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ROLE OF THE AMYGDALA IN CHROMATIN REMODELING EFFECTS UNDERLYING LONG TERM MEMORY

HASSIBA BELDJOUD


217 AMYGDALA AND CHROMATIN REMODELING EFFECTS IN LONG TERM MEMORY

Hassiba Beldjoud
I have the age of my scientific knowledge; with all the weakness and strength that one can have at this stage of his carrier. Eagerness, passion, and determination are my Drive.

So, to be or not to be. That is the question, and I decided to be a Neuroscientist!
Role of the amygdala in chromatin remodeling effects underlying long-term memory

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Donders Graduate School for Cognitive Neuroscience Series

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Publication list

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chapter
GENERAL INTRODUCTION
GENERAL INTRODUCTION

Chromatin remodeling (DNA packaging) is part of a complex mechanism named epigenetics. The term epigenetics, first coined by Conrad Waddington in the 1940s, refers to changes that occur above (‘epi-’) the genome. Waddington defined epigenetics as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being” (Waddington, 1942), in a word the phenotypes are produced by the interplay between genes and their environment.

Advances in molecular biology research have clarified this first definition by Allis and collaborators (2007b) defined epigenetics as “the sum of the alterations to the chromatin template that collectively establish and propagate different patterns of gene expression (transcription) and silencing from the same genome.”

How does chromatin remodeling influence gene expression?

In the eukaryotic cell, the nucleus contains the chromatin that conserves genetic information. In its simplest form, chromatin consists of the nucleosome core particle, composed of 147 base pairs of DNA wrapped around the nucleosome. The nucleosome is an octamer of core histone proteins H2A, H2B, H3, and H4. The histone protein H1 links the DNA between single nucleosomes (Kornberg, 1974). The chromatin can undergo dynamic physical changes, as for example during cell cycle progression, where it can be either loose, named euchromatin, or compact, named heterochromatin. This ability for chromatin to remodel its architecture confers to it a critical role in controlling gene expression. In fact, heterochromatin is characterized by a highly compacted state, which prevents the transcriptional machinery from reaching the DNA, and thereby is restrictive to transcription. On the other hand, euchromatin is characterized by an open state permitting the transcriptional machinery to reach the DNA and thereby transcription to occur (Tamaru, 2010).

Each core of histones displays long C-terminal and N-terminal tails consisting of an amino-terminal protruding from the nucleosome. Crystal structure of the nucleosome core particle reveals that the N-terminal tails of the four core histones are targets for posttranslational modifications (PTMs) (Luger et al., 1997). These include acetylation, methylation and ubiquitination of lysine (K) residues, phosphorylation of serine (S) and threonine (T) residues, and methylation of arginine (R) residues (Davie, 1998; Fischle et al., 2003) and many others such as, sumoylation, ADP-ribosylation, deimination, and proline isomerization (Allfrey et al., 1964; Kouzarides, 2007). Specific antibodies and mass spectrometry have shown over 60 different residue modifications on histones (Kouzarides, 2007) reflecting the multiple possibilities of modification of a histone tail, which we will return to in more detail while dealing with the histone code hypothesis in the next section.

Histone molecules have a highly positive charge due to the high number of lysine residues, which favors their binding to DNA. Changes in histone residues alter their charge and binding properties to the DNA (Mühlbacher et al., 2006) which in turn alters chromatin structure that will allow, or not, access to the DNA. These PTMs also influence the recruitment of effecter proteins that contain biological information. These modifications, along with the methylation of cytosine residues in DNA (not dealt with here) will “define” the switch off/on of a particular gene. Figure 1 shows a schema of nucleosome histone tails based on Crosio and collaborators (2003).
Mechanisms of Histone Modification and Function

Two mechanisms are implicated in chromatin remodeling: the first one is that histone acetylation can weaken the affinity between the histone and the DNA, which relieves the chromatin accessibility to the transcription machinery (Choi and Howe, 2009). The second mechanism is the recruitment of specific binding proteins by histone marks (Turner, 1993; Strahl and Allis, 2000) that read them and convert them into specific functional chromatin states and regulate downstream responses (Izzo and Schneider, 2010). An example, acetylation serves as a molecular tag (Peixoto and Abel, 2013) which is recognized by proteins with bromodomains that include transcriptional coactivators such as CBP, p300, and PCAF (Zeng and Zhou, 2002; Mujtaba et al., 2007). Phosphorylation represents a tag for 14-3-3 proteins and methylation at lysines is recognized by chromo-like domains (Winter et al., 2008; Izzo and Schneider, 2010).

Enzymes related to histone modifications

PTMs of histone residues are both multiple and reversible. All four histones undergo acetylation, phosphorylation and ubiquitination of lysine residues, phosphorylation of serine and threonine residues, and methylation of arginine residues (Davie, 1998; Fischle et al., 2003) and many others and all these modifications can be reversed, making them highly dynamic.

Histone acetylation

In the previous section we saw how the positive charge of histones due to high lysine content favors histone-DNA contacts which results in compact chromatin and gene repression. Here we will see how acetylation relaxes chromatin structure. Histones are targets for acetylation. The family of enzymes responsible for this acetylation of histone molecules are known as histone acetyltransferases (HATs), which catalyze the direct transfer of an acetyl group from acetyl-CoA to the e-amino nitrogen of lysine side chains of the N-terminal tails of histones (Marmorstein, 2001). Thus, it is perfectly accepted that histone acetylation results in an opening of the chromatin fiber which, in turn, favors gene activation (Brownell and Allis, 1996). Figure 2 shows the histone acetylation-deacetylation dynamic.

The best-known HATs are cAMP response-element binding protein (CREB) binding protein (CBP), p300, p300/CREB binding protein-associated factor (PCAF) and HIV Tat interactive 60-kDa protein (Tip60). Steroid receptor coactivators 1 and 3 (SRC-1 and -3, respectively) are HATs recruited by steroid receptors (Spencer and Davie, 1999; Davie and Moniwa, 2000; Sterner and Berger, 2000; Davie, 2003).

Inhibition of histone deacetylase activity with butyrate

In our study, we inhibited histone deacetylation by using sodium butyrate (NaB) as a histone deactylase inhibitor. NaB inhibits most HDACs except class III HDAC and class II HDAC 6 and 10. It should be noted that when using HDAC inhibitors, the activity of HAT continues, which leads to an accumulation of acetylation and therefore to a hyperacetylation state (Davie, 2003). The existence and extent of this hyperacetylation therefore must depend on the amount of on-going HAT activity. It has also been shown that both HAT and HDAC also target other substrates (Davie, 2003).

Histone phosphorylation

In the case of histone phosphorylation modifying enzymes, while a great deal is known in relation to the cell cycle and during mitosis, much less is known about any specific role played by these enzymes in the context of the brain. Therefore, it is difficult to dissociate and know whether kinases implicated in mitotic phosphorylation are the same as that which phosphorylates histones in the brain. Histone phosphorylation can occur on serine, threonine and tyrosine residues. In general, histone phosphorylation is catalyzed by distinct and multiple kinases where each enzyme is specific for an individual residue. Any one histone residue can, however, be phosphorylated by several kinases (Oki et al., 2007).

Histone H3 phosphorylation at serine (10) (pH3S10) is an extensively studied histone phosphorylation and has been demonstrated to play a role in gene activation. Various kinases phosphorylate pH3S10. Phosphorylation of pH3S10 by mitogen- and stress-activated protein kinases 1 and 2 (MSK1 and MSK2) as well as RSK2 kinase have been shown to play a role in the activation of...
mitogen-stimulated immediate-early response genes, such as c-fos and c-jun (Mahadevan et al., 1991; Thomson et al., 1999; Sola and Corces, 2003; Nowak and Corces, 2004). More details can be found in Sawicka and Seiser (2012).

Histone methylation

Methylation of histone tails occurs mainly at lysines (K) and arginines (R) which are catalyzed by histone methyltransferases (HMTs) that also methylate other proteins (Di Lorenzo and Bedford, 2011). Histone methylation depends on a precise methylation site and the degree of modification: Lysine residues can be mono-, di- or tri-methylated, whereas arginines can be mono- or di-methylated. In addition, arginines can be symmetrically or asymmetrically di-methylated (Kouzarides, 2007).

Until recently histone methylation was considered to be a stable and irreversible epigenetic mark. The discovery of the first histone demethyltransferase (HDMs) (Shi et al., 2004) showed that, like acetylation, histone methylation is dynamic and reversible (Mossammamparast and Shi, 2010).

Histone methylation can result in either transcription or repression. For example, methylation of H3K9, and H3K27 are related to gene repression whereas H3K4 trimethylation is related to gene expression (Izzo and Schneider, 2010).

Dynamics of histone PTMs

One important feature that needs to be addressed is that histone modifications are highly dynamic. In all four groups of histone, the histone can be rapidly acetylated and then rapidly deacetylated with a half-life of only 3-7 minutes (Davie, 2003). A second population has a lower acetylation – deacetylation rate with a half-life of 30 min (Davie, 2003). Not only is histone acetylation highly dynamic but so are other HPTMs. In an elegant review Barth and Imhof (2010) described the dynamics of histone modifications and the kinetic differences between methylation, phosphorylation and acetylation. Phosphorylation of serine, threonine and tyrosine are also highly dynamic with a half-life comparable to histone acetylation. Interestingly histone methylation turn-over is much slower (Barth and Imhof, 2010).

Regarding histone acetylation, studies have shown that fast turnover of acetylation is tied to transcriptional activation (Waterborg, 2002). This idea was extended by others who suggested that it is the rapid turnover rather then simply acetylation that directs transcription activation (Clayton et al., 2006). This interesting feature requires further investigation. Figure 2, shows Histone post-translational modifications dynamics, illustrated by the histone acetylation- deacetylation turnover.

Histone cross-talk and the histone code

An important aspect of histone modifications is that they can occur in concert on the same histone tail (Cheung et al., 2000; Clayton et al., 2000) or on the tails of different histones (Turner et al., 1992; Zeitlin et al., 2001; Sun and Allis, 2002). One histone modification can promote or block another modification, or one modification can stimulate or block the removal of another modification. It is these combinatorial modifications that will define the transcription or the repression of distinct sets of genes. The existence of this histone cross-talk led to the histone code hypothesis enunciated by Strahl and Allis in 2000. For reviews see Strahl and Allis, 2000; Kouzarides, 2007; Izzo and Schneider, 2010; Bannister and Kouzarides, 2011. As an example, methylation of lysine 9 interferes with phosphorylation of serine 10, but is also influenced by pre-existing modifications in the amino terminus of H3 (Rea et al., 2000).

Another example is that acetylation of histone H3K14 can be stimulated by the phosphorylation of histone H3 at serine 10 (pH3S10). Interestingly, neuronal stimulation that induced histone H3K14 acetylation was shown to be also associated with pH3S10 (Chwang et al., 2006, 2007; Ciccarelli and Giustetto, 2014). Moreover, Zippo and colleagues found that H3 phosphorylation at the promoter of FOSL1 gene occurs in parallel with H3 acetylation (Zippo et al., 2007). Other studies have shown that pH3S10 can be induced simultaneously (Li et al., 2004; Chwang et al., 2007; Ciccarelli and Giustetto, 2014) but also independently of H3K14 acetylation (Crosio et al., 2003; Brami-Cherrier et al., 2005; Bertran-Gonzalez et al., 2008) depending on the ongoing stimulation (Cheung et al., 2000; Lo et al., 2000). Whether histone acetylation of H3K14 occurs before pH3S10 still a debate.
THE ROLE OF CHROMATIN-MODIFYING ENZYMES IN EARNING 
AND MEMORY

Memory consolidation is the process by which newly learned information stabilizes into long-term memory (LTM). Long-term memory is thought to rely on de novo RNA and protein synthesis (Flexner et al., 1963; Agranoff and Klinger, 1964; Davis and Squire, 1984; Dudai and Morris, 2000; McCaugh, 2000; Kandel, 2001) which has also been demonstrated in models of synaptic plasticity such as long-term potentiation (LTP) (Nguyen et al., 1994; Stanton and Sarvey, 1984). Furthermore, in order for this RNA and protein synthesis to occur following learning, it requires regulation of gene expression involving access to the DNA, which is normally highly compacted in chromatin (Alberini, 2009; Barrett and Wood, 2008).

In this section I will describe the latest data regarding the growing evidence that chromatin remodelling affects learning and memory.

An early study by Schmitt and Matthis, used histone acetylation by radioactive acetate incorporation ([14C]-acetate) during the training session. Trained animals compared to their passive controls showed a decrease in histone acetylation at both 5 min as well as 120 min post-training (Schmitt and Matthis, 1979).

One of the first studies that described the implication of chromatin remodelling in memory formation was reported by Swank and Sweatt in which they studied the activity of an enzyme that permits acetylation of lysine residues on histones (histone acetyltransferase or HAT) in the insular cortex during novel taste learning. The novel taste learning induced activation of lysine acetyl transferase activity together with ERK/MAPK cascade. They concluded that lysine–histone acetyl transferase activation may play a role in regulating gene expression in single-trial learning and long-term memory formation (Swank and Sweatt, 2001).

Approximately at the same time, in aplasia, histone acetylation in the promoter region of the immediate early gene C/EBP was shown to follow serotonin facilitation of synaptic plasticity activation (Cuan et al., 2002).

These two studies indicated that histone acetylation known to relax chromatin and related to gene expression (Brownell and Allis, 1996) has something to do in gene expression related to synaptic plasticity and memory expression.

A few years after the first study by David Sweat's group, the work of Crosio et al. (2003) on hippocampal cell stimulation showed a transient phosphorylation of histone H3 with a rapid activation of the mitogen-activated protein kinase pathway together with an induction of c-fos transcrption. This study was probably the first to show a direct link between neuronal cell stimulation and chromatin modification that could take part in learning and memory.

The real groundbreaking study regarding chromatin remodelling and its relation to learning and memory came from the work of Levenson and colleagues (2004) who investigated hippocampal histone acetylation in a contextual fear conditioning paradigm. They showed that contextual fear conditioning regulates acetylation of histone H3 in the CA1 area of the hippocampus and that this was coupled with ERK signaling activation. Histone acetylation was also enhanced by the use of the histone deacetylase inhibitors (HDAC) trichostatin A or NaB, which enhanced and thus facilitated the induction of LTP at Schaffer-collateral synapses in the CA1 area of the hippocampus. In vivo injection of HDAC inhibitors to rats before contextual fear conditioning training enhanced the formation of long-term memory. This was the first study demonstrating a direct link between a learning task and the induction of chromatin changes (Levenson et al., 2004).

From that time, a myriad of studies on the relation of chromatin remodeling as well as other markers of epigenetics changes such as cytosine DNA methylation that was thought to be a more stable epigenetic mark (Law and Jacobsen, 2010) but also microRNAs followed this early study by David Sweat's group.

Chromatin remodeling was investigated using several memory tasks such as fear conditioning (Levenson et al., 2004; Chwang et al., 2006; Kishibui et al., 2011) inhibitory avoidance (Blank et al., 2014; Caglio et al., 2014) object recognition (Kishibui et al., 2009; Stefañko et al., 2009; Roozendaal et al., 2010; Zhao et al., 2012, Beldjoud and al., 2015) object location memory (Haertig et al., 2011), spatial water maze (Bousiges et al., 2010; Dagnas et al., 2013; Caglio et al., 2014) conditioned taste aversion (Kwon and Houpt, 2010; Morris et al., 2013). Thus, depending on the memory task used, a number of brain regions were investigated to identify histone modifications (and therefore chromatin remodeling) and their relation to memory formation: the hippocampus and its sub-regions (Levenson et al., 2004; Chwang et al., 2006; Fischer et al., 2007; Kishibui et al., 2009; Gupta et al., 2010; Graff et al., 2012; Dagnas et al., 2013), amygdala (Kwon and Houpt, 2010), lateral sub-region of the amygdala (Monsey et al., 2011; Ota et al., 2010), insular cortex (Roozendaal et al., 2010; Beldjoud et al., 2015), médial préfrontal cortex (Graff et al., 2012; Sui et al., 2012; Zovkic et al., 2014) and the striatum (Gaglio et al., 2014; Dagnas and Mons, 2013).

**HDAC inhibitors as a pharmacological tool for modulating the epigenome**

Histone deacetylase (HDAC) inhibitors prevent the removal of the acetyl group from histones, thereby increasing histone acetylation (because of the HAT activity that is still active). Histone acetylation is a useful epigenetic mark related to gene expression since histone acetylation is well accepted to be linked to gene expression (Guan et al., 2009). Thus, HDAC inhibitors represent a useful and valid tool in the field of neuroscience.

HDAC inhibitors such as SAHA, NaB, TSA, valporic acid, or situin administered either systemically or locally into the targeted brain region have been revealed to have powerful actions on memory enhancement. Early studies from Sweatt's group used HDAC inhibitors such TSA and NaB to enhance acetylation in hippocampal neurons in vitro. NaB injected prior to fear condition enhanced long-term memory (Levenson et al., 2004).

Intracerebroventricular injections of the HDAC inhibitors NaB and TSA significantly facilitated fear-conditioning learning in mice and rescued memory deficits in an animal model of neurodegenerative disease (Fischer et al., 2007).

The administration of the HDAC inhibitors NaB systemically or TSA intrahippocampally prior to an extinction session of contextual fear was shown to facilitate contextual fear extinction (Lattal et al., 2007).

In the same line of research, the administration of an HDAC inhibitor was found to improve memory in aged rats. In an object recognition memory task a systemic injection of NaB improved an aging-associated deficit of memory (Reolon et al., 2011). The administration of one of the most used HDAC inhibitors NaB was shown to improve memory formation (Levenson et al., 2004; Fischer et al., 2007; Lubin and Sweatt, 2007; Cuan et al., 2009).
Stefanko and colleagues (2009) showed that NaB administered immediately after 3 min object recognition training enhanced long-term memory of the object assessed 24 h after training and drug treatment. This memory was maintained for 1 week after the treatment (Stefanko et al., 2009). Local administration of NaB into the insular cortex or the hippocampus differentially enhanced memory retention when tested 24 h later. In fact, NaB administered into the insular cortex enhanced memory of the object per-se but not of the location of the object whereas administration of NaB into the hippocampus enhanced memory of the location of the object but not that of the object itself (Roozenendaal et al., 2010).

Post-training local infusion of the HDAC inhibitor SAHA into the ventral striatum of mice trained on a one-trial inhibitory avoidance task improved memory retention and increased acetylation of specific residues previously acetylated by the one-trial inhibitory avoidance training alone (Caglio et al., 2014).

Because classical HDAC inhibitors are known to have a broad action on the different classes of HDAC (pan-HDAC), it is difficult to target a specific class of HDAC and determine its specific role in cognition. Morris and collaborators, by using mice lacking the class I HDACs (HDAC1 or HDAC2), could investigate the specific role of these two HDACs in relation to learning and synaptic plasticity. They specifically showed that mice lacking HDAC2 displayed a better extinction of the conditioned fear memory and conditioned taste aversion (Morris et al., 2013).

Interestingly, as discussed above in the section related to histone cross-talk, HDAC inhibitors not only have an action on histone acetylation but also seem to influence other histone marks such as methylation. In fact, Gupta and collaborators showed that inhibition of HDACs with NaB resulted in an increased H3K4 trimethylation and decreased H3K9 dimethylation in the hippocampus following contextual fear conditioning (Gupta et al., 2010). These results indicate that HDAC inhibitors might influence other histone marks by an indirect mechanism possibly related to complex details of the histone code.

In the amygdala NaB was also observed to induce a phospho-acetylation of histone H3 (Kwon and Houpst, 2010).

Whereas histone investigations are mainly focusing on histone acetylation and phosphorylation, other groups tend to investigate other histone modifications such as histone methylation and their relation to memory formation. Gupta et al. (2010) described that 1 h following contextual fear conditioning, trimethylation of histone H3 at lysine 4 as well as dimethylation of histone H3 at lysine 9 were increased in the hippocampus, indicating that other histone modifications besides histone acetylation take part in memory formation.

In the same line of thinking, using new approaches, other groups have enlarged the investigation of histone modifications to other types and subtypes of histones. Saito’s group also investigated other types of histones but also introduced a novel mechanism implicating histone variant exchange, in which canonical histones are replaced with their variant counterparts in which the histone H2A.Za variant of histone H2A, is actively exchanged in response to fear conditioning in the hippocampus and the cortex, where it mediates gene expression for the maintenance of the memory (Zovkic et al., 2014).

**HAT modulator usage in learning and memory**

Far fewer studies have examined the effect of HAT inhibitors or activators on memory because few HAT-specific inhibitors have been isolated or synthesized. Further, the use of drugs that are known to inhibit HAT activity such as p300/CBP and PCAF is challenging because of their cell impermeability that prevents their proper use in vivo (Dal Piaz et al., 2010). So far, the HATs that are the most studied are p300/CBP and PCAF (p300/CBP-associated factor) and their relation to hippocampal memory (Zhao et al., 2012).

An early study used a mouse model of Rubinstein-Taybi syndrome (RTS) lacking the CREB-binding protein (CBP), which is known to have intrinsic histone acetyl-transferase (HAT) activity. This study demonstrated a deficiency in LTP as well as learning and memory, whereas short-term memory was maintained. Administration of HDAC inhibitors rescued the memory deficiency seen in those mice indicating that histone acetylation is essential for long-term memory (Alarcón et al., 2004; Korzus et al., 2004).

Mice having specific CBP knockout in the CA1 subregion of the hippocampus showed a reduction in long-term memory for two hippocampus–dependent tasks, contextual fear conditioning and object location memory. This reduction was correlated with a decrease in the acetylation at lysine residues in the hippocampus, whereas upstream signaling p300 or CREB phosphorylation was unchanged (Barrett et al., 2011).

Mice with mutations of p300 (Oliveira et al., 2007) or PCAF (Duclot et al., 2010; Maurice et al., 2008) also showed an impaired hippocampal memory and LTP.

HAT investigations were extended to other brain regions involved in memory.

Wei and collaborators (2012) demonstrated that in the infralimbic prefrontal cortex of C57BL/6 mice, the activity of p300/CBP-associated factor (PCAF) is required for LTP as well as for fear memory extinction but not for the acquisition of fear memory and that a PCAF activator administered systemically enhanced memory for fear extinction and prevents fear reconsolidation, indicating the importance of HAT activity in memory formation (Wei et al., 2012).

New and more specific HAT inhibitors have been tested for their efficiency in vitro (Zhao et al., 2012) showed that Garcinol blocked the memory-enhancing effects of oestradiol on object recognition memory. Furthermore, Garcinol also reversed the oestradiol-induced increase in dorsal hippocampus histone H3 acetylation, HAT activity, as well as the oestradiol-induced decrease of histone deacetylase 2, known for its memory repressor effect.

Local infusion of the selective p300/CBP inhibitor c646 into the lateral amygdala (LA) was shown to impair memory consolidation and reconsolidation of fear conditioning indicating that p300/CBP HAT activity is critical for amygdala–dependent Pavlovian fear memories (Maddox et al., 2013).

Using RNA-sequencing to screen the levels of all known histone acetyltransferases (HATs) in the hippocampal CA1 region, Stilling and collaborators identified K-acetyltransferase 2a (Kat2a) as being involved in learning and memory. In fact, mice that lack (Kat2a) showed impaired synaptic plasticity as well as reduced hippocampal memory consolidation (Stilling et al., 2014).
THE BASOLATERAL AMYGDALA PLAYS A PIVOTAL ROLE IN MEMORY CONSOLIDATION

Amongst the most of powerful forms of long-lasting memory are those containing emotional imprinting, be it positive such as the first lover or a graduation ceremony, or negative such as remembering an accident or loss of a loved one. In fact, it is well documented that emotional memories are vivid and long lasting (for review Roozendaal and McGaugh, 2011) and that stress hormones released from the adrenal glands are critically involved in memory consolidation of emotionally arousing experiences. Arousal-induced memory enhancement is known to involve noradrenergic activation within the basolateral complex of the amygdala (BLA) (McGaugh, 2000; 2015; McGaugh et al., 2002) which in turn, facilitates the storage of information in its many target regions (Setlow et al., 2000; Roesler et al., 2002; Miranda and McGaugh, 2004; Roozendaal et al., 2004; LaLumiere et al., 2005; Malin et al., 2007).

Experimental approaches that manipulate BLA activity have shown that post-training BLA activation by norepinephrine or a β-adrenoceptor agonist enhanced memory consolidation for several types of training (Introini-Collison et al., 1991; Ferry et al., 1999; Hatfield and McGaugh, 1999; LaLumiere et al., 2003; Huff et al., 2005; Roozendaal et al., 2008). In contrast, inactivation of the BLA by a β-adrenoceptor antagonist impairs the consolidation of memory (Hatfield and McGaugh, 1999; Miranda et al., 2003; Roozendaal et al., 2008).

In object recognition training, which is a non-invasive memory task, it has been reported that corticosterone enhanced memory consolidation of the object when administered to rats that were “aroused ” by the novelty of the unfamiliar training apparatus. The corticosterone treatment, however, had no effect when administered to rats that had extensive prior habituation to the training apparatus context thereby reducing novelty-induced arousal (Okuda et al., 2004).

Further monitoring norepinephrine release in the amygdala using microdialysis and high-performance liquid chromatography (HPLC), showed that Footshock training increases amygdala norepinephrine release. This increase in norepinephrine was correlated with better retention (Galvez et al., 1996; Quirarte et al., 1998). Interestingly NE release was not increased in rats that were placed in the inhibitory avoidance apparatus and given no footshock, indicating that it is the emotionally arousing training that induces the endogenous release of norepinephrine into the amygdala (McIntyre et al., 2002).

Thus, the memory enhancement in emotional memories requires BLA noradrenergic activity, which in turn enables memory consolidation in its many target brain regions, specifically involved in the training experience (McGaugh, 2000; McGaugh et al., 2002; Roozendaal and McGaugh, 2011). Neurobiological systems regulating memory consolidation are summarized in Figure 3.

BLA activity influences various memory tasks

Numerous studies have reported the modulatory effect of BLA on several memory tasks. Using an inhibitory avoidance task Introini-Collison et al. (1991) showed that post-training intra-amygdala infusion of the β2-adrenoceptor agonist clenbuterol enhanced memory retention. Another study showed that infusions of norepinephrine or β-adrenoceptor agonists into the BLA enhance memory for inhibitory avoidance as well as water maze training and that this norepinephrine-induced retention enhancement for the location of a hidden platform in a spatial version of the water maze task was dose-dependent (Ferry et al., 1999).

In a taste aversion task where intraperitoneal injections of lithium chloride (LiCl) produce a strong taste aversion, blocking BLA activity with the -adrenergic antagonist propranolol before LiCl administration was reported to impair conditioned taste aversion memory (Miranda et al., 2003).

BLA activity was also reported to be important in contextual fear conditioning (LaLumiere et al., 2003) as well as contextual fear conditioning where post-training inactivation of the BLA with muscimol, a GABA-A agonist, was also shown to impair memory for contextual fear conditioning (as measured by freezing) whereas intra-BLA norepinephrine enhanced that memory (Huff et al., 2005).

A recent study also indicated that BLA activity is important for memory consolidation of a low-arousing training experience. Norepinephrine administered after 3 min of object recognition training (a duration that is normally not sufficient to produce long-term memory) produced dose-dependent enhancement of 24-h object recognition memory whereas propranolol administered after 10 min of training (a duration known to produce a strong memory when tested 24 h later) produced dose-dependent impairment of memory. Thus post-training noradrenergic activation of the BLA enhances object recognition memory in exposure conditions that would otherwise not induce long-term memory (Roozendaal et al., 2008).

Taken together, these data clearly indicate that BLA noradrenergic activity modulates the consolidation of memory in a large number of behavioural memory tasks (McGaugh, 2000; McGaugh et al., 2002; Roozendaal and McGaugh, 2011).
Interactions of the basolateral amygdala with other brain regions in mediating emotional arousal-induced memory consolidation

Experiences initiate memory consolidation in many brain regions involved at that particular time. As described earlier, stress hormones released during an emotionally arousing or stressful experience activate the release of norepinephrine in the BLA. This activation of the BLA, in turn, modulates memory consolidation by influencing neuroplasticity in its target brain regions (for review: “Memory: a century of consolidation” McGaugh, 2000).

Early studies indicated that BLA activation modulates memory in many brain regions. In an elegant study Packard and White (1991) indicated that the hippocampus and striatum are implicated in different forms of memory. Post-training infusion of amphetamine into the dorsal hippocampus enhances memory of spatial localization of a non-visible submerged platform in a water maze whereas amphetamine infused into the striatum selectively enhances memory of visual cues associated with the location of an escape platform placed in different places during the different training trials. When amphetamine was infused into the BLA after training, memory of both types of training was enhanced. Indicating that the amygdala modulates consolidation by altering processing in these two brain regions (Packard and White, 1991). The β-adrenoceptor antagonist propranolol infusion into the BLA significantly impaired hippocampal long-term potentiation in vivo, which is one form of synaptic plasticity that may underlie learning and memory (Ikegaya et al., 1997). Infusions of a memory-enhancing dose of the β-adrenoceptor agonist clenbuterol into the BLA immediately after training on an inhibitory avoidance task significantly increased Arc protein levels in the dorsal hippocampus. This experiment is one of the first to indicate neuroplasticity changes in a BLA target brain region (McIntyre et al., 2005).

The BLA also interacts with the medial prefrontal cortex as described in an experiment where systemic injections of the β-adrenoceptor antagonist propranolol given before testing prevented corticosterone-induced working memory impairment (Roozendaal et al., 2004).

A study by Miranda and McGaugh (2004) also indicated that the insular cortex was involved in memory consolidation of both inhibitory avoidance as well as conditioned taste aversion and this requires intact noradrenergic activity in the BLA (Miranda and McGaugh, 2004).

Taken together, all these data provide extensive evidence that the BLA interacts with other brain regions, in modulating memory consolidation of emotionally arousing experience.

How does BLA activity influence neuroplasticity in its target brain regions? Possible role of chromatin remodeling

Changes in gene expression are thought to be involved in neuronal plasticity associated with learning and memory. Coming back to emotional memories that are known to last in some cases for the entire life, and with the extensive evidence that BLA activity plays a pivotal role in such “strong” memories, the molecular mechanism underlying this BLA influence on memory in its target regions remains to be completely elucidated.

Few research groups have investigated and reported molecular changes in brain regions targeted by the BLA after the BLA has been activated during an emotional memory or stress at the time of memory consolidation. McIntyre and colleagues (2005) reported that stimulation of β-adrenoceptors within the BLA or glucocorticoid–induced elevation of norepinephrine activity in the amygdala after inhibitory avoidance training resulted in enhanced memory and an increased expression of the activity-regulated cytoskeletal-associated (Arc) protein in the dorsal hippocampus. As immediate early genes (IEGs) such as ARC are rapidly induced in the brain in response to synaptic activity, they could be an indicator of critical molecular processes involved in synaptic plasticity (McIntyre et al., 2005). The same group later reported similar data in hippocampal synapses for an aversive (inhibitory avoidance) as well as a non-aversive (object recognition) memory task (McReynolds et al., 2010; 2014).

A new approach in neuroscience came with the epigenetics concept (see previous section) that experience and environmental stimuli have the capacity to alter gene expression and thus the behavioural outcome. In fact, gene modulation could be an excellent way to explain how the brain expresses its plasticity and might explain, at least in part, how emotional memories related to BLA activity are long lasting. During the last decade several studies emphasize and suggest that chromatin structure must be altered to allow for robust and lasting changes in gene expression related to memory retention. Histone PTMs (described in the previous section) are a means by which chromatin structure may be remodelled between open or closed states, which controls the access of the transcriptional machinery to reach the DNA, thereby allowing gene transcription or repression to occur.

OBJECT RECOGNITION

Because object recognition is the main behavioural task used in all experiments constituting the present thesis, I present an overview of the behavioural task as well as brain regions that it involves.

The object recognition task, also known as novel object preference or novel object recognition is a one-trial learning test for neurobiological studies introduced by Ennaceur and Delacour, (1988). The task is based on the natural tendency of rodent to explore a novel object and this means that the task is relatively free of stress.

Recognition memory uses different types of information such as object familiarity, the location of the object or when an object was encountered.

In a typical object recognition task, animals are allowed to freely explore two identical objects (A1 and A2) presented in an arena for a certain time. This constitutes the learning task. After a certain delay depending on experiment requirements, the animal is returned to the arena and is presented with a copy of the previously presented object (A3) and a new, unknown object (B). If the rats show more time exploring the novel object (B) at the testing trial this reflects that they have remembered that they have “already seen” the object (A) and the difference in time exploration both objects is taken as an indicator of memory.

Brain regions involved in object recognition memory

Recognition memory, the ability to recognize that an item has been encountered previously, depends on the integrity of the medial temporal lobe which includes, the hippocampus, the dentate gyrus and the subicular complex, together with the entorhinal, perirhinal and parahippocampal cortices, which lie along the adjacent parahippocampal gyrus (Squire et al., 2007).
A number of studies have revealed the essential role of the perirhinal cortex in novel object recognition. Lesions of the perirhinal cortex lead to a severe disruption of object recognition (Mumby and Pinsel, 1994; Ennaceur et al., 1996; Bussey et al., 1999; Brown and Aggleton, 2001; Brown et al., 2012) but not object location memory (Ennaceur et al., 1996; Glenn and Mumby, 1998; Barker et al., 2007).

The role of the hippocampus in object recognition memory is still controversial as some studies revealed no effect of hippocampal or fornix lesions on object recognition (Bussey et al., 2000; Mumby et al., 2002; Forwood et al., 2005; Good et al., 2007) whereas others reported a significant impairment (Clark et al., 2000). Therefore the role of the hippocampus is thought to be related to processing of contextual information, rather than object representation per se (Gaffan, 1994; Cassaday and Rawlins, 1997; Aggleton and Brown, 2005; Balderas et al., 2008; Winter et al., 2011).

Studies of lesions of the medial prefrontal cortex have shown no effect on object recognition memory whereas they disrupt temporal order and object in place (Barker et al., 2007). Earlier studies, however, revealed that microinfusion of the protein synthesis inhibitor anisomycin or an N-MNDA receptor antagonist into the ventromedial prefrontal cortex immediately after training resulted in impairment of long-term recognition memory, indicating a role of this brain region in object recognition memory (Akirav and Maroun, 2006). Studies from our laboratory have shown that micro-infusion of glucocorticoids agonists enhanced both object characteristics and it location (Barsayan and collaborators, unpublished data).

The insular cortex (IC) was also shown to take part in object recognition memory. Immediate post-training infusions of the muscarnic cholinergic receptor antagonist scopolamine administered into the IC produced impairment of object recognition memory (Bermudez-Rattoni et al., 2005). The same group also showed that long-term memory of the object was impaired when the protein synthesis blocker anisomycin was applied into the IC or perirhinal cortex indicating that both brain regions are involved in object recognition memory formation. This study also demonstrated regional and memory-type specificity. When the anisomycin was injected into the hippocampus, the memory for the location of the object was affected, but not that of the object per se. However, when the site of anisomycin injections was the IC, the memory deficits concerned the type of the object and not its location (Balderas et al., 2008). As already mentioned above, another recent study has also described that a post-training infusion of NaB into the IC, but not the hippocampus, enhanced memory of the object per-se but not memory of the location of the object, whereas an infusion of NaB into the hippocampus, but not the IC, enhanced memory of the location of the object and had no effect on the memory of the characteristics of the object, reinforcing the implication of the IC in object recognition memory (Roozendaal et al., 2010).

OUTLINE

In this introduction we have described how the concept of emotional memories rely on BLA activation and how chromatin remodeling is involved in memory consolidation. There is extensive evidence that emotional enhancement of memory depends essentially on noradrenergic activation of the BLA and its modulatory influences on information storage processes in its many target regions. However, the molecular basis underlying these BLA effects on other brain regions remains to be elucidated.

Therefore in the present work we aimed to investigate whether the modulatory effect of BLA activity on memory consolidation in brain regions involved at the time of memory consolidation implies chromatin remodeling, which by definition alters gene expression and which, at least in part, might explain such strong memories. Because long-term memory is thought to rely on persistent changes in synaptic strength we also aimed to study synaptic plasticity molecules, known to sustain synaptic plasticity and long-term memory at the time when memory consolidation is established and see whether the memory maintenance observed 24-h after BLA noradrenergic activation relies on synaptic plasticity proteins.

In CHAPTER 2 of the present thesis we described the main molecular method, which we developed for histone isolation and identification. The method was refined from other methods, in order to specifically work with minute quantities of tissue from specific brain regions for reproducible and reliable results for satisfactory histone post-translational identification and quantification without losing anatomical precision.

In Chapters 3 to 6 we described the experimental approaches, results and the conclusions in investigating the role of BLA in chromatin remodeling and brain plasticity.

In all studies we used object recognition memory as a memory task and we looked at the insular cortex as a brain region known to be involved in object recognition memory. We combined behavioural, and pharmacological approaches together with a molecular study to examine whether noradrenergic activation of the basolateral amygdala regulates memory consolidation of object recognition training by enabling chromatin modification as well as protein related plasticity in the insular cortex. In brief, using a sub-threshold object recognition training time, (known to produce a poor memory retention 24-h later) we can “boost” the memory pharmacologically. Thus, the memory was either boosted with an i.p. injection of a memory-enhancing dose of histone deacetylase inhibitor sodium butyrate or by a local micro infusion into the BLA of memory-enhancing dose of norepinephrine. Inactivating BLA activity with a local injection of the β-adrenoceptor antagonist propranolol also prevented the memory. We first looked at the behavioural outcome of this modulatory drug effect on memory consolidation. Then, using the same experimental approaches, chromatin remodeling and plasticity related protein changes were investigated in the insular cortex, which has recently been shown to be implicated in the memory of characteristics of the object.

CHAPTER 3 named “Chromatin remodelling in the insular cortex associated with the consolidation of object recognition memory requires basolateral amygdala noradrenergic activity” constitutes the main chapter in this thesis in which we showed the role and implication of the BLA in promoting chromatin remodeling in its target brain regions involved at the time of memory formation. We combined behavioural, and pharmacological approaches together with a molecular study to examine whether noradrenergic activation of the BLA regulates memory consolidation of object recognition training by enabling chromatin modification in the insular cortex. In this study memory was boosted with a histone deacetylase inhibitor and BLA activity was either maintained intact or blocked. We then assessed the long-term memory retention after drug manipulation as well as a battery of histone marks to evaluate chromatin remodeling. The present study is the first to show a direct implication of the BLA in chromatin alteration in brain regions involved at the time of memory consolidation.
In CHAPTER 4 we investigated the effect of the HDAC inhibitor Sodium Butyrate in a different brain region. Because the medial prefrontal cortex is thought to be involved in infralimbic (IL), prelimbic (PL) and anterior cingulate cortex one may expect that these regions are differentially involved in recognition memory. We addressed the question as to whether the facilitatory effect of HDAC inhibitor on memory altered histone methylation marks in the IL and PL cortex and whether BLA activity is necessary for this alteration.

In CHAPTER 5 of this study the BLA output was boosted by a local infusion of a memory-enhancing dose of noradrenaline immediately after object recognition training, and behavior as well as histone modifications were investigated. This study is of particular interest as it shows that for the same behavioural outcome molecular changes can differ. We have explored the significance of this result in the discussion.

In CHAPTER 6 we investigated, downstream from histone effects on gene expression, whether the plasticity-related proteins such as GLUR2, PSD95 and PKMζ, that are known to sustain synaptic plasticity and long-term memory, play a role in the maintenance on the long-term memory for object recognition memory in synaptoneurosomes from the insular cortex. 24-h after BLA noradrenergic activation. 24 hours after training, GLU2 and PSD-95 showed increased expression, whereas PKMζ did not vary.

Finally in CHAPTER 7 we conclude the thesis with the general discussion and the possible perspectives and insight related to our investigation.

REFERENCES


CHAPTER 1

GENERAL INTRODUCTION


GENERAL INTRODUCTION

30

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EXTRACTION, IDENTIFICATION AND QUANTIFICATION OF HISTONES FROM SMALL QUANTITIES OF SPECIFIC BRAIN TISSUE
ABSTRACT

Histone post-translational modifications (PTMs), by their action on chromatin conformation, play a central role in the regulation of gene expression. The discovery that some PTMs in the brain are regulated highly dynamically by experience and environmental factors make them an important subject for the study of plasticity changes in learning and memory, addiction and neurological disorders. Current histone isolation protocols, however, require large amounts of tissue, which limit their application for analyzing small tissue samples from a specific brain region. We describe here a step-by-step protocol for histone extraction and isolation from 1 mm³ of tissue from brain punches, which allows reproducible and reliable results for histone PTM identification and quantification without losing anatomical precision.

INTRODUCTION

It is becoming increasingly evident that histone post-translational modifications (PTMs) act as a code in the regulatory mechanism of gene expression, be it in developmental biology (LaSalle et al., 2013), the onset of disease (Di Cerbo and Schneider, 2013) or brain plasticity and pathology (Rudenko and Tsai, 2014). A great deal of attention during the last decade has focused on the state of the chromatin within the nucleus, and histone PTMs have been directly linked to this state (Cheung et al., 2000; Strahl and Allis, 2000; Fischle et al., 2003; Kouzarides, 2007) constituting a part of a complex mechanism called epigenetics.

The DNA in the nucleus is wrapped around an octamer of histone molecules (together termed the nucleosome), consisting of two copies of each of the histone H2A, H2B, H3 and H4 molecules. A fifth histone molecule, H1, links the nucleosome to the DNA, providing a tight association (Kornberg, 1974). The chromatin can be either in an open or relaxed form, referred to as euchromatin, allowing the transcriptional machinery to reach the DNA, leading to gene transcription, or in a condensed form, called heterochromatin, that prevents the transcriptional machinery from reaching the DNA, and thereby preventing gene transcription (Tamaru, 2010). Whether the chromatin is open or closed depends on the relationship between the DNA and the histone molecules, which in turn is linked to the PTMs of histones.

It has been reported that different histone PTMs (acetylation, phosphorylation, methylation, ubiquitination or sumoylation) are associated either with gene transcription or gene silencing. For example, evidence indicates that hyper-acetylation of histones is linked to gene transcription whereas methylation can be associated with either transcriptional activation (histone H3 at lysine 4) or repression (di- and tri-methylation on histone H3 at lysine 9) (Fischle et al., 2003; Feng et al., 2007). Interestingly, several modifications can occur on the same tail of a particular histone molecule. For example, a lysine residue on histone H3 can be phosphorylated or acetylated. Other modifications can occur even within the same amino acid. For example, a single lysine or arginine can be mono, di or tri-methylated (Fischle et al., 2003; Kouzarides, 2007). One modification on a particular histone can also define other modifications on the same or a different histone, resulting in histone modification cross-talk (Fischle et al., 2003; Kouzarides, 2007; Bannister and Kouzarides, 2011). These multiple combinations of histone PTMs define which genes are expressed and which are repressed, which results in the concept of a “histone code” (Strahl and Allis, 2000).

Histone modifications can be stable throughout the lifespan, such as organ-specific stem cells that maintain their identity and thereby function as an epigenetic memory, or highly dynamically regulated by experience or environmental factors (Cohen et al., 2011) which makes them a tool of choice to study brain plasticity (Levenson et al., 2004; Martin and Sun, 2004; Fischer et al., 2007; Gupta et al., 2010; Graff and Tsai, 2013). From this perspective, the study of histone PTMs has become a major topic in neuroscience and understanding the histone code within the nervous system remains an essential element in understanding various functional characteristics of the nervous system, especially in the field of plasticity. In the past decade, several research groups have demonstrated the importance of this approach in understanding plastic changes in the brain, such as involved in learning and memory (Koržus et al., 2004; Levenson et al., 2004; Fischer et al., 2007; Gupta et al., 2010; Graff and Tsai, 2013), addiction (Maze and Nestler, 2011) and neural disorders (Tsankova et al., 2007).
Western blotting remains a powerful method for the investigation of histone PTMs and the first step requires histone isolation. Several methods are available for the isolation of histones from cell culture or brain tissue (Levenson et al., 2004; Shechter et al., 2007; Rodriguez-Collazo et al., 2009; Rumbaugh and Miller, 2011). These protocols require a relatively large amount of tissue, such as the entire CA1 region of the rat hippocampus, obtained by dissection. However, if the experimental question focuses on small brain regions or a subregion of a larger brain region, we need to refine and adjust the usual methods because of the very small amounts of tissue available. Some researchers have overcome this problem by working on whole-tissue fractions, leading to a loss of nuclear-specific protein content. Others pool specific brain regions from two or more animals in order to obtain sufficient tissue to work with.

Here, we provide an improved method for investigating histone PTMs in specific brain regions or subregions of the rat brain. The method is an adaptation of several well-established histone-isolation methods (Levenson et al., 2004; Shechter et al., 2007; Rodriguez-Collazo et al., 2009; Rumbaugh and Miller, 2011) with the advantage of being adapted for the purpose of working with very small amounts of tissue (1 mm³ total volume of tissue), using the Palkovits’ punch technique (Palkovits, 1973). Anatomical precision is preserved and depends on visual anatomical landmarks in 350-µm thick frozen brain slices. This protocol describes the step-by-step procedure for histone isolation and permits reproducible and reliable results for histone extraction from small amounts of nuclear-specific brain tissue. The method has been used with satisfactory results for various histone modification antibodies with some illustrations in the text.

**MATERIALS**

**Equipment**

- Palkovits’ punch technique tools (0.75-1.25 mm) (Stoelting, Dublin; cat. no. 57401).
- Tubes (1.5, 2 ml, 10 ml, 50 ml)
- Micropipette and tips (10, 20, 100 and 1000 µl)
- Tissue grinding Pestles
- Bench-top centrifuge for 1.5-ml microcentrifuge tubes with cooling system
- Vortex
- Heater block
- Roller or shaker
- SDS-PAGE apparatus
- Odyssey IR scanner (Li-cor Biosciences), for quantification

**Histone isolation reagents**

- Hypotonic lysis buffer: 250 mM sucrose, 50 mM Tris and 25 mM KCl, pH 7.5. Store at 4°C. Prior to use: for 10 ml homogenization buffer add one complete protease inhibitor cocktail tablet (Roche), one complete phosphatase inhibitor cocktail tablet (Roche) and 0.9 mM sodium butyrate (Sigma-Aldrich).
- Acid extraction buffer: 0.2 N HCl. Store at 4°C
- Trichloroacetic acid (TCA) precipitation buffer: 100% TCA containing 4 mg/ml deoxycholic acid. To prepare 100% TCA: add 227 ml ddH2O to 500 g TCA. Store at 4°C.
- Washing buffer 1: Acetone 100% containing 0.1% HCl. Store at -20°C.
- Washing buffer 2: Acetone 100%. Store at -20°C.
- Storage buffer: 50 mM Tris (pH 8.0) or 50 mM Tris containing 3% SDS (pH 8.0)
- 5x Laemmli sample buffer: 10% SDS, 250 mM Tris (pH 6.8), 1 mg/ml bromphenol blue, 0.5 mM dithiothreitol (DTT) and 50% glycerol. Store aliquots at -20°C
- Detergent-compatible colorimetric assay Kit (DC Protein assay kit Bio-Rad).

**Western blotting reagents**

- Running buffer: For 10 x stock solution: 10 g SDS; 30.3 g Tris and 144 g glycine in 1 liter ddH2O. Store at room temperature. Use running buffer (1x) by diluting the 10x stock solution in ddH2O.
- Transfer buffer: 5.82 g Tris and 2.93 g glycine in 1 liter ddH2O. Freshly prepared and cooled at 4°C prior to use.
- Methanol for wetting of PVDF.
- Blocking buffer: Licor blocking buffer (Li-cor odyssey. cat. no. 927-40000) diluted in phosphate-buffered saline (PBS) 1:1.
- Phosphate-buffered saline (PBS): For 10x stock: 80 g NaCl, 2 g KCl; 14.4 g Na2HPO4 and 2.4 g KH2PO4 in 1 liter ddH2O. Store at room temperature. Use PBS (1x, pH 7.4) by diluting the 10x stock solution in ddH2O.
- Stripping buffer: 25 mM glycine and 1-2% SDS, pH 2.0.
- Polyvinylidene difluoride (PVDF) membrane.
- Primary antibody to histone PTM of interest.
- Secondary antibody, fluorescently labeled

**CAUTION!** As several of the reagents are highly corrosive (Hydrochloric acid (HCl), trichloroacetic acid (TCA), acetone), it is recommended to employ appropriate safety procedures (e.g., wearing gloves and protective clothing and working under a fume hood).
**METHOD**

**Histone isolation from frozen tissue**

**Brain isolation**

Tissue is collected from fresh-frozen rat brains. Deeply anesthetize the animal with an overdose of anesthetic (pentobarbital) and rapidly remove the brain and flash freeze it by submersion in a beaker filled with pre-cooled isopentane placed on dry ice for 2 min. The temperature needs to be between -30 and -40°C. Lower temperatures could result in cracking of the brains. The brains can then be wrapped in aluminum foil and stored immediately at -80°C.

**Slice preparation**

Make 350 µm-thick coronal sections of the brain region of interest with a cryostat or freezing microtome (between -13 and -17°C). Collect and place the brain slices on a pre-cooled glass slide. Smoothe the brain slices by briefly placing the glass slide on a flat surface at room temperature just long enough for slices to be flattened (about 10 s). Immediately afterwards, place the slide on dry ice and store at -80°C until proceeding with the next steps.

**Tissue dissection and histone extraction (Fig. 1)**

All procedures should be performed on ice. All solutions, tubes and centrifuge need to be chilled to 4°C prior to use. All procedures should be performed on ice. All solutions, tubes and centrifuge need to be chilled to 4°C prior to use.

**Tissue collection and cell lysis**

1. Tissue from frozen 350-µm coronal slices is dissected on a cold flat surface using the Palkovits’ punch technique and collected in 100 µl of ice-cold hypotonic lysis buffer.

   *With a 1.25-mm diameter punch size, 4-6 punches are enough to get a good Western-blotting signal, even with an antibody that shows poor affinity to the antigen. With a 0.75-mm diameter punch size, 10-12 punches are required. See: Table 1 and Fig. 4 for tissue yield (volume in mm³).*

2. Tissue punches are easily collected in the cap of a 1.5-ml Eppendorf tube. Open the tube, turn the cap over and fill the cap with 100 µl hypotonic lysis buffer and carefully put the tissue in the buffer (in the cap) to permit the release of the punch into the buffer. Putting the buffer directly in the tube would not guarantee it, as the puncher is usually too short to reach the bottom of the tube. See: Fig. 1.

3. After collection of the punches, carefully close the tube with the cap down. Keep it this way on ice and collect the next sample. See: Fig. 1.

   *We recommend to not make more than 10 samples per isolation to keep all the steps standard such that the first sample is not waiting too long compared to the last one.*

4. Perform a quick centrifugation of the tubes (7,800 x g for 10 s) in order to concentrate the tissue at the bottom of the tube.

5. With a pestle, grind the tissue for 15-20 quick strokes and incubate for 5 min on ice.

   *The pestle needs to fit the tube perfectly. This step is critical to perform a good lysis of the cells (Shechter et al., 2007) and to ensure optimal histone isolation. Pestles should be chilled to 4°C to avoid overheating and foam formation.*

6. Centrifuge the homogenate at 7,800 x g for 1 min to separate the nucleus and cell debris from the cytoplasm.

7. Discard the supernatant (cytoplasmic fraction) and keep the pellet.

   *If also investigating cytoplasmic molecules, the supernatant can be stored at -20 or -80°C for later use.*

8. Acid extraction. Re-suspend the pellet in 100 µl of 0.2 N HCl (Rodriguez-Collazo et al., 2009) for 1 hour on ice, and vortex every 10 min (3 x 1 s at one-second intervals), then centrifuge at 16, 000 x g for 15 min.

9. TCA precipitation. Collect the supernatant in a fresh 1.5 ml tube and add 30 µl of 100% TCA containing 4 mg/ml deoxycholic acid drop by drop. The solution will appear milky. Briefly vortex (0.5 s, once) and let the proteins precipitate for 15 min, then centrifuge at 16, 000 x g for 15 min.

   *Always keep the tubes oriented in the same direction in the centrifuge. The final pellet size can be very small or just a “smear” on the tube that you can hardly see. By keeping the tubes oriented in the same direction during ALL centrifugation steps, the pellet will not be discarded by inadvertence during the different steps.*

10. Discard the supernatant carefully.

   *Position and hold the tube so that the pellet is above the supernatant, and carefully place the pipette tip opposite to the pellet. This way, even if the pellet is not visible it will not be discarded during the pipetting. See: Fig. 1.*

11. **Washing step 1.** Wash the pellet by carefully adding 100 µl ice-cold acidified acetone (0.1% HCl) (Washing buffer 1). Gently invert the tube to wash the pellet. Do not disturb the pellet, do not vortex. Keep on ice for 5 min, then centrifuge at 16, 000 x g for 5 min.

12. **Washing step 2.** Discard the supernatant; then wash the pellet with 100 µl ice-cold 100% acetone (Washing buffer 2) for 5 min and centrifuge at 16, 000 x g for 5 min in step 11.

13. Discard the supernatant and dry the remaining histone pellet for 15 min at room temperature for the remaining acetone to evaporate.

   *Do not overdry the pellet, as this will prevent its re-suspension.*

14. Re-suspend the pellet in 30 µl 50 mM Tris (pH 8.0) containing 3% SDS. Let the pellet dissolve at room temperature for 1 hour with frequent vortexing. This will help the pellet to dissolve and get enough histone for protein estimation (Rumbaugh and Miller, 2011).

   *After TCA precipitation it is difficult to dissolve the histones. Adding 3% SDS will help dissolving the pellet.*

15. Estimate protein concentrations using a detergent-compatible colorimetric assay kit (DC Protein assay kit, Bio-Rad).
16. Store histone samples in 5X Laemmli sample buffer to protect histones from degradation (Rumbaugh and Miller, 2011).

Use 5X Laemmli sample buffer rather 2x, as this will prevent excessive dilution of your samples and help concentrating the antigen.

Alternative for very small amount of tissue

This alternative can be used when protein estimation cannot be performed because of low re-suspension of the histone pellet that leads to a low yield of histone protein or when using smaller or fewer number of punches reaching no more then 1 mm³ of tissue. See: Table 1 and Fig. 4 for tissue yield (volume in mm³). It is possible to skip the protein essay without losing precision and the reliability of the method as shown in figure 3 and 5.

17. Histone pellet after step 13 can be re-suspended directly in 30 µl 50 mM Tris (pH 8.0) then boiled in 5x sample buffer: vortex and boil at 95°C for 5 min and vortex again. The reliability of this method is shown in Fig. 3 and Fig. 4.

We prefer this method that immediately protects histone PTMs from any kind of degradation that can occur when leaving histone to dissolve for 1 hour at room temperature.

18. Make aliquots: re-suspend the sample by vortexing, then make aliquots of 10 µl and store at -20°C or for a longer period at -80°C.

Do not dilute the histones (final pellet) too much. We found that a volume of 30 µl of Tris buffer is optimal for 4-6 punches of 1.00-1.25 mm. When using smaller punches (e.g. 0.75 mm) on brain tissue with a structural organization that makes large diameter punches impossible, 20-25 µl of buffer is optimal. Using 5X sample buffer keeps the samples less diluted.

Western blotting

All equipment needs to be clean and rinsed with ddH2O prior to use. Only critical steps are mentioned here.

19. Take the samples from -80°C and let them thaw at room temperature.

20. Quickly spin and vortex sample, then load 10 µl of sample, corresponding to approximately 6 µg of protein, on the gel.

Depending on the specificity and quality of the antibody, a good signal can be detected with 5µl.

21. When preparing resolving gel use narrowest possible well size when loading your protein (1 mm or smaller to maximize the antigen concentration with a minimum of volume or amount of protein loaded).

22. Run samples on a discontinuous polyacrylamide gel consisting of 15% or 20% gel acrylamide resolving and 4% acrylamide stacking gel. Run at 200 V for about 1 hour until you see a good separation of the lowest molecular weight markers to ensure that histone proteins are nicely separated.

A 20% gel allows you to get histones H3 and H2B on the same blot with two sharp bands that will not overlap. See: Fig. 6.

23. Pre-wet PVDF membrane in methanol for 15 s and equilibrate in blotting buffer for at least 5 min. Handle membranes carefully and with clean forceps.

From this time make sure to not allow the membrane to dry as this will prevent the blotting or the binding of the primary or secondary antibody at the time of antibody incubation.

24. Do not equilibrate the gel. Just wash the gel in blotting buffer to remove SDS and gel debris that prevent a good blotting and perform the blotting immediately. Run the blot at 100 V for 1 hour.

25. After the blotting is finished, rinse the PVDF membrane briefly in ddH2O.

26. Air-dry the PVDF for 1-2 hours or overnight after transfer (we dry the membrane overnight) to ensure that proteins are retained in the membrane (Li-core troubleshooting guide (Rumbaugh and Miller, 2011)).

27. Blocking the membrane: re-wet the PVDF membrane in methanol and rinse twice briefly with ddH2O.

Figure 1. Outline of the different steps for histone isolation.
28. Block the PVDF in licor-blocking buffer diluted in PBS (Licor–PBS buffer 1:1) at room temperature for 1 hour with gentle shaking. Use sufficient blocking buffer to cover the membrane. 5% (w/v) non-fat milk can also be used as blocking buffer.

Licor–PBS buffer 1:1 blocking buffer can be reused once if stored at 4°C to prevent bacterial development.

29. Incubate the PVDF membrane in primary antibody (in Licor–PBS buffer 1:1) on rotator overnight at 4°C (see primary antibodies tested and dilutions in Table 1).

30. Wash the PVDF membrane 4 times for 5 min in PBS at room temperature with gentle shaking.

31. Incubate the PVDF membrane in secondary antibody on rotator at room temperature for 2 hours (see secondary antibodies dilutions listed in Table 1).

As the secondary antibodies used in this protocol are fluorescent, PVDF membrane must be protected from light during secondary antibody incubations and washes. Never dry the membrane after the antibody incubation, as it will not be possible to strip it later, if desired. (See stripping step)

32. Wash the PVDF membrane 4 times for 5 min in PBS at room temperature with gentle shaking.

33. Detection: Band intensity can be determined and quantified using Odyssey IR scanner (Li-cor Biosciences). Chemiluminescence detection can also be used in this case make sure you use an appropriate secondary antibody compatible with chemiluminescence detection such as HRP-conjugated secondary antibodies.

If next step is to strip the PVDF membrane, do not dry the membrane after detection, as stripping is ineffective after the membrane has dried.

34. Cover the membrane with sufficient stripping buffer and incubate at room temperature for 10 to 15 min with shaking.

35. Incubate blot with fresh stripping buffer for another 10-15 min with shaking. Wash the membrane in PBS with 0.1% Tween-20 for 5 min, with shaking.

36. Rinse the membrane with PBS. As intense bands or strong antibodies are stripped with difficulty, scan the membrane at low resolution to check the stripping efficiency. If some signal remains, repeat stripping procedure.

If using the same blot for more than one primary antibody, we recommend starting with the primary antibody that gives the weakest signal for the first detection before stripping the membrane and re-probing with the second primary antibody. The stripping will be more efficient. However, we recommend not to strip the membrane more than twice as this can lead to a loss of protein.

37. Once the stripping is completed, re-block the membrane in Licor–PBS buffer 1:1 for 30 min and proceed with antibody incubation. Steps 30-31 and 32.

Analysis of western blots, Odyssey imagine software
The bands of a given histone PTM are normalized to total histone H3 or H4 or another total histone protein.

1. With Odyssey software, draw a square around each band avoiding being too close to the band, as this will be counted as background.

Do not forget to subtract the background. Odyssey software gives the option to subtract the background automatically.

2. Using the menu of the Odyssey software, the integrated intensity value can be exported to an excel file for analysis.

3. Calculating the ratio: divide each given band intensity corresponding to a histone PTM by its corresponding total histone intensity. For example, AcH3K14 band intensity from sample 1 will be divided by its corresponding total histone H3 (TH3). Fig. 2.

Figure 2. (A). The blot was scanned using Odyssey IR scanner (Li-cor Biosciences) after incubation overnight in AcH3K14. (B). The blot was then stripped and re-probed in TH3.

TROUBLESHOOTING
Problem
No bands visible on the blot.

Solution
1. First, make sure that your histone isolation was performed correctly by incubating the blot in a total histone antibody (e.g TH3). The presence of sharp bands will ensure that your histone isolation was correct. Two cases:

2. Presence of sharp bands: your histone isolation was successful, check your primary antibody for histone modification, increase amount or incubation time of primary and secondary antibody, optimizing for best performance.

3. No bands with the TH3 antibody. Your histone isolation was not correctly performed:
Check your isolation procedure: try first to isolate histone from a larger amount of tissue to master the procedure, then go for a smaller volume of tissue.

Check all your buffers.

Make sure that you run the gel long enough to get the band of interest. Histone H3 is at 17 kDa and histone H4 at 10 kDa.

If using a 2x sample buffer, go for 5x. This will prevent diluting your sample too much and will concentrate antigen.

Use narrowest possible well size (1 mm or smaller) to concentrate antigen.

Dry the PVDF membrane to avoid losing antigen during the blocking step or use 0.2 µm PVDF membrane that is preferable for low molecular weight proteins.

Increase the amount of protein to load, if necessary. This will depend on the affinity of your primary antibody. Some antibodies have a high affinity, which allows the use of a low amount of protein whereas others have less affinity, which requires more protein.

**VALIDATION OF THE HISTONE EXTRACTION AND PURIFICATION METHOD FOR VERY SMALL SAMPLES**

Accurate targeting of small brain regions implies by definition that only very small amounts of brain tissue will be available. This inevitably results in a very low yield of histone, making the estimation of protein concentration difficult or impossible. In this protocol, the standardization relies on using the same amount of tissue as starting material without the need of protein assay estimation: Steps 1 to 13 followed by 17 to 19 of the protocol.

The standardization of the samples starts at the tissue collection stage as every sample will have a similar amount of tissue (size and number of punches), see table then all samples are treated the same way with high precision during every pipetting step to minimize variation.

The ratio is stable across varying volumes from the same sample

Here we show that loading different volumes from the same sample, independently of the protein estimation, is a valid procedure. To confirm that this does not create inconsistencies, we examined the consistency of results across different volumes loaded from the same sample.

Different volumes (5 µl, 10 µl, 15 µl or 20 µl) from the same histone sample were loaded and histone acetyl H3 at lysine 14 (AcH3K14) was detected. The blot was stripped then re-probed with total histone H3 (TH3) antibody as a protein control. We then calculated the AcH3K14 to TH3 ratio. The results showed that, independently of the volume loaded (10 µl, 15 µl or 20 µl), the percentage of AcH3K14 normalized to TH3 was the same (Fig. 3). This finding demonstrates that when the yield of histone protein is too low (less then 0.60 µg/µl) to perform protein estimation, the method is valid with no need to perform a protein assay when using the total histone of the given histone PTM as a protein control.

Figure 3. After histone isolation, different volumes from the same sample (5 µl, 10 µl, 15 µl, 20 µl) were run on a 15% SDS gel. (A). The blot was probed in histone AcH3K14 antibody overnight and scanned using Odyssey IR scanner (Li-cor Biosciences) (B). The blot was then stripped and re-probed in TH3 (C). Each band intensity is represented by a bar on the histogram from the different volumes (5 µl, 10 µl, 15 µl, 20 µl). Note that low histone yield (5 µl corresponding to less than 5 µg of histone loaded) results in a non-reliable result.
Histone isolation from different size and number of punches

The main difficulty of working with small quantities (specifically when the starting material is only 1 mm³ of tissue) is to obtain a satisfactory signal with the primary antibody. To determine how much tissue is required to obtain a satisfactory signal with the AcH3K14 antibody that in our experience has a poor affinity to its antigen, we used different numbers and diameters of punches from one cortical brain area (350-µm thick coronal slice) and isolated the histones with the described method. See corresponding volume of tissue related to the number of punches (Table II). Ten microliter (10 µl) of histone sample (independently of protein assay estimation) was loaded in each condition. The blot was probed in acetyl Histone H3 at lysine 14 (AcH3K14) antibody (1:10000). A satisfactory signal required a minimum volume of 1 mm³ of tissue as shown in Fig. 4A (see the corresponding punch number – volume in table I). The linearity of the points in the graph demonstrates that the intensity of the signal detected is proportional to the amount of histone isolated from different quantities of tissue (Fig. 4B).

Table I. Punch volume (mm³)

<table>
<thead>
<tr>
<th>Inner diameter of punch tool (mm)</th>
<th>Thickness slice (µm)</th>
<th>Volum of tissue per punch (mm³)</th>
<th>Volum of tissue for 2 p</th>
<th>Volum of tissue for 4 p</th>
<th>Volum of tissue for 6 p</th>
<th>Volum of tissue for 8 p</th>
<th>Volum of tissue for 10 p</th>
<th>Volum of tissue for 12 p</th>
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</thead>
<tbody>
<tr>
<td>0.75</td>
<td>350</td>
<td>0.35x10⁻⁶</td>
<td>0.31</td>
<td>0.62</td>
<td>0.93</td>
<td>1.24</td>
<td>1.55</td>
<td>1.86</td>
</tr>
<tr>
<td>1</td>
<td>350</td>
<td>0.275x10⁻⁶</td>
<td>0.55</td>
<td>1.10</td>
<td>1.65</td>
<td>2.20</td>
<td>2.75</td>
<td>3.30</td>
</tr>
<tr>
<td>1.25</td>
<td>350</td>
<td>0.430x10⁻⁶</td>
<td>0.86</td>
<td>1.72</td>
<td>2.58</td>
<td>3.44</td>
<td>4.30</td>
<td>5.15</td>
</tr>
</tbody>
</table>

Figure 4. Histone isolation from different size and number of punches. (A): The blot was probed in histone AcH3K14 antibody. Note that a satisfactory signal starts with 6-8 punches of 0.75 mm puncher or 4 punches of 1 mm or 1.25 mm corresponding to a volume of about 1 mm³. (B): The linearity of the points in the graph demonstrates that the intensity of the signal detected is proportional to the amount of histone isolated from different quantities of tissue. Quantification used Odyssey IR scanner (Li-cor Biosciences).

Results from several comparable samples

We next investigated the reproducibility of the histone extraction procedure from tissue taken from different animals. The histone pellet was isolated from 10 punches of 0.75 mm diameter taken from the dorsal part of hippocampal CA1 region in a series of 10 male rats. The histone pellet was re-suspended directly in 25 µl 50 mM Tris (pH 8.0) as described in step 17. An equal sample volume (10 µl) was loaded and the blot was probed in TH3 as a nuclear housekeeping protein. Signal intensity was measured using Odyssey IR scanner (Li-cor Biosciences) and compared across animals. As is shown in Fig. 5, the between–subject variability of band intensity is very small, indicating that the amount of protein loaded varied little from one sample to another.

This shows that normalizing histone PTMs to a total amount of a particular histone corrects for small variations due to loading. In general, it is better to normalize to a given histone PTM to its total histone. For example, phosphorylation of histone H3 at lysine 10 (pH3S10) will be normalized to its total H3 and acH2B can be normalized to its total H2B (Levenson et al., 2004; Bousiges et al., 2010). The blot can be stripped and re-probed with the total histone antibody.

It is also well accepted that any of the total histone subunits (TH3, TH4, TH2B, TH2A or TH1) could be used as a nuclear housekeeping protein for normalization (Rumbaugh and Miller, 2011; Graff et al., 2012). In this case, the blot is re-probed with a total histone antibody that corresponds to a different molecular weight from the histone PTM of interest. In this way, the bands do not overlap, because of the difference in molecular weight, eliminating the need to strip the membrane. For example, if looking at pH3S10, the blot can be re-probed with a different total histone antibody (TH4 or TH1) for normalization.

Figure 5. Each band intensity is represented by a bar on the histogram coming from a single rat (B). Each bar of the histogram represents the integrated intensity of an individual band of the blot (A). The intensity of the signal was measured using Odyssey IR scanner (Li-cor Biosciences).
Representative blots using different histone post-translational antibodies

Here we show that the method gives reliable results with different antibodies. The described method and the quality of histone isolation have been tested with several primary antibodies for histone detection as shown in Fig. 6. In this example, 6 punches (1.25 mm) thickness from the insular cortex were taken from 350 µm thick brain slices. Histone isolation was performed using the described method and the blots were probed with antibodies against either trimethylated H3 at lysine 27 (3meH3K27), histone H3 phosphorylated at serine (10) (pH3S10), histone H3 acetylated at lysine 14 (AcH3K14) or total acetyl histone H2B (AcH2B). The blots where then stripped and reprobed in TH3. See antibody dilutions in Table 1. As shown in Fig. 6, a satisfactory signal was seen with all antibodies used, which indicates good histone isolation. Similar results have been obtained with comparable tissue punches from other brain regions (data not shown).

![Figure 6. Representative Western blotting images showing the quality of signal of different histone -PTM antibodies from the insular cortex (6 punches of 1.25 mm). The blot was scanned using Odyssey IR scanner (Li-cor Biosciences).](image)

**Table 2. Primary and secondary antibodies.**

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Used in the protocol</th>
<th>Tested with success</th>
<th>Dilution</th>
<th>Incubation time</th>
<th>Company</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-AcH3K14</td>
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<td>1:100</td>
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<td>Millipore</td>
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<td>+</td>
<td>1:100</td>
<td>Overnight at 4°C</td>
<td>Millipore</td>
<td>06-989</td>
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</tr>
<tr>
<td>Anti-pH3S10</td>
<td>+</td>
<td>1:100</td>
<td>Overnight at 4°C</td>
<td>Millipore</td>
<td>05-998</td>
<td></td>
</tr>
<tr>
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<td>1:100</td>
<td>Overnight at 4°C</td>
<td>Millipore</td>
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<td></td>
</tr>
<tr>
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<td>2h Room temperature</td>
<td>Millipore</td>
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</tr>
<tr>
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<td>Overnight at 4°C</td>
<td>Millipore</td>
<td>07-373</td>
<td></td>
</tr>
<tr>
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<td>Anti-acH4</td>
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<td>Overnight at 4°C</td>
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<table>
<thead>
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<th>Secondary antibody</th>
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<th>Dilution</th>
<th>Incubation time</th>
<th>Company</th>
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<tbody>
<tr>
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<td>1:25,000</td>
<td>1-2 h: Room temperature</td>
<td>Li-cor</td>
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<tr>
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<td>1:25,000</td>
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<td>Li-cor</td>
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<tr>
<td>IRDye 680LT Donkey</td>
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</table>

**DISCUSSION**

Investigating histone PTMs from a small brain area requires a refinement of the classical methods, which can take more time than expected to obtain reliable results. That is why we propose this refined method for researchers who have limited amount of starting brain tissue. Most of the methods for histone isolation make use of large amounts of starting tissue, like the entire CA1 area of the hippocampus that represents approximately 40 mg of starting material, and requires approximately 20 times more tissue than one can get with punches. Here, we have shown that histones can be isolated with precision from small brain regions and subregions of rodent brain using a punching tool and that a minimum volume of 1 mm³ of tissue is sufficient for a detectable signal, which gave reliable results with all of the primary antibodies tested in the protocol.

One of the major problems with histone isolation is that the final pellet is hard to re-suspend after TCA precipitation which adds a layer of complication when working with a limited amount of tissue. With this small amount of tissue the final pellet is hardly visible. Re-suspension of the final pellet is improved by keeping the sample after TCA precipitation at room temperature for 1 hour (Rodriguez-Collazo et al., 2009; Rumbaugh and Miller, 2011). This might lead to sufficient histone to perform a protein assay when working with a large yield of tissue 40 mg or more of starting brain tissue (Rumbaugh and Miller, 2011), but not with punches. We overcame this limitation by using 50 mM Tris (pH 8.0) containing 3% SDS which permits a good re-suspension of the histone pellet and the possibility to estimate protein concentration using a detergent-compatible colorimetric assay Kit. However, even with punches this has a limitation, as the starting volume of brain tissue needs to be at least 2.5 mm³ (corresponding to approximately 0.60 µg/µl).

Using 5x sample buffer together with the narrowest possible well size (0.75 or 1 mm) helped to concentrate antigen for a better detection. Drying the PVDF membrane after the blotting also helps to retain protein on the membrane, preventing the loss of histones from the membrane during the blocking step.

We have shown that when working with a very small amount of tissue it is possible to use an alternative step, which permits reliable results. The alternative relies on the standardization of the samples that starts at the tissue collection stage as every sample will have similar amounts of tissue (size and number of punches). All samples are then treated the same way with high precision during every pipetting step to minimize variation. We demonstrate that this method can overcome the limitations relative to protein assay estimations when working with quantities of tissues that are below the threshold for protein assay estimation. Using the total histone of the given PTM as a housekeeping protein (in our case TH3), we have shown that the signal intensity of the bands are below the threshold for protein assay estimation. Using the total histone of the given PTM as a housekeeping protein (in our case TH3), we have shown that the signal intensity of the bands from a series of 10 different samples varies little from one sample to another (Fig. 5), which is the result of a good standardization starting at tissue isolation (same size and number of punches). With this standardization, the small variation in a given experiment due to the small variation of protein between the different samples is then corrected by normalizing each histone PTMs to its own total histone (Fig. 4). This suggests that the described method can compensate for the lack of the use of a protein assay for standardization when working with small amounts of tissue from punches.

Experimental results showing histone PTMs variation in the insular cortex after an arousal-“like-situation” have been recently published using the present method (Beldjoud and al., 2015). The method has been validated with different brain regions, like the dorsal CA1 subdivision of the hippocampus as well as insular cortex, respectively (Fig. 5 and Fig. 6).
REFERENCES


chapter 3

CHROMATIN REMODELING IN THE INSULAR CORTEX ASSOCIATED WITH THE CONSOLIDATION OF OBJECT RECOGNITION MEMORY REQUIRES BASOLATERAL AMYGDALA NORADRENERGIC ACTIVITY

Beldjoud et al.

Manuscript in preparation
ABSTRACT

Extensive evidence indicates that arousal-induced noradrenergic activation of the basolateral complex of the amygdala (BLA) facilitates information storage processes in its many target regions. In the current study, we examined whether such noradrenergic activation of the BLA regulates memory consolidation of object recognition training by enabling chromatin modification in the insular cortex, a brain region that plays a major role in object recognition memory. Male Sprague-Dawley rats were trained for 3-min on an object recognition task during which they could freely explore two identical objects. Immediately after the training, they received an intraperitoneal injection of either the histone deacetylase (HDAC) inhibitor sodium butyrate (NaB) or a saline control together with bilateral intra-BLA infusions of the β-adrenoceptor antagonist propranolol or saline control. The NaB administration enhanced 24-h object recognition memory whereas intra-BLA infusions of propranolol blocked this NaB effect. At the molecular level, we investigated several histone post-translational modifications within the insular cortex 1-h after the training that are associated with either gene expression [acetylation of histone H3 at lysine 14 (AcH3K14), acetylation of histone H2B (AcH2B) and phosphorylation of histone H3 at serine 10 (pH3S10)] or gene repression [tri-methylation of histone H3 at lysine 27 (3meH3K27)]. NaB treatment after object training, but not for home cage controls, increased AcH3K14 levels as well as pH3S10 levels whereas it decreased 3meH3K27 levels. Propranolol administration into the BLA did not block the NaB-induced increase in AcH3K14 levels, but did block the NaB effect on pH3S10 and 3meH3K27 levels. These findings indicate that noradrenergic activity within the BLA is a co-requisite to enable the effects of direct HDAC inhibition on chromatin modifications and object recognition memory.

INTRODUCTION

Extensive evidence indicates that emotionally arousing experiences are remembered more vividly than emotionally neutral experiences (McGaugh, 2000). This arousal-induced memory enhancement is known to involve noradrenergic activation within the basolateral complex of the amygdala (BLA) (McGaugh, 2000; McGaugh and Roozendaal, 2002; McGaugh, 2004) which, in turn, facilitates the storage of information in its many target regions (Setlow et al., 2000; Roesler et al., 2002; Miranda and McGaugh, 2004; Roozendaal et al., 2004; Lampe et al., 2005; Malin et al., 2007). Norepinephrine administration into the BLA also enhances the consolidation of low-arousing object recognition memory, whereas the β-adrenoceptor antagonist propranolol impairs memory of this training (Roozendaal et al., 2008). Memory of objects was known to depend on synaptic plasticity within the perirhinal cortex (Barker and Warrington, 2011) and insular cortex (IC) (Balderas et al., 2008). Long-term memory of the object is impaired when the protein synthesis blocker anisomycin was applied into either the IC or perirhinal cortex whereas short-term memory was not significantly affected (Balderas et al., 2008). On the other hand, anisomycin injected into the IC did not impair memory for the location of the object. In contrast, when the anisomycin was administered into the hippocampus, the memory deficits concerned the location of the object and not the object per se. Early studies showed that the IC and BLA share dense reciprocal connections (McDonald and Jackson, 1987; Shi and Cassell, 1998). It was also reported that high-frequency stimulation of the BLA induces long-term potentiation at synapses in the IC (Escobar et al., 1998). Paré and colleagues (1995) showed that the BLA sends glutamatergic projections to the IC. Furthermore, studies based on conditional taste aversion have demonstrated that the IC and BLA are functionally interconnected (Miranda and McGaugh, 2004; Rodríguez-Durán et al., 2011; Moraga-Amaro and Stehberg, 2012).

Memory consolidation is the process by which newly encoded information is stabilized into a long-term memory trace (Dudai, 2004) and is thought to rely on persistent changes in synaptic efficacy architecture that require gene expression and protein synthesis (McGaugh, 1966; Davis and Squire, 1984; Goelet et al., 1986; McGaugh, 2000). During the last decade investigations concerning the mechanism of gene expression have shed light on epigenetic modifications, i.e., histone post-translational modifications (PTMs), DNA methylation and non-coding RNAs, that are involved in different kinds of learning and memory (Levenson et al., 2004; Miller et al., 2008; Stefanko et al., 2009; Miller et al., 2010; Roozendaal et al., 2010; Reolon et al., 2011; Griggs et al., 2013). In the case of object recognition memory, Stefanakos et al. (2009) and Reolon et al. (2011) demonstrated that systemic post-training adámministration of the histone deacetylase (HDAC) inhibitor sodium butyrate (NaB) enhanced long-term object recognition memory in mice. Recently, we reported that post-training administration of NaB directly into the IC enhanced memory of the object, but not of the location of the object (Roozendaal et al., 2010). Conversely, when the NaB was administered into the hippocampus, it enhanced memory of the location of the object, but not of the object itself.

Although it is well established that arousal-induced noradrenergic activation within the BLA facilitates information storage processes in its many target regions, the neural mechanism underlying this memory facilitation remains largely unexplored. Recently, Blank and collaborators (2014) investigated whether BLA activity interacts with chromatin remodeling in its target regions. They showed that the HDAC inhibitor trichostatin A (TSA) increased inhibitory
avoidance memory when injected into the hippocampus, and that BLA inactivation by the GABA-A receptor agonist muscimol blocked this TSA-induced facilitation of memory formation. However, it is unknown whether BLA inactivation blocks the effects of TSA on inhibitory avoidance memory by preventing TSA-induced chromatin remodeling within the hippocampus. In the current study we investigated whether the memory-enhancing effect of NaB requires concurrent noradrenergic activity within the BLA and its potential participation in chromatin remodeling in its target regions. Rats were trained on an object recognition task for 3-min and received an immediate post-training systemic injection of NaB either alone or together with bilateral infusions of the β-adrenoceptor antagonist propranolol administered into the BLA. Object recognition memory was assessed 24-h later. Furthermore, we investigated in separate groups of rats the consequence of attenuation of noradrenergic activity within the BLA on the effects of NaB on chromatin remodeling in the IC 1-h after the training. We investigated drug and training effects on different histone PTMs that are either involved in gene expression, such as acetylation of histone H3 at lysine 14 (AcH3K14), acetylation of histone H2B (AcH2B) and phosphorylation of histone H3 at serine 10 (pH3S10), or gene repression such as tri-methylation of histone H3 at lysine 27 (3meH3K27).

MATERIAL AND METHODS

Subjects

Male adult Sprague-Dawley rats (280–320 g at time of surgery) from Charles River Breeding Laboratories (Kisstegg, Germany) were housed individually in a temperature-controlled (22°C) vivarium room and maintained on a 12-h:12-h light:dark cycle (lights on: 7:00 – 19:00 h) with ad libitum access to food and water. Training and testing were performed during the light phase of the cycle between 10:00 and 15:00 h. 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.

Surgery

Rats, adapted to the vivarium for 1 week, were anesthetized with a subcutaneous injection of ketamine (37.5 mg/kg of body weight; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion) and received the non-steroidal analgesic carprofen (4 mg/kg; Pfizer). Oxygen (35%) mixed with ambient air was administered during surgery such that blood oxygenation levels would not drop below 90% (Fornari et al., 2012). After surgery, the rats were allowed for 1-2 min per day for 5 days preceding the training day.

Object Recognition Training and Testing Procedures

The experimental apparatus was a gray open-field box (in cm: 40w × 40d × 40h) with the floor covered with sawdust and placed in a dimly illuminated room. The objects to be discriminated were transparent glass vials (5.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). On the training trial, each rat was placed individually in the training apparatus at the opposite end from the objects and was allowed to explore the identical objects (A1 and A2) for 3-min, which by itself is insufficient to induce long-lasting memory of the objects (Okuda et al., 2004; Bermudez-Rattoni et al., 2005; Roozendaal et al., 2006; 2008). Rats’ behavior was recorded with a video camera positioned above the experimental apparatus. Videos were analyzed off-line by a trained observer blind to treatment condition. The total time spent exploring the two objects during the training trial was taken as a measure of object exploration. Rats showing a total exploration time of <10 s on the training trial were removed from analyses, because previous findings indicated that such rats do not acquire the task (Okuda et al., 2004). To avoid the presence of olfactory cues, the sawdust was stirred and the objects were cleaned with 70% ethanol after each animal.

Some rats were sacrificed for tissue collection 1-h after training and immediate post-training drug treatment. Other rats were tested for retention 24-h after the training trial. During the retention test, one copy of the familiar object (A3) and a new object (B) were placed in the same location as stimuli during the training trial. All combinations and locations of objects were used in a balanced manner to reduce potential biases due to preference for particular locations or objects. The rat was placed in the experimental apparatus for 3-min and the time spent exploring each object and the total time spent exploring both objects were recorded. Exploration of an object was defined as pointing the nose to the object at a distance of <1 cm and/or touching it with the nose. Turning around, climbing or sitting on an object was not considered as exploration. In order to analyze cognitive performance, a discrimination index was calculated as the difference in time exploring the novel and familiar object, expressed as the ratio of the total time spent exploring both objects (i.e., [Time Novel – Time Familiar / Time Novel + Time Familiar] x 100%).

Local and systemic drug administration

Immediately after object recognition training, rats received bilateral BLA infusions of saline or the β-adrenoceptor antagonist propranolol (0.3 µg in 0.2 µl, Sigma-Aldrich), dissolved in saline (Roozendaal et al., 2006). For infusions, animals were gently restrained and bilateral infusions of drug or an equivalent volume of saline were administered into the BLA via 30-gauge injection needles connected to 10-µl Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 2.0 mm beyond the cannula tips and a 0.2-µl injection volume per hemisphere was infused over a period of 30-s by an automated syringe pump (Stoelting Co. Dublin Ireland). The injection needles were retained within the cannulae for an additional 20 s 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 drug infusions into the adjacent central amygdala do not affect memory consolidation (Roozendaal and McLaugh, 1996; 1997).

The HDAC inhibitor NaB (0.4 g/kg; Sigma-Aldrich) or saline was administered intraperitoneally, in a volume of 2 ml/kg, immediately after the training trial (Kwon and Houpt, 2010). Other rats received NaB (0.4 g/kg) or saline without training. All drug solutions were freshly prepared before each experiment.

**Cannula Placement Verification**

After object recognition memory testing, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, ip) and perfused transcardially with 0.9% saline followed by 4% formaldehyde. After decapitation, the brains were removed and immersed in fresh 4% formaldehyde.

At least 24-h before sectioning, the brains were transferred to a 30% sucrose solution in saline for cryoprotection. Coronal sections of 50 mm 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 within the BLA was made according to the atlas plates of Paxinos and Watson (2007) by an observer blind to drug treatment condition. Rats with injection needle tip placements outside the BLA or with extensive tissue damage at the injection needle site were removed from analyses.

**Insular Cortex Tissue Collection and Histone Preparation**

Rats for the molecular investigations were deeply anesthetized with an overdose of pentobarbital (100 mg/kg, ip) 1-h after training and drug treatment. Within 90 s after the pentobarbital injection, the rats were decapitated, the brains rapidly removed and flash frozen by submersion for 2 min in a beaker filled with pre-cooled isopentane on dry ice. Flash-frozen brains were stored at -80°C until tissue processing.

The anterior part of the brain was cut on a cryostat into 350-µm-thick coronal slices for IC tissue collection. The rest of the brain, containing the BLA, was immersed in 4% formaldehyde for 3 days, and then transferred to a 30% sucrose solution for cryoprotection. Coronal sections of 50 µm were cut on a cryostat, collected on gelatin-coated slides, and fixed in 100% acetone before staining with cresyl violet. Determination of injection needle placement within the BLA was performed as previously described.

IC tissue was dissected from frozen 350-µm thick coronal slices using a 1.25-mm brain puncher (Stoelting Co.). Bipolar punches from the anterior IC were collected from three consecutive slices for a total of six punches (approximate range of coordinates: AP, +2.7 to -0.3 mm; ML, -4.0 to 6.0 mm; DV, -5.0 to -7.0 mm). Histones were isolated according to the Levenson et al. (2004) protocol with some modifications as described in chapter II. All procedures were performed on ice and all solutions and centrifugations were chilled to 4°C prior to use. Tissue was homogenized in 100 µl of hypotonic lysis buffer (250 mM sucrose, 50 mM Tris, 25 mM KCI, 1 Complete protease inhibitor cocktail tablet (Roche), 1 phosphatase inhibitor tablet (Roche), 0.9 mM NaB, pH 7.5) and ground for 10 s. The homogenate was centrifuged at 7,800 x g for 1 min. The supernatant was removed and the pellet (nuclear fraction) was resuspended in 100 µl of 0.2 N HCl (Rodriguez-Collazo et al., 2009) for 1-h on ice and vortexed every 10 min, and then centrifuged at 16,000 x g for 15 min.

The supernatant was transferred into a fresh tube and 30 µl of trichloroacetic acid containing 4 mg/ml deoxycholic acid was added to precipitate the proteins for 15 min and then centrifuged at 16,000 x g for 15 min. The supernatant was discarded and the pellet was washed with 100 µl of ice-cold acidified acetone (0.1% HCl) for 5 min and then centrifuged at 16,000 x g for 5 min, washed again with 100 µl of ice-cold 100% acetone for 5 min, and centrifuged at 16,000 x g for 5 min. Finally, the supernatant was removed and the remaining histone pellet was dried for 15 min for the remaining acetone to evaporate. The pellet was resuspended in 30 µl of 50 mM Tris (pH 8.0) and sample buffer (5x) was added to prevent over-dilution of the samples. The samples were then boiled and 10-µl aliquots were stored at -80°C to protect histones from degradation (Rumbaugh and Miller, 2011).

**Western Blotting**

The samples were run on a discontinuous polyacrylamide gel consisting of a 15% acrylamide resolving and a 4% acrylamide stacking gel. The gel was then blotted onto a polyvinylidene difluoride (PVDF) membrane for immunoblotting (Millipore). The membranes were blocked for 1-h in Li-coro blocking buffer (Li-COR), diluted 1:1 in phosphate-buffered saline (PBS) [or Tris-buffered saline (TBS) for phosho-antibodies], then incubated in primary antibody overnight at 4°C, followed by incubation with the appropriate secondary antibody for 2 h at room temperature. Primary and secondary antibodies were dissolved in the same blocking buffer. Band intensity was determined and quantified using an Odyssey IR scanner (Li-cor Biosciences). The blot was stripped and reprobed in total histone H3. The signal of the different histone PTMs was normalized to total histone H3.

**Antibodies**

The primary antibodies and their dilution were: anti-acetylated histone H3 at lysine 14 (AcH3K14, 1:1,000, Millipore), anti-acetylated histone H2B (AcH2B, 1:2,000, Millipore), anti-phosphorylated histone H3 at serine 10 (pH3S10, 1:1,000, Millipore), anti-trimethylated histone H3 at lysine 27 (3meH3K27, 1:2,000, Millipore) and total H3 (1:2,000, Millipore). The secondary antibodies were goat anti-rabbit (1:25,000, LI-COR Biosciences) and donkey anti-mouse (1:20,000, LI-COR Biosciences).

**Statistics**

Data are expressed as mean ± SEM. The discrimination index and total object exploration time were analyzed with unpaired t-tests. One-sample t-tests were used to determine whether the discrimination index was different from zero (i.e., chance level) and thus whether memory was expressed. Normalized histone PTMs data are expressed as the percentage of the mean of the saline-treated control group and analyzed with two-way ANOVAs with training condition and drug treatment as between-subject variables, followed by post-hoc comparison tests, when appropriate. A probability level of < 0.05 was accepted as statistical significance for all tests. The number of rats per group is indicated in the figure legends.
RESULTS

Post-training infusion of the β-adrenoceptor antagonist propranolol into the BLA blocks NaB-induced enhancement of object recognition memory

This experiment investigated whether noradrenergic activity within the BLA is required to enable the enhancing effect of a post-training systemic injection of the HDAC inhibitor NaB on the consolidation of object recognition memory. For this, rats were subjected to a single 3-min training trial during which they could freely explore two identical objects. Immediately afterwards, they received a systemic injection of saline or NaB (0.4 g/kg) together with bilateral infusions of saline or the β-adrenoceptor antagonist propranolol (0.3 µg in 0.2 µl) into the BLA. During the 24-h retention test trial, one object was familiar and the other object was novel. Figure 1A shows the timeline of the object recognition experiment. Figure 2 shows a representative needle tip terminating within the BLA and the placement of all needle tips of rats included in the analyses.

Training trial: Two-way ANOVA for total exploration time during the 3-min training trial, before drug treatment, indicated no differences between later systemic NaB treatment (F(1,42) = 0.05; p > 0.83), intra-BLA propranolol infusion (F(1,42) = 0.07; p > 0.79) or an interaction between these two parameters (F(1,42) = 2.88; p > 0.10) (Figure 1B).

Retention trial: Figure 1C shows the discrimination index of rats during the 24-h retention test trial. Two-way ANOVA indicated a marginal NaB effect (F(1,42) = 3.49; p = 0.06), a significant propranolol effect (F(1,42) = 9.39; p < 0.003) and a significant interaction between both factors (F(1,42) = 13.09; p = 0.0008). One-sample t-test indicated that the discrimination index of control (saline-saline) rats did not differ significantly from zero (i.e., chance level, t12 = -0.08; p = 0.94), indicating that they did not show any evidence of retention of the training. The HDAC inhibitor NaB administered systemically immediately after object recognition training enhanced the discrimination index (p < 0.01). Moreover, one-sample t-test indicated that the discrimination index was significantly different from zero (t12 = 8.35; p < 0.0001), indicating that these rats readily discriminated the novel object at the 24-h retention test. This enhanced memory was seen following NaB treatment in animals with intact BLA activity but antagonism of noradrenergic activity with post-training intra-BLA infusions of propranolol blocked the memory enhancement induced by NaB (p < 0.01). One-sample t-test indicated that the discrimination index of rats given NaB systemically together with propranolol into the BLA did not differ significantly from zero (t12 = 1.09; p > 0.30).

Two-way ANOVA for the total time spent exploring the two objects during the retention test trial indicated no significant NaB effect (F(1,42) = 2.57; p = 0.12), intra-BLA propranolol effect (F(1,42) = 0.24; p = 0.63) or interaction between these two parameters (F(1,42) = 1.47; p = 0.23). This confirmed that overall exploration time or activity levels were not affected by treatments.

Figure 1. Effect of post-training systemic NaB injection and intra-BLA propranolol infusion on the consolidation of object recognition memory. (A) Diagram of the behavioral protocol and drug administration. Rats were trained on an object recognition task for 3-min and given a post-training systemic injection of NaB (0.4 g/kg, ip) and bilateral infusions of the β-adrenoceptor antagonist propranolol (0.3 mg in 0.2 ml) into the BLA. Memory for the object was tested 24-h later. (B) Total exploration time during the 3-min training trial, before drug treatment, did not differ between groups. (C) Discrimination index during the 3-min retention test trial. Saline controls displayed no evidence of 24-h memory whereas the group given the HDAC inhibitor NaB immediately after training displayed a significant exploration preference for the novel object. With intact BLA activity, injections of NaB enhanced memory (● ●, p < 0.01), compared with the Saline-Saline group. Post-training infusions of the β-adrenoceptor antagonist propranolol into the BLA blocked the memory-enhancing effect of NaB (● ● ●, p < 0.01) compared with the corresponding intra-BLA saline group. The discrimination index was calculated as the difference in time exploring the novel and familiar objects, expressed as the ratio of the total time spent exploring both objects. N = 10–13 rats/group.

Figure 2. Histological analyses. (A) Representative photomicrograph illustrating placement of a cannula and needle tip terminating in the BLA. Arrow points to the needle tip. (B) Diagram representing the different nuclei of the BLA, the lateral nucleus (L), basal nucleus (B) and accessory basal nucleus (AB), and central nucleus (CEA). (C) The location of needle tips within the BLA of all rats included in the analysis.
Effects of BLA noradrenergic activity on histone PTMs after object recognition training

After we demonstrated that BLA noradrenergic activity is necessary for enabling the facilitating effect of the HDAC inhibitor NaB on memory consolidation of object recognition training, the next step was to assess the action of NaB together with manipulation of BLA activity on chromatin remodeling. We chose to look at histone PTMs in the IC because of its known involvement in memory consolidation of object recognition training (Balderas et al., 2008, 2014; Roozendaal et al., 2010).

We investigated histone PTMs that reveal either gene expression [i.e., acetylation of histone H3 at lysine 14 (AcH3K14), acetylation of histone H2B (AcH2B) and phosphorylation of histone H3 serine 10 (pH3S10)] or gene repression [tri-methylation of histone H3 at lysine 27 (3meH3K27)]. For this, rats were trained on the object recognition task and administered a post-training systemic injection of NaB together with intra-BLA infusion of propranolol as described above. Animals were sacrificed 1-h after training and drug administration for tissue collection and histone isolation. The procedure is summarized in Figure 3A.

NaB increased AcH3K14 levels in the IC, which does not depend on noradrenergic BLA activity

Figure 4A shows AcH3K14 levels (normalized to total H3 levels) within the IC, assessed 1-h after object recognition training and drug treatment. Two-way ANOVA revealed a significant systemic NaB effect ($F_{1,39} = 15.44; p < 0.001$), but no intra-BLA propranolol effect ($F_{1,39} = 0.05; p = 0.82$) or interaction between both factors ($F_{1,39} = 0.56; p = 0.57$). Post-training systemic injection of NaB induced hyperacetylation of H3K14 within the IC relative to controls ($p < 0.05$). Propranolol infusions alone did not change acetylation levels of H3K14 ($p = 0.78$) and, furthermore, did not block the hyperacetylation induced by NaB ($p = 0.48$). Acetylation levels of rats administered NaB together with propranolol were significantly higher than those of their control group ($p < 0.05$). Levels of total H3 remained unchanged in the different experimental conditions (data not shown).

NaB and propranolol did not alter AcH2B levels in the IC

Figure 4B shows AcH2B levels (normalized to total H3 levels) within the IC, assessed 1-h after object training and drug treatment. Two-way ANOVA revealed no systemic NaB effect ($F_{1,39} = 0.006; p = 0.94$), no intra-BLA propranolol effect ($F_{1,39} = 1.84; p = 0.18$) and no interaction between both factors ($F_{1,39} = 0.39; p = 0.54$). Post-training NaB administration did not induce any notable increase in histone H2B acetylation levels ($p = 0.41$). Moreover, intra-BLA infusions of propranolol did not alter AcH2B levels by themselves ($p = 0.41$) or when administered together with NaB ($p = 0.62$).

Figure 4. Effects of post-training NaB injection and intra-BLA propranolol infusion on histone PTMs in the IC. Histones were isolated from the IC 1-h after the training session, and histone PTMs were determined by Western blot and summarized in Figure 4A. Representative immunoblots for the different histone PTMs and total histone H3 in the IC were shown for each condition.
The main finding of the present experiments is that noradrenergic activity within the BLA controls the effect of HDAC inhibition on chromatin remodeling in the IC during memory consolidation of an object recognition task. Our study is integrated within a large experimental framework that shows that arousal-induced BLA activation facilitates the consolidation of memory by influencing information storage processes in its many target regions (for review see, McGaugh, 2000). Besides the fact that this postulate has been largely and extensively explored, it remains that the neural and molecular mechanisms underlying this memory facilitation remain largely unknown.

Our findings indicate that the HDAC inhibitor NaB administered systemically immediately after object recognition training enhanced long-term memory of the objects when tested 24-h later. Moreover, the NaB injection increased levels of AcH3K14 and pH3S10 within the IC, which are both histone alterations related to gene activation (Kouzarides, 2007; Bannister and Kouzarides, 2011) and memory enhancement (Levenson et al., 2004; Chwang et al., 2006; Graf et al., 2010).

Propranolol infusions into the BLA blocked NaB-induced increases in pH3S10 levels in the IC
Figure 4C shows the relative changes in phosphorylation of histone H3 at serine 10 (normalized to total H3 levels) in the IC 1-h after object recognition training and systemic NaB treatment, with or without concomitant intra-BLA infusions of propranolol. Two-way ANOVA revealed no overall NaB ($F_{1,39} = 0.86; p = 0.36$) or propranolol effect ($F_{1,39} = 1.63; p = 0.21$). However, a significant interaction between NaB and propranolol was found ($F_{1,39} = 7.10; p = 0.01$). Systemic NaB administration induced hyper-phosphorylation of H3S10 when compared to the saline-saline control group ($p < 0.05$). However, this NaB-induced hyper-phosphorylation was prevented when BLA noradrenergic activity was concurrently blocked with the β-adrenoceptor antagonist propranolol ($p < 0.05$).

Suppression of noradrenergic activity in the BLA blocked the NaB-induced decrease in 3meH3K27 levels in the IC
Figure 4D shows the relative changes in trimethylation of histone H3 at lysine 27 (normalized to total H3 levels) in the IC 1-h after object recognition training and systemic NaB treatment, with or without concomitant intra-BLA infusions of propranolol. Two-way ANOVA revealed significant NaB ($F_{1,39} = 4.55; p = 0.04$) and propranolol effects ($F_{1,39} = 7.44; p = 0.009$), but no significant interaction between NaB and propranolol ($F_{1,39} = 0.58; p = 0.58$). Systemic NaB administration reduced trimethylation levels of H3K27 when compared to the saline-saline control group ($p < 0.05$). However, this NaB-induced hypomethylation effect was blocked when BLA noradrenergic activity was blocked concurrently with the β-adrenoceptor antagonist propranolol ($p < 0.01$ relative to saline-NaB group).

NaB administration to non-trained home cage control rats did not alter histone PTMs in the IC
Finally to control for non-specific effects of the drugs, we investigated the effect of systemic NaB injection on histone acetylation and phosphorylation within the IC of animals that did not experience any object training. We therefore examined the effect of systemic NaB injection on Ach5H3K14 and pH3K10 levels in home cage controls (procedure summarized in Figure 5A). Unpaired t-test comparison tests revealed that NaB did not change Ach5H3K14 nor pH3S10 levels in non-trained control rats when compared to the corresponding saline groups ($p = 0.96; p = 0.22$).

DISCUSSION
The main finding of the present experiments is that noradrenergic activity within the BLA controls the effect of HDAC inhibition on chromatin remodeling in the IC during memory consolidation of an object recognition task. Our study is integrated within a large experimental framework that shows that arousal-induced BLA activation facilitates the consolidation of memory by influencing information storage processes in its many target regions (for review see, McGaugh, 2000). Besides the fact that this postulate has been largely and extensively explored, it remains that the neural and molecular mechanisms underlying this memory facilitation remain largely unknown. Our findings indicate that the HDAC inhibitor NaB administered systemically immediately after object recognition training enhanced long-term memory of the objects when tested 24-h later. Moreover, the NaB injection increased levels of Ach5H3K14 and pH3S10 within the IC, which are both histone alterations related to gene activation (Kouzarides, 2007; Bannister and Kouzarides, 2011) and memory enhancement (Levenson et al., 2004; Chwang et al., 2006; Graf et al., 2010).
Roozendaal et al., 2010). On the other hand, the NaB treatment decreased 3meH3K27 levels in the IC, a histone mark that has been associated with gene repression (Kouzarides, 2007). When noradrenergic activity within the BLA was blocked post-training by propranolol, the enhancing effect of NaB on the consolidation of object recognition memory was prevented. This behavioral outcome was paralleled at the molecular level as the propranolol infusion also blocked the NaB effect on pH3S10 and 3meH3K27 levels. In contrast, BLA inactivation did not block the effect of NaB on AcH3K14 level. These findings indicate that BLA noradrenergic activity is necessary to induce NaB effects on some types of chromatin remodeling within the IC as well as on the enhancement of memory consolidation.

NaB, like all butyrate, inhibits most HDACs, except class III HDAC and class II HDACα and class 10 (Davie, 2003). While HDAC activity is inhibited after NaB administration, histone acetyl transferase (HAT) activity continues, which results in histone hyperacetylation (Davie, 2003). At first sight, it might appear surprising that in our study the administration of an HDAC inhibitor not only induced increases in histone acetylation levels but also changed pH3S10 and 3meH3K27 levels. Although NaB is normally used to increase histone acetylation in the context of learning and memory (Levenson et al., 2004; Chwang et al., 2006; Fischer et al., 2007; Stefanko et al., 2009; Roozendaal et al., 2010), several studies have indicated that the NaB effect on inducing histone hyperacetylation could result in secondary effects on other histone PTMs. For example, findings indicated that NaB administration results increased H3K4 tri-methylation and decreased H3K9 di-methylation in hippocampus 1-h after contextual fear conditioning (Gupta et al., 2010) or histone H3 phosphorylation in conditioned taste aversion (Kwon and Houpt, 2010). Another study reported that HDAC inhibition by its global histone hyperacetylation effect was accompanied by increased H3K4 methylation levels in HeLa and HL60 cells (Nightingale et al., 2007). Such findings that the HDAC inhibitor NaB induces changes in other histone PTMs such as phosphorylation and methylation, reflects a complex interplay between primary and secondary, or indirect, changes in histone PTMs (Mathew et al., 2010).

At the behavioral level, our findings are consistent with previous evidence that post-training systemic NaB treatment enhances object recognition memory in mice (Stefanko et al., 2009; Reolon et al., 2011). Our findings are reinforced by a recent and closely related study indicating that the HDAC inhibitor TSA increased inhibitory avoidance memory when infused directly into the hippocampus, and that BLA inactivation by the GABA-A receptor agonist muscimol blocked this TSA-induced facilitation of memory formation (Blank et al., 2014). However, this prior study did not investigate the molecular changes underlying this dual interaction between BLA activity and HDAC inhibition. Our finding that propranolol infusion into the BLA blocked the systemic NaB effect on memory consolidation provides strong evidence for the view that NaB treatment alone is insufficient to enhance memory consolidation and that the memory facilitation requires concurrent arousal-induced brain activity, in this case arising from noradrenergic activity within the BLA. These findings are comparable with previous findings from our laboratory indicating that the enhancing effect of NaB infusion directly into the IC on object recognition memory was blocked when a glucocorticoid receptor antagonist or protein kinase A inhibitor was co-administered into the IC (Roozendaal et al., 2010). Thus, these findings show that chromatin remodeling effects on memory consolidation require arousal-induced changes in noradrenergic and/or glucocorticoid signaling. Roozendaal et al (2010) concluded that the effect of HDAC inhibition on gene expression and memory consolidation require co-activation of cAMP response element-binding (CREB) protein and CREB-binding protein (CBP) or other transcriptional coactivators which are activated by arousal-associated neural mechanisms. A surprising finding of the present study was that although the propranolol infusion blocked the NaB effect on memory consolidation, it did not block the NaB effect on increased AcH3K14 levels. Such findings could be interpreted as indicating that AcH3K14 changes are not critically involved in mediating the NaB effect on memory consolidation. However, this is unlikely, as extensive evidence has indicated a critical role for AcH3K14 in synaptic plasticity and memory. Another interpretation of the current findings is that NaB effects on memory enhancement require a simultaneous elevation in both AcH3K14 and pH3S10 levels. Such a requirement of dual modifications of histone phospho-acetylation has also been shown in chromatin remodeling of stress-related responses (Chandramohan et al., 2007; 2008). We need to emphasize here, however, that even if this histone phospho-acetylation is necessary for memory consolidation, these modifications might not be the only ones required. This adds a layer of complication to the investigation of histone PTMs related to memory consolidation. In fact, several histone PTMs can occur concomitantly and or subsequent to a previous modification, involving a cross talk between and within histones (Strahl and Allis, 2000; Fischle et al., 2003; Kouzarides, 2007; Izzo and Schneider, 2010; Bannister and Kouzarides, 2011). With this perspective in mind, chromatin remodeling is the result of multiple histone PTMs, referred to as the histone code (Cheung et al., 2000; Lee et al., 2010; Banerjee and Chakravarti, 2011), which will result in a cocktail of histone modifications induced by a particular environmental condition. The resulting combination of histone PTMs will define the molecular as well as behavioral outcome.

Our findings further showed that the HDAC inhibitor NaB was able to change AcH3K14 and pH3S10 levels in the IC only in animals that received the NaB injection post-training and not in home cage control rats that received the NaB injection without training. This finding indicates that the NaB injection does not induce any hyperacetylation action by itself and that the acetylation and phosphorylation changes observed are not the result of non-specific effects of NaB, such as the pain due to the intraperitoneal injection of hypertonic NaB, but due to the conjunction of NaB with either the encoding of new information by the training or the emotional arousal associated with the training experience. We propose that the hyperacetylation observed might be due to the action of a histone acetyl-transferase activity (HAT) induced by the training and that the role of NaB is limited to maintaining the acetylation by its HDAC inhibition action. The NaB effects on acetylation also require an activity-dependent process sufficient to permit the activation of HAT and therefore histone acetylation. Previous work from Roozendaal and colleagues (2010) has shown that memory facilitation by HDAC inhibitor administration into the IC was abolished when CR activity was blocked. Furthermore a PKA inhibitor also blocked the ability of HDAC inhibition to enhance memory in the insular cortex. They suggested that hyper acetylation induced by HDAC inhibition is not sufficient to enhance long-term memory and that upstream signaling are necessary for the activation of transcription factors such as CREB and CBP (Vecsey et al., 2007, Roozendaal et al., 2010). Figure 6 shows a diagram that summarizes, in a simplified form, the dual information coming from the encoding of the object as well as arousal-associated information initiated by BLA activation, toward the IC associating their molecular and behavioral aspects.

In summary, the present findings show that noradrenergic activation of the BLA is required to enable the memory-enhancing effect of systemic HDAC inhibitor administration. Moreover, such arousal-induced noradrenergic activation interacts in a complex manner with HDAC inhibition on
ultimately such changes in histone PTMs alter the state of the chromatin and are associated with transcriptional genes. Therefore, it is important in future studies to investigate altered gene transcription following the different drug treatments and memory conditions. Chromatin immunoprecipitation (ChIP) analyses could be a starting point to determine histone mark changes at some candidate genes. But the best would be to employ the latest technology to determine genome-wide histone modifications such as chromatin immunoprecipitation coupled to deep sequencing (ChIP-seq) and microarray technology.

Figure 6. Schematic representation of histone alterations after object recognition training and BLA manipulation. Epigenetic modifications observed in a context of object recognition memory in the IC are the result of dual information coming from the characteristics of the object (green dashed) as well as the BLA efferents (red dashed) to the IC. The HDAC inhibitor NaB (yellow dots) induces a hyper-acetylation and phosphorylation in the IC only in animals that had undergone a change in their “steady state” due to the training procedure. BLA noradrenergic activity directed to the IC is specifically necessary for the elevation of pH3S10. Together this dual information is necessary for the behavioral outcome, revealed by the enhanced memory performance where noradrenergic activity is necessary for pH3S10 elevation and 3meH3K27 reduction (not shown in the diagram).
REFERENCES


chapter 4

EFFECT OF HISTONE DEACETYLASE INHIBITION AND NORADRENERGIC SUPPRESSION OF THE BASOLATERAL AMYGDALA ON HISTONE METHYLATION IN PREFRONTAL CORTEX SUBREGIONS IN THE CONTEXT OF OBJECT RECOGNITION TRAINING

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Manuscript in preparation
ABSTRACT

Chromatin remodeling via post-translational modification of histone proteins represents a mechanism by which the brain expresses its plasticity. While histone H3 acetylation and phosphorylation are well documented in the context of memory formation, little is known about the role of methylation of histone molecules in memory. Previously we have shown that systemic administration of a memory-enhancing dose of the histone deacetylase inhibitor sodium butyrate (NaB) after object recognition training altered acetylation as well as methylation levels of histone molecules in the insular cortex. Here we examined whether systemic administration of this memory-enhancing dose of NaB altered histone 3meH3K4, 2meH3K9 as well as 3meH3K27 levels in different subregions of the medial prefrontal cortex 1 h after object recognition training. Moreover, based on extensive evidence that noradrenergic activity of the basolateral complex of the amygdala (BLA) regulates memory consolidation by influencing neural plasticity changes within different brain regions, we further investigated whether inhibition of noradrenergic activity within the BLA by a post-training infusion of propranolol would alter the effect of NaB administration on histone methylation changes within mPFC subregions. NaB administration did not significantly alter 3meH3K4, 3meH3K27 or 2meH3K9 levels in either the prelimbic or infralimbic cortex. Inactivation of BLA noradrenergic activity increased di-methylation of histone H3 at lysine 9 (H3K9) within the infralimbic cortex whereas it had no effect on any of the other methylation marks. No changes in histone methylation levels were observed in the prelimbic cortex after BLA inactivation. These findings indicate that noradrenergic activity within the BLA affects certain aspects of chromatin remodeling within the prefrontal cortex after object recognition training.

INTRODUCTION

Memory consolidation is thought to rely on persistent changes in synaptic efficiency that requires protein synthesis (McGaugh, 1986; Davis and Squire, 1984; Goelet et al., 1986; McGaugh, 2000), which in turn relies on gene expression. During the last decade, new investigations concerning the mechanism of gene expression have shed light on the role of epigenetic modifications, such as histone post-translational modifications (PTMs) and DNA methylation, in neural plasticity and memory (Levenson et al., 2004; Miller et al., 2008; Stefanko et al., 2009; Gupta et al., 2010; Roozendaal et al., 2010). Accumulating evidence has shown that PTMs of histone tails such as acetylation and phosphorylation play an important role in memory formation by regulating gene expression (Levenson et al., 2004; Chwang et al., 2006; Koshibu et al., 2009; Stefanko et al., 2009; Gupta et al., 2010; Roozendaal et al., 2010; Haetting et al., 2011; Graff et al., 2012). In a previous study, we demonstrated that systemic post-training administration of the histone deacetylase (HDAC) inhibitor sodium butyrate (NaB) enhanced 24-h memory of object recognition training and induced chromatin remodeling in the insular cortex, a brain region known to be important for object recognition (Chapter 3). Interestingly, the HDAC inhibitor not only increased histone acetylation levels, as might be expected from an HDAC inhibitor, but it also initiated a cascade of secondary histone PTMs such as changes in the phosphorylation and methylation levels of histone molecules. Another recent study showed that NaB administration regulates histone methylation and memory formation in the hippocampus after a fear conditioning task (Gupta et al., 2010). However, the precise contribution of histone methylation changes to memory consolidation is at present not clear.

Another interesting finding of our previous study is that inactivation of the basolateral complex of the amygdala (BLA) by local infusions of the β-adrenoceptor antagonist propranolol blocked the facilitating effect of systemic NaB administration on object recognition memory and blocked the NaB-induced changes in chromatin remodeling in the insular cortex. Another recent study demonstrated that the HDAC inhibitor trichostatin A (TSA) increased inhibitory avoidance memory when injected after the training into the hippocampus and that BLA inactivation by the GABA-receptor agonist muscimol blocked this TSA-induced influence on memory formation (Blank et al., 2014). These findings indicating that BLA activity is required to enable the effect of HDAC administration on both memory enhancement and chromatin remodeling in the insular cortex and hippocampus are in agreement with a large and consistent literature indicating that BLA neural activity, normally induced during emotionally arousing conditions, can modulate memory consolidation processes by influencing neural plasticity changes in different brain regions (McGaugh, 2000; Roozendaal and McGaugh, 2011).

The present study investigated further whether systemic NaB administration after object recognition training can alter histone methylation levels in different subdivisions of the medial prefrontal cortex (mPFC) and whether these effects depend on BLA noradrenergic activity. Several recent findings have indicated that the mPFC is not only involved in higher cognitive functions such as executive control but also appears to be critically involved in the regulation of memory consolidation (Akirav and Maroun, 2006; Roozendaal et al., 2009; Barsegyan et al., 2010). Moreover, the mPFC has been shown to be involved in regulating memory of object recognition training (Akirav and Maroun, 2006; Barsegyan et al., unpublished observation). Other findings indicate
that the mPFC and BLA critically interact in regulating memory consolidation for emotionally arousing training experiences (Roozendaal et al., 2009) as well as in influencing performance on other affectively motivated tasks (Timms, 1977; Pérez-Jarayan and Vives, 1991; Garcia et al., 1999; Quirk and Gehlert, 2003; Roozendaal et al., 2004). Here, we investigated the effect of systemic NaB administration immediately after object recognition training on three different methylation marks within the mPFC based on the diversity of their function. However, as we previously found that NaB altered acetylation and phosphorylation of histone H3, these different investigated methylation marks were also located on histone H3. We examined tri-methylation of histone H3 K4 (3meH3K4), which is associated with gene transcription (Lee et al., 2010) tri-methylation of H3 K27 (3meH3K27), which has been associated with developmental repression of gene transcription (Kouzarides, 2007), and di-methylation of H3 at lysine 9 (2meH3K9) which has been demonstrated to be a repressive mark of transcription (Horn and Peterson, 2006).

MATERIALS AND METHODS

Subjects

Male adult Sprague-Dawley rats (280–320 g at 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 12-h:12-h light:dark cycle (lights on: 7:00 – 19:00 h) with ad libitum access to food and water. Training was performed during the light phase of the cycle between 10:00 and 15:00 h. 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.

Surgery

Rats, adapted to the vivarium for 1 week, were anesthetized with a subcutaneous injection of ketamine (37.5 mg/kg of body weight; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion) and received the non-steroidal analgesic carprofen (4 mg/kg; Pfizer). Oxygen (35%) mixed with ambient air was administered during surgery such that blood oxygenation levels would not drop below 90% (Fornari et al., 2012). The rats were positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA), and two stainless-steel guide cannulae (15 mm; 23 gauge; SKU Solutions, Fort Meade, FL) were implanted bilaterally with the cannula tips 2.0 mm above the skull surface; incisor bar: −3.3 mm from interaural. The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (15-mm-long 00 insect dissection pins) were inserted into each cannula to maintain patency. After surgery, the rats were administered atipamezole hydrochloride (0.25 mg/kg sc; Orion) to reverse anesthesia and were subsequently injected with 3 ml of sterile saline to facilitate clearance of drugs and prevent dehydration. The rats were allowed to recover for a minimum of 10 days prior to training and were handled for 1-2 min per day for 5 days preceding the training day.

Object Recognition Training Procedure

The experimental apparatus was a gray open-field box (in cm: 40w × 40d × 40h) with the floor covered with sawdust and placed in a dimly illuminated room. The objects to be discriminated were transparent glass vials (5.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). The rats were not habituated to the experimental context prior to the training trial. Previously, we have shown that this produces novelty-induced arousal during the training (Okuda et al., 2004). On the training trial, each rat was placed individually in the training apparatus at the opposite end from the objects and was allowed to explore two identical objects (A1 and A2) for 3 min, which by itself is insufficient to induce long-lasting memory of the objects (Okuda et al., 2004; Bermudez-Rattoni et al., 2005; Roozendaal et al., 2006, 2008). The two different objects were used in a balanced manner to reduce potential biases due to preference for particular objects. Rats’ behavior was recorded with a video camera positioned above the experimental apparatus. Videos were analyzed off-line by a trained observer blind to treatment condition. The total time spent exploring the two objects during the training trial was taken as a measure of object exploration. Exploration of an object was defined as pointing the nose to the object at a distance of <1 cm and/or touching it with the nose. Turning around, climbing or sitting on an object was not considered as exploration. Rats showing a total exploration time of <10 s on the training trial were removed from analyses, because previous findings indicated that such rats do not acquire the task (Okuda et al., 2004). To avoid the presence of olfactory cues, the sawdust was stirred and the objects were cleaned with 70% ethanol after each animal.

Local and systemic drug administration

Immediately after object recognition training, rats received bilateral BLA infusions of saline or the β-adrenoceptor antagonist propranolol (0.3 µg in 0.2 µl; Sigma-Aldrich), dissolved in saline (Roozendaal et al., 2006). For infusions, animals were gently restrained and bilateral infusions of drug or an equivalent volume of saline were administered into the BLA via 30-gauge injection needles connected to 10-µl Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 2.0 mm beyond the cannula tips and a 0.2-µl injection volume per hemisphere was infused over a period of 30 s by an automated syringe pump (Stoelting Co. Dublin Ireland). The injection needles were retained within the cannulae for an additional 20 s 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 drug infusions into the adjacent central amygdala do not affect memory consolidation (Roozendaal and McCaugh, 1996; 1997).

The HDAC inhibitor NaB (0.4 g/kg; Sigma-Aldrich) or saline was administered intraperitoneally, in a volume of 2 ml/kg, immediately after the training trial (Kwon and Houpt, 2010). All drug solutions were freshly prepared before each experiment.

Prefrontal Cortex Tissue Collection and Histone Preparation

Rats were deeply anesthetized with an overdose of pentobarbital (100 mg/kg, ip) 1 h after training and drug treatment. Within 90 s after the pentobarbital injection, the rats were decapitated, the brains rapidly removed and flash frozen by submersion for 2 min in a beaker filled...
with pre-cooled isopentane on dry ice. Flash-frozen brains were stored at -80°C until tissue processing. The anterior part of the brain was cut on a cryostat into 350-µm-thick coronal slices for mPFC tissue collection. The rest of the brain, containing the BLA, was immersed in 4% formaldehyde for at least 3 days, and then transferred to a 30% sucrose solution for cryoprotection. Coronal sections of the BLA of 50 µm were cut on a cryostat, collected on gelatin-coated slides, and fixed in 100% acetic acid before staining with cresyl violet. The sections were examined under a light microscope and determination of the location of injection needle tips in the BLA was made according to the atlas plates of Paxinos and Watson (2007) by an observer blind to drug treatment condition. Rats with injection needle tip placements outside the BLA or with extensive tissue damage at the injection needle site were removed from analyses.

The prelimbic (PL) and the infralimbic (IL) subregions of the mPFC were dissected from frozen 350-µm-thick coronal slices using a 1.0-mm brain puncher (Stoelting Co.). Bilateral punches from three consecutive slices (approximately A2, +2.20 to +1.0 mm relative to Bregma) to a total of 6 punches were taken separately from the PL and the IL cortex. Histones were isolated according to the Levenson et al. (2004) protocol with some modifications as outlined in chapter 1. All procedures were performed on ice and all solutions and centrifugations were chilled to 4°C prior to use. Tissue was homogenized in 100 µl of hypotonic lysis buffer [250 mM sucrose, 50 mM Tris, 25 mM KCl, 1 M HCl (Rodriguez-Collazo et al., 2009)] for 1 h on ice and vortexed every 10 min, and then centrifuged at 16,000 × g for 15 min. The supernatant was transferred into a fresh tube and 30 µl of trichloroacetic acid containing 4 mg/ml deoxycholic acid was added to precipitate the proteins for 15 min and then centrifuged at 16,000 × g for 15 min. The supernatant was discarded and the pellet (nuclear fraction) was resuspended in 100 µl of 0.2 M NaB, pH 7.5 and grinded for 10 s. The homogenate was centrifuged at 7,800 × g for 1 min. The supernatant was removed and the pellet (nuclear fraction) was resuspended in 30 µl of 0.2 M NaB (Rodriguez-Collazo et al., 2009) for 1 h on ice and vortexed every 10 min, and then centrifuged at 16,000 × g for 15 min. The supernatant was transferred into a fresh tube and 30 µl of trichloroacetic acid containing 4 mg/ml deoxycholic acid was added to precipitate the proteins for 15 min and then centrifuged at 16,000 × g for 15 min. The supernatant was discarded and the pellet was washed with 100 µl of ice-cold acidified acetone (0.1% HCl) for 5 min and then centrifuged at 16,000 × g for 5 min, washed again with 100 µl of ice-cold 100% acetone for 5 min, and centrifuged at 16,000 × g for 5 min. Finally, the supernatant was removed and the remaining histone pellet was dried for 15 min for the remaining acetone to evaporate. The pellet was resuspended in 30 µl of 50 mM Tris (pH 8.0) and sample buffer (5x) was added to prevent over-dilution of the samples. The samples were then boiled and 10-µl aliquots were stored at -80°C to protect histones from degradation (Rumbaugh and Miller, 2011).

Western Blotting
Histone samples were run on a discontinuous polyacrylamide gel consisting of a 15% acrylamide resolving and a 4% acrylamide stacking gel. The gel was then blotted onto a polyvinylidene difluoride (PVDF) membrane for immunoblotting (Millipore). The membranes were blocked for 1 h in blocking buffer (LI-COR), diluted 1:1 in phosphate-buffered saline (PBS), then incubated in primary antibody overnight at 4°C followed by incubation with the appropriate secondary antibody for 2 h at room temperature. Primary and secondary antibodies were dissolved in the same blocking buffer. Band intensity was determined and quantified using an Odyssey IR scanner (LI-COR Biosciences). The blot was stripped and reprobed in total histone H3. Levels of 3meH3K4, 3meH3K27 and 2mH3K9 were normalized to total histone H3 levels for each sample.

Antibodies
The primary antibodies and their dilution were as follows: 3meH3K4 (1:1,000; Millipore), 3meH3K27 (1:2,000; Millipore), 2meH3K9 (1:1,000; Millipore), total H3 (1:2,000; Millipore). The secondary antibodies were goat anti-rabbit (1:25,000; LI-COR) and donkey anti-mouse (1:20,000; LI-COR).

Statistics
Data are expressed as mean ± SEM. Normalized histone PTM data are expressed as the percentage of the mean of the Saline-Saline control group and analyzed with two-way ANOVAs with systemic NaB injection and intra-BLA drug infusion as between-subject variables. When appropriate, additional independent-sample t-tests were performed between the two different groups sharing the same condition of the other variants, using the appropriate error term of the ANOVA as the variance. A probability level of < 0.05 was accepted as statistical significance for all tests. The number of rats per group is indicated in the figure legends.

RESULTS
In this experiment rats were trained on an object recognition task and given an immediate post-training systemic administration of a memory-enhancing dose of NaB either alone or together with intra-BLA infusions of the β-adrenergic antagonist propranolol. The rats were sacrificed 1 h after training and drug administration for mPFC tissue collection and histone isolation. The behavioral effects, indicating that NaB administration enhanced long-term memory and that BLA noradrenergic inhibition blocked this NaB effect, have been described in Chapter 3. In this Chapter we investigated the effect of these drug manipulations on three different methylation marks within the PL and IL cortex: Tri-methylation of histone H3 at lysine 4 (3meH3K4); tri-methylation of H3 at lysine 27 (3meH3K27) and di-methylation of H3 at lysine 9 (2meH3K9). An outline of the experimental procedure is shown in Figure 1A.
Effect of the HDAC inhibitor NaB and intra-BLA propranolol administration after object recognition training on histone 3meH3K4 levels in the PL and IL cortex

Figure 1 shows 3meH3K4 levels (normalized to total H3 levels) within the PL and IL cortex assessed 1 h after object recognition training and drug treatment. In the PL cortex, two-way ANOVA for 3meH3K4 levels showed no significant NaB effect ($F_{1,34} = 1.73, p = 0.20$) or propranolol effect ($F_{1,34} = 0.00, p = 0.99$) or interaction between the two parameters ($F_{1,34} = 0.89, p = 0.35$) (Figure 1B).

In the IL cortex, two-way ANOVA also revealed no NaB effect ($F_{1,32} = 2.63, p = 0.11$), propranolol effect ($F_{1,32} = 2.12, p = 0.15$) or interaction between the two factors ($F_{1,32} = 0.41, p = 0.52$) (Figure 1C). Thus, these findings indicate that 3meH3K4 levels within the PL and IL after object recognition training were not significantly altered by the NaB injection or intra-BLA propranolol infusion.

Effect of the HDAC inhibitor NaB and intra-BLA propranolol administration after object recognition training on histone 2meH3K9 levels in the PL and IL cortex

Figure 2 shows di-methylation levels at the H3K9 site (2meH3K9) in the PL and IL cortex as a result of systemic NaB administration and intra-BLA propranolol infusion 1 h after object recognition training.

As shown in Figure 2A, two-way ANOVA for 2meH3K9 levels in the PL revealed no NaB effect ($F_{1,32} = 0.19, p = 0.89$), propranolol effect ($F_{1,32} = 0.67, p = 0.42$) or interaction between the two factors ($F_{1,32} = 0.84; p = 0.37$).

In the IL cortex, two-way ANOVA for 2meH3K9 levels also revealed no NaB effect ($F_{1,32} = 0.06, p = 0.81$; Figure 2B). However, there was a significant propranolol effect ($F_{1,32} = 5.56, p = 0.05$), but no interaction between NaB and propranolol treatment ($F_{1,32} = 1.53, p = 0.22$). Post hoc comparison tests indicated that the propranolol infusion alone after training significantly increased 2meH3K9 levels ($t_a = 2.34, p < 0.05$) relative to the Saline-Saline control group. The propranolol infusion did not significantly increase 2meH3K9 levels in animals that were also given NaB systemically ($t_a = 0.81, p = 0.42$).
**Effect of the HDAC inhibitor NaB and intra-BLA propranolol administration after object recognition training on histone 3meH3K27 levels in the PL and IL cortex**

Figure 3 shows 3meH3K27 levels in PL (A) and IL (B). The gene repressor 3meH3K27 did not vary in either the PL or the IL cortex and irrespective of treatment with NaB and/or propranolol.

For the PL cortex, two-way ANOVA for 3meH3K27 levels did not reveal a significant NaB effect ($F_{1,36} = 3.25, p = 0.08$), propranolol effect ($F_{1,36} = 0.54, p = 0.47$) or interaction between both factors ($F_{1,36} = 1.44, p = 0.23$). Similarly, two-way ANOVA for 3meH3K27 levels in the IL cortex also did not reveal a significant NaB ($F_{1,36} = 0.04, p = 0.84$), propranolol ($F_{1,36} = 0.10, p = 0.76$) or NaB x propranolol interaction effect ($F_{1,36} = 0.28, p = 0.56$).

DISCUSSION

In the present study we addressed the question as to whether a memory-enhancing dose of NaB administered systemically after object recognition training altered histone methylation levels in the PL and IL subregions of the mPFC, and whether BLA noradrenergic activity is necessary for enabling such alterations. We chose to look at histone methylation marks because of previous findings indicating that NaB not only induces changes in histone acetylation levels, but also influences histone methylation in the hippocampus 1 h after contextual fear conditioning (Gupta et al., 2010) and in the insular cortex after object recognition memory (Chapter 3). Our findings indicate that NaB administration did not significantly alter histone methylation levels in the PL or IL, but that the BLA propranolol infusion significantly increased levels of 2meH3K9 in the IL.

In Chapter 3 we showed that treatment with the HDAC inhibitor NaB immediately after object recognition training enhanced 24 h retention. Interestingly, the facilitating effect of NaB on memory was entirely blocked when BLA noradrenergic activity was inhibited with the $\beta$-adrenoceptor antagonist propranolol. At the molecular level, we focused in this previous study on the insular cortex, a brain region critically involved in object recognition memory (Bermudez-Rattoni et al., 2005), and found that NaB treatment increased histone H3 acetylation at lysine 14 (acH3K14) and phosphorylation of histone H3 at lysine 10 (pH3S10) but decreased histone methylation at lysine 27 in the insular cortex. Moreover, we found that inactivation of the BLA with the $\beta$-adrenoceptor antagonist propranolol did not have any effect on acetylation of histone H3K14, but blocked the effect of NaB on pH3S10 and 3meH3K27 levels. Thus, these findings indicate that that BLA noradrenergic activity is necessary to mediate the memory-enhancing effects of NaB administration and also blocked, at least in part, the molecular changes within the IC induced by NaB. Another interesting finding of our previous study was that NaB did not only alter acetylation levels but also induced a cascade of subsequent secondary changes, such as phosphorylation and methylation of histone tails. In fact, such cross-regulation between histone acetylation and histone methylation has been well documented and might be required for regulating neuroplasticity changes (Latham and Dent, 2007). Moreover, we demonstrated in Chapter 3 that the NaB administration did not induce any changes in histone PTMs in the insular cortex in non-trained home cage control rats, indicating that the NaB effect requires the training experience or the emotional arousal response associated with the training.

In the present study, we found that the memory-enhancing effect of NaB did not induce any significant changes in methylation levels within the mPFC. Therefore, it is appears that NaB did not induce any chromatin changes in this brain region in the context of object recognition training. Future studies should also examine the effect of NaB administration on acetylation levels within mPFC after object recognition training. However, it is also possible that this result is a false negative due to insufficient statistical power. When the effect of systemic NaB versus saline was analyzed alone, thus not taking into account the BLA manipulation, we found that NaB significantly increased 3meH3K4 (two-tailed t-test: $p = 0.02$) levels in the PL. The effect was not seen in the IL. In rats with an inactivated BLA, the NaB administration did not change 3meH3K27 levels in the PL, however it is close to significant (two-tailed t-test: $p = 0.06$). Interestingly, and consistent with these findings, Gupta et al. (2010) have shown increased 3meH3K4 levels in the CA1 subregion of the hippocampus when NaB was administered 1 h before fear conditioning, a result that was correlated with an increased fear...
memory. Currently, novel classes of drugs are being developed that can directly increase or inhibit methylation levels of particular histone sites. Future studies should employ direct administration of such drugs in order to assess the causal involvement of histone methylation changes within these and other brain regions in mechanisms of neural plasticity and memory consolidation.

The NaB administration did not change 2meH3K9 levels in the PL or IL, nor when the NaB effect was analyzed in isolation. Gupta et al. (2010) also investigated the effect of NaB administration on methylation of this histone site after fear conditioning and reported a decrease in 2meH3K9 levels in the CA1 in rats treated with NaB 1 h before fear conditioning. Thus, these findings do suggest that NaB administration via its primary effects on histone acetylation is able to induce changes in histone methylation at this particular site. Therefore, the absence of a significant NaB effect on 2meH3K9 levels in the present study suggests that changes in this histone tail within the mPFC might not play a significant role in mediating the modulatory effects of NaB on object recognition memory.

However, we further found that inactivation of β-adrenoceptor activity within the BLA significantly increased immunoreactivity of the 2meH3K9 in the IL but not in the PL. In the present study, rats were only trained for 3 min on the object recognition task, which is insufficient to induce long-term memory of the training (Roozendaal et al., 2008), and thus the propranolol infusion did not have a significant behavioral effect. However, in general, noradrenergic blockade of the BLA is known to exert impairing effects on memory consolidation. Therefore the increased 2meH3K9 levels after propranolol administration are consistent with the findings by Gupta et al. indicating that 2meH3K9 might have a memory-suppressing action.

Thus, our findings indicate that NaB administration after 3 min object recognition training had no significant effect on the histone methylation marks investigated in the PL or IL cortex. On the other hand, BLA noradrenergic inactivation induced a significant increase in 2meH3K9 levels within the IL, but had no effect within the PL. Very little is known about the role of mPFC sub-regions in memory consolidation of object recognition training and even less is known about the role of methylation marks within the mPFC (or other brain regions) related to memory formation. The only study that investigated histone methylation marks on memory is the one by Gupta et al. (2010) investigating the effect of NaB after aversive fear conditioning. However, as some evidence indicates that stressful experiences can also influence histone methylation (Hunter et al., 2009) it is possible that the differential effects between this prior study and the present one are attributable to differences in the aversiveness of the respective learning tasks. Further, most studies investigating the roles of the PL and IL have examined their effect on fear memory. A major role of the PL, rather than IL, cortex in has been shown in the expression of conditioned fear memory (Sierra-Mercado et al., 2011). On the other hand, the IL cortex has been primarily implicated in the consolidation of fear extinction and inhibitory learning (Milad and Quirk, 2002; Laurent and Westbrook, 2009). As indicated, little is known about a possible differential involvement of these two subdivisions in object recognition memory. In one study, (Akirav and Maroun, 2006) showed that protein synthesis and NMDA receptor activity are required for consolidation of recognition memory in the ventromedial part of the PFC. Interestingly, the infusion sites were mainly in the IL cortex, but it cannot be excluded that the drugs diffused up the cannula track and had their effect in the PL. In another study, lesions of either the PL or IL did not disturb object recognition memory, whereas selective IL lesions did disrupt performance on an hippocampus-dependent object location task (Nelson et al., 2011). In an ongoing study, we found evidence for a more prominent role of the PL in memory consolidation of object recognition. We found that direct infusions of a glucocorticoid receptor agonist into the PL cortex immediately after object recognition training enhanced long-term memory of both the object per se (object recognition) as well as the location of the object during training (object location), whereas similar infusions into the IL cortex were ineffective (Barsegyan et al., unpublished observations). Moreover, simultaneous propranolol infusions into the BLA blocked the memory-enhancing effect of the PL drug manipulation. Thus, these findings clearly show the necessity of further examination the specific contribution of the PL and IL, and the role of histone modifications and neural plasticity changes in object recognition memory.
REFERENCES

NORADRENERGIC ACTIVATION OF THE BASOLATERAL AMYGDALA ENHANCES OBJECT RECOGNITION MEMORY AND INDUCES CHROMATIN REMODELING IN THE INSULAR CORTEX

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CHAPTER 5

ABSTRACT

It is well established that arousal-induced memory enhancement requires noradrenergic activation of the basolateral complex of the amygdala (BLA) and modulates influences on information storage processes in its many target regions. While this concept is well accepted, the molecular basis of such BLA effects on neural plasticity changes within other brain regions remains to be elucidated. The present study investigated whether noradrenergic activation of the BLA after object recognition training induces chromatin remodeling through histone post-translational modifications in the insular cortex (IC), a brain region that is importantly involved in object recognition memory. Male Sprague-Dawley rats were trained on an object recognition task, followed immediately by bilateral microinfusions of norepinephrine (1.0 µg) or saline administered into the BLA. Saline-treated control rats exhibited poor 24-h retention, whereas norepinephrine treatment induced robust 24-h object recognition memory. Most importantly, this memory-enhancing dose of norepinephrine induced a global reduction in the acetylation levels of histone H3 at lysine 14, H2B and H4 in the IC 1 h later, whereas it had no effect on the phosphorylation of histone H3 at serine 10 or tri-methylation of histone H3 at lysine 27. Norepinephrine administered into the BLA of non-trained control rats did not induce any changes in the histone marks investigated in this study. These findings indicate that noradrenergic activation of the BLA induces training-specific effects on chromatin remodeling mechanisms, and presumably gene transcription, in its target regions, which may contribute to the understanding of the molecular mechanisms of stress and emotional arousal effects on memory consolidation.

INTRODUCTION

Enhanced memory for emotionally arousing events is a well-recognized phenomenon, which has obvious adaptive value in evolutionary terms, as it is vital to remember both dangerous and favorable situations (Roozendaal and McGaugh, 2011). Extensive evidence indicates that noradrenergic activation of the basolateral complex of the amygdala (BLA) is critically involved in mediating emotional arousal effects on memory enhancement by influencing synaptic plasticity and information storage processes in other brain regions (Introini-Collison et al., 1991; Ferry et al., 1999; Hatfield and McGaugh, 1999; Roozendaal et al., 2002, 2009; Lalumiere et al., 2003; Huff et al., 2005; Barsegyan et al., 2014). Noradrenergic activation of the BLA also enhances the consolidation of low-arousing object recognition memory (Roozendaal et al., 2008), a naturalistic task based on the spontaneous tendency of rodents to explore a novel object more than a familiar one (Ennaceur and Delacour, 1988). Memory for object recognition training is known to depend on synaptic plasticity changes within the perirhinal cortex (Albasser et al., 2009; Barker and Warburton, 2011) and insular cortex (IC; Bermúdez-Rattoni et al., 2005; Balderas et al., 2008; Roozendaal et al., 2010; Bermúdez-Rattoni, 2014). For example, long-term memory of an object, but not of the location of the object, is impaired when the protein-synthesis blocker anisomycin is applied into either the perirhinal cortex or IC, whereas short-term memory is not affected (Balderas et al., 2008). Considerable evidence indicates that the IC, which has traditionally been investigated mostly with respect to its involvement in taste memory (Berman et al., 2000; Bermúdez-Rattoni, 2004; Shema et al., 2007; Núñez-Jaramillo et al., 2010; Stehberg et al., 2011), is an important node of the rodent brain network involved in emotional regulation of learning and memory (Bermúdez-Rattoni and McGaugh, 1991; Bermúdez-Rattoni et al., 1991; Nerd et al., 1996; Fornari et al., 2012b). The IC is also densely interconnected with the BLA (McDonald and Jackson, 1987; Paré et al., 1995; Shi and Cassel, 1998) and several studies indicated functional interactions between these two brain regions (Escobar et al., 1998; Rodríguez-Durán et al., 2011; Moraga-Amaro and Stehberg, 2012). Critically, the finding that noradrenergic blockade of the BLA prevents the effect of drug administration into the IC on conditioned taste aversion as well as inhibitory avoidance memory (Miranda and McGaugh, 2004), provides important support for the view that noradrenergic activity of the BLA regulates neural plasticity and memory consolidation processes within this brain region.

Whereas the behavioral consequences of noradrenergic activity of the BLA on memory consolidation are well established, the molecular mechanism(s) underlying this BLA influence on information storage processes in efferent brain regions are yet to be determined. During the last decade new investigations concerning the mechanism of gene expression have shed light on different forms of epigenetic modifications, i.e., histone post-translational modifications (PTMs), DNA methylation and non-coding RNAs, that are involved in learning and memory (Levenson et al., 2004; Miller et al., 2008, 2010; Stefanko et al., 2009; Gupta et al., 2010; Roozendaal et al., 2010; Redlon et al., 2011). An impressive body of literature indicates that the chromatin state through histone PTMs, such as acetylation, phosphorylation or methylation of histone tails, must be altered to allow for changes in gene expression related to memory consolidation (Levenson et al., 2004; Chwang et al., 2006; Koshibu et al., 2009; Stefanko et al., 2009; Gupta et al., 2010; Roozendaal et al., 2010; Haettig et al., 2011; Kye et al., 2011; Graff et al., 2012; Griggs et al., 2013). In a prior study,
we reported that inducing a histone hyperacetylated state within the IC with local posttraining infusions of the histone deacetylase (HDAC) inhibitor sodium butyrate (NaB) enhanced memory of object recognition training (Roozendaal et al., 2010). Here, we investigated whether a memory-enhancing dose of norepinephrine administered into the BLA after object recognition training triggers chromatin modifications within the IC. We examined different histone PTM marks that have been reported to be involved in learning and memory and/or stress adaptation such as acetylation of histone H3 at lysine 14 (acH3K14), acetylation of histone H2B (acH2B), acetylation of histone H4 (acH4), phosphorylation of histone H3 at serine 10 (pH3S10) and tri-methylation of histone H3 at lysine 27 (3meH3K27) (Chwang et al., 2006; Fischer et al., 2007; Hunter et al., 2009; Koshibui et al., 2009, 2011; Bousiges et al., 2010; Gräff et al., 2012). To determine whether the norepinephrine effect on histone PTMs depends on the object recognition training experience, changes in these histone marks were also assessed after norepinephrine administration into the BLA of non-trained control rats. Furthermore, as some findings indicated an important role for extracellular signal-regulated kinase 1/2 (ERK1/2) protein in regulating histone PTMs in memory formation (Levenson et al., 2004; Chwang et al., 2006; Chwang et al., 2008; Gutierrez-Mecinas et al., 2011; Mifsud et al., 2011), we also investigated whether noradrenergic activation of the BLA after object recognition training changes the phosphorylation status of ERK1/2 in the IC.

MATERIALS AND METHODS

Subjects
Male adult Sprague-Dawley rats (280–320 g at 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 12-h:12-h light:dark cycle (lights on: 7:00–19:00 h) with ad libitum access to food and water. Training and testing were performed during the light phase of the cycle between 10:00 and 15:00 h. 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, Netherlands.

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Rats, adapted to the vivarium for 1 week, were anesthetized with a subcutaneous injection of ketamine (37.5 mg/kg of body weight; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion) and received the non-steroidal analgesic carprofen (4 mg/kg; 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 rats were positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA), and two stainless-steel guide cannulae (15 mm; 23 gauge; Component Supply Co/SKU Solutions, Fort Meade, FL) were implanted bilaterally with the cannula tips 2.0 mm above the BLA. The coordinates were based on the atlas of Paxinos and Watson (2007): anteroposterior (AP), -2.8 mm from Bregma; mediolateral (ML), ±5.0 mm from the midline; dorsoventral (DV), -6.5 mm from skull surface; incisor bar: -3.3 mm from interaural. The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (15-mm-long 00 insect dissection needles) protruded 2.0 mm beyond the cannula tips and a 0.2-µl injection volume per hemisphere was infused over a period of 30 s by an automated syringe pump (Stoelting Co., Dublin, Ireland).

Object Recognition Training and Testing Procedures
The experimental apparatus was a gray open-field box (in cm: 40 w × 40 d × 40 h) with the floor covered with sawdust and placed in a dimly illuminated room. The objects to be discriminated were transparent glass vials (5.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). The rats were not habituated to the experimental context prior to the training trial. Previously, we have shown that this produces novelty-induced arousal during the training (Okuda et al., 2004). On the training trial, each rat was placed individually in the training apparatus at the opposite end from the objects and was allowed to explore two identical objects (A1 and A2) for 3 min, which by itself is insufficient to induce long-lasting memory of the objects (Okuda et al., 2004; Bermúdez-Rattoni et al., 2005; Roozendaal et al., 2006, 2008). Rats’ behavior was recorded with a video camera positioned above the experimental apparatus. Videos were analyzed off-line by a trained observer blind to treatment condition. The total time spent exploring the two objects during the training trial was taken as a measure of object exploration. Rats showing a total exploration time of <10 s on the training trial were removed from analyses, because previous findings indicated that such rats do not acquire the task (Okuda et al., 2004). To avoid the presence of olfactory cues, the sawdust was stirred and the objects were cleaned with 70% ethanol after each animal.

Some rats were sacrificed for tissue collection 1 h after training and immediate posttraining drug treatment. Other rats were tested for retention 24 h after the training trial. During the retention test, one copy of the familiar object (A3) and a new object (B) were placed in the same location as stimuli during the training trial. All combinations and locations of objects were used in a balanced manner to reduce potential biases due to preference for particular locations or objects. The rat was placed in the experimental apparatus for 3 min and the time spent exploring each object and the total time spent exploring both objects were recorded. Exploration of an object was defined as pointing the nose to the object at a distance of <1 cm and/or touching it with the nose. Turning around, climbing or sitting on an object was not considered as exploration. In order to analyze cognitive performance, a discrimination index was calculated as the difference in time exploring the novel and familiar object, expressed as the ratio of the total time spent exploring both objects (i.e., [Time Novel – Time Familiar]/Time Novel + Time Familiar)×100%

Drug Administration
Norepinephrine (1.0 µg; Sigma-Aldrich) was dissolved in saline and administered into the BLA immediately after the object recognition training trial (Roozendaal et al., 2008). Bilateral infusions of drug or an equivalent volume of saline were administered into the BLA via 30-gauge injection needles connected to 10-µl Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 2.0 mm beyond the cannula tips and a 0.2-µl injection volume per hemisphere was infused over a period of 30 s by an automated syringe pump (Stoelting Co., Dublin, Ireland).
The injection needles were retained within the cannulae for an additional 20 s 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 drug infusions into the adjacent central amygdala do not affect memory consolidation (Roozendaal and McGaugh, 1996, 1997). Drug solutions were freshly prepared before each experiment.

**Cannula Placement Verification**

After object recognition memory testing, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, ip) and perfused transcardially with 0.9% saline followed by 4% formaldehyde. After decapitation, the brains were removed and immersed in fresh 4% formaldehyde. At least 24 h before sectioning, the brains were transferred to a 30% sucrose solution in saline for cryoprotection. Coronal sections of 50 μm 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 BLA was made according to the atlas plates of Paxinos and Watson (2007) by an observer blind to drug treatment condition. Ten rats with injection needle tip placements outside the BLA or with extensive tissue damage at the injection needle site were removed from behavioral analyses.

**Insular Cortex Tissue Collection and Histone Preparation**

Rats for the molecular investigations were deeply anesthetized with an overdose of pentobarbital (100 mg/kg, ip) 1 h after training and drug treatment. Within 90 s after the pentobarbital injection, the rats were decapitated, the brains rapidly removed and flash frozen by submersion for 2 min in a beaker filled with pre-cooled isopentane on dry ice. Flash-frozen brains were stored at −80°C until tissue processing. The anterior part of the brain was cut on a cryostat into 350-μm-thick coronal slices for IC tissue collection. The rest of the brain, containing the BLA, was immersed in 4% formaldehyde for at least 3 days, and then transferred to a 30% sucrose solution for cryoprotection. Coronal sections of 50 μm were cut on a cryostat, collected on gelatin-coated slides, and fixed in 100% acetone before staining with cresyl violet. Determination of injection needle placement within the BLA was performed as previously described. Fifteen rats for the molecular experiments were removed from analyses on histological grounds.

IC tissue was dissected from frozen 350-μm-thick coronal slices using a 1.25-mm brain puncher (Stoelting Co., Dublin, Ireland). Bilateral punches from the anterior IC were collected from three rats for the molecular experiments. Fifteen rats for the molecular experiments were deeply anesthetized with an overdose of pentobarbital (100 mg/kg, ip) 1 h after training and drug treatment. Within 90 s after the pentobarbital injection, the rats were decapitated, the brains rapidly removed and flash frozen by submersion for 2 min in a beaker filled with pre-cooled isopentane on dry ice. Flash-frozen brains were stored at −80°C until tissue processing. The anterior part of the brain was cut on a cryostat into 350-μm-thick coronal slices for IC tissue collection. The rest of the brain, containing the BLA, was immersed in 4% formaldehyde for at least 3 days, and then transferred to a 30% sucrose solution for cryoprotection. Coronal sections of 50 μm were cut on a cryostat, collected on gelatin-coated slides, and fixed in 100% acetone before staining with cresyl violet. Determination of injection needle placement within the BLA was performed as previously described. Fifteen rats for the molecular experiments were removed from analyses on histological grounds.

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**Western Blotting**

Histone samples (10 μl) or equal protein concentrations of cytoplasmic samples (5 μg) for pERK1/2 identification were resolved on a discontinuous polyacrylamide gel consisting of a 20% acrylamide resolving gel for histone proteins, or 10% acrylamide resolving gel for ERK1/2 protein, and 4% acrylamide stacking gel. The gel was then blotted onto a polyvinylidene difluoride (PVDF) membrane for immunoblotting (Millipore, Amsterdam, Netherlands). The membranes were blocked for 1 h in blocking buffer (LI-COR Biosciences, Bad Homburg, Germany), diluted 1:1 in phosphate-buffered saline (PBS) [or Tