box A en box B). Maar, de schok wordt alleen in één van de boxen toegediend (box A). Tijdens de geheugenretentie test worden ratten opnieuw blootgesteld aan box A, B en een onbekende box (C). Als de herinneringenvan de twee boxen (A en B) nauwkeurig zijn opgeslagen, zal het dier bij een volgende blootstelling aan de boxen alleen onderdrukkingsgedrag vertonen in box A. In deze studie werd gevonden dat dieren,tijdens IAD leren, geen onderscheid kunnen maken tijdens de retentie test wanneer er sprake is van een kort tijdsinterval (1 minuut) tussen box A en B tijdens geheugenvorming. Beide boxen werden door de dieren geassocieerd met een schok, de nieuwe box (C), werd echter wel als veilig beschouwd. Dit toont aan dat zowel de schok als de boxen A en B onthouden zijn, maar dat er geen duidelijk box-schok associatie is gevormd. Activatie van het noradrenerge systeem, wat normaal gesproken onderdeel is van de respons op een stressvolle gebeurtenis, liet een verbetering en versterking van geheugen zien, waarbij de specifieke box-schok associatie wel werd gevormd. De IAD taak ontwikkeld in **hoofdstuk 3** is geschikt om nauwkeurigheid van geheugen te meten en te moduleren. De bevinding dat het stress systeem de nauwkeurigheid van geheugen kan beïnvloeden heeft daarnaast belangrijke implicaties voor humaan onderzoek naar leren en geheugen van emotionele gebeurtenissen.

Onderzoek naar de kwaliteit van emotioneel geheugen

De kwaliteit en nauwkeurigheid van geheugen kan onderzocht worden met behulp van de IAD taak ontwikkeld in **hoofdstuk 3**. In **hoofdstuk 4** is deze taak gebruikt om de rol van de basolaterale amygdala (BLA) te bestuderen in nauwkeurigheid van geheugen. De amygdala is betrokken bij angst en emotioneel geheugen en speelt een belangrijke rol in noradrenerge modulatie hiervan. In **hoofdstuk 4** is gevonden dat activatie van het noradrenerge systeem, door directe toediening van norepinephrine in de BLA direct na training, zowel de nauwkeurigheid als

de sterkte van de herinnering deed toenemen. Daarnaast zorgde toediening van norepinephrine in de BLA voor moleculaire veranderingen in de dentate gyrus (DG) van de hippocampus, een gebied betrokken bij geheugenvorming. Om precies te zijn, het resulteerde in een verlaging van microRNA-134, een molecuul dat betrokken is bij plasticiteit. In een vervolgexperiment werd met behulp van farmacologische manipulaties aangetoond dat directe onderdrukking van microRNA-134 in de DG hetzelfde effect op geheugen had; door infusie van een antisense oligonucleotide werden herinneringen nauwkeuriger. Tenslotte is aangetoond het mechanisme waarmeenoradrenerge activatie van de BLA geheugen nauwkeuriger maakt, inderdaad gemedieerd wordt door microRNA-134. Dit is gedaan door tegelijkertijd met infusie van norepinephrine in de BLA, microRNA-134 in de DG toe te dienen. Hierdoor werd de natuurlijke verlaging van microRNA-134 teniet gedaan, en resulteerde daardoor niet in een verbetering van geheugen nauwkeurigheid.

Deze resultaten laten zien dat de BLA de nauwkeurigheid van emotionele herinneringen verbetert, door een directe verlaging van microRNA-134 in een ander hersengebied, de dentate gyrus van de hippocampus.

Langetermijngeheugen

Dagelijkse ervaringen worden vaak makkelijk vergeten, zeker naarmate er meer tijd verstrijkt. Dit is anders voor emotionele gebeurtenissen; vaak worden deze juist -tot in detail- onthouden gedurende meerdere dagen, maanden en zelfs jaren. Van de hippocampus wordt gedacht dat deze betrokken is bij het ophalen van recentelijk opgeslagen herinneringen, maar er is geen consensus over hoe lang, in welke mate, en onder welke condities herinneringen afhankelijk blijven van de hippocampus.Er zijn dan ook meerdere theorieën geformuleerd over

de betrokkenheid van de hippocampus bij het ophalen van herinneringen. In **hoofdstuk 5** wordt onderzocht hoe lang herinneringen afhankelijk blijven van de hippocampus, in hoeverre de zulke herinneringen nauwkeurig blijven, en hoe het noradrenerge systeem hier invloed op uitoefent. Hiervoor wordt opnieuw de IAD geheugentaak gebruikt, waarbij na 2 en 28 dagen wordt geëvalueerd of ratten onderscheid kunnen maken tussen boxen A en B. Ratten werden getraind op een makkelijkere versie van de IAD taak, waarbij het interval tussen box A en B langer was (2 minuten), tijdens de consolidatie werden dieren behandelt met norepinephrine in de BLA of een zoutoplossing (controleconditie). Na 2 dagen konden zowel saline- en norepinephrine behandelde dieren goed onderscheid maken tussen beide boxen, wat erop duidt dat er een accurate associatie is gevormd tussen box A en de schok. Na 28 dagen echter, konden dieren waarbij saline in de BLA was toegediend, geen onderscheid meer maken tussen box A en B, terwijl norepinephrine behandelde dieren nog steeds een nauwkeurige herinnering hadden aan de associatie tussen box A en de schok. Om te bestuderen of nauwkeurigheid (en de verandering hiervan over tijd) afhankelijk is van hippocampus activatie, werd neurotransmissie in de hippocampus geblokkeerd door toediening van de GABA-agonist muscimol tijdens de retentie test. In de dieren die behandeld waren met saline in de BLA had hippocampus inactivatie geen effect op retentie na 28 dagen. Geheugenprestaties 2 dagen na training waren wel veranderd: zonder hippocampus inactivatie hebben ratten een accurate herinnering van de schok in context A, maar als hippocampus activiteit geblokkeerd werd, was deze herinnering niet aanwezig. Dieren generaliseren tussen context A en B. Echter, in ratten die behandeld waren met norepinephrine in de BLA (wat resulteerde in accurate herinneringen, zelfs na 28 dagen) zorgde hippocampus inactivatie ervoor dat dieren geen onderscheid konden maken tussen de boxen, op beide tijdstippen. Dit duidt erop dat de herinnering aan een emotionele gebeurtenis door norepinephrine langer afhankelijk blijft van de hippocampus.

De bevindingen in dit hoofdstuk lijken erop te duiden dat de betrokkenheid van verschillende hersengebieden in emotionele herinneringen kan veranderen, door activatie van het noradrenerge systeem. Daarnaast werden, op moleculair niveau, veranderingen gevonden in de DG van norepinephrine-behandelde ratten.

Deel II

Nieuwe medicatie voor veranderd geheugen door stress

De stressreactie wordt voornamelijk gereguleerd door de hypothalamus-hypofyse-bijnier (HPB), activatie van dit systeem resulteert in de productie van glucocorticoïde hormonen. Deze hormonen reguleren de (gedrags)respons van een organisme tijdens en na een stressvolle gebeurtenis, wat resulteert in een optimale adaptatie aan de omgeving. Deze hormonen kunnen aan mineralocorticoid receptoren (MR) en glucocorticoid receptoren (GR) binden, die in veel hersengebieden worden aangetroffen. . Blootstelling aan hoge concentraties van glucocorticoïde hormonenworden echter geassocieerd met psychopathologie., Antagonisten van de glucocorticoide receptor, zoals RU-486, zijn niet altijd specifiek. RU-486 bindt bijvoorbeeld ook aan de progesterone receptor en kan daardoor bijwerkingen vertonen. Daarnaast kan GR-antagonisme leiden tot een 'disinhibitie' van de HHB-as, het systeem dat hormoonproductie reguleert. Disinhibite van de HHB-as resulteert in verhoogde glucocorticoide-hormoon niveaus. Het is daarom nodig om meer selectieve medicijnen te ontwikkelen. Selectieve glucocorticoïdreceptor modulators (SGRM) beïnvloeden meer specifiek nucleaire receptoren zoals GR. Deze specifieke modulatie kan er voor zorgen dat er minder schadelijke bijwerkingen dan klassieke GR antagonisten optreden. In **hoofdstuk 6 en 7** worden SGRMs beschreven die een specifiek GR-coregulator interactieprofiel tonen, en zouden potentieel als therapeutische interventie kunnen fungeren in de behandeling van veranderd geheugen door stress.

In **hoofdstuk 6** wordt een nieuwe SGRM getest (C108297) met een specifiek GR-coregulatie interactie profiel. Deze verbinding liet een gedeeltelijke agonistische en antagonistische activiteit en toonde een unieke interactie profiel tussen GR en zijn downstream effector moleculen (nucleaire receptor coregulatoren). Aan de ene kant C108297 onderdrukt hypothalamus CRH genexpressie en tegengewerkt GR gemedieerde vermindering van neurogenese in de hippocampus, maar het leidde niet tot ontremming van de HBB-as. Aan de andere kant posttraining systemische injecties van C108297 verbeterde het geheugen consolidatie van een emotioneel wekken taak in een GR-afhankelijke wijze. C108297 combineert antagonistische en agonistische GR eigenschappen die kunnen leiden tot een betere therapeutische medicatie.

In **hoofdstuk 7**,wordt een andere nieuwe GR ligand beschreven, C118355, een SGRM met een specifiek interactie profiel. C118355 induceert selectieve GR modulatie, maar heeft een antagonistische werking op de mineralocorticoid receptor (MR). C118355 leidt niet tot HBB-as disinhibitie maar blokkeerde genexpressie ten gevolge van verhoogde corticosteron niveaus. C118355 injecties na leren resulteerde in verminderde consolidatie van emotionele gebeurtenissen. Daarnaast blokkeerde C118355 de positieve effecten van corticosterone op geheugen in dezelfde taak. C118335 toonde een antagonistisch profiel met weinig disinhibitie van de HBB-as, en zou daarom een geschikt geneesmiddel kunnen zijn voor de negatieve gevolgen van stress.

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Publication list

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The Journey

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MEXICO
VIENNA
GRONINGEN
NIJMEGEN
BOCHUM

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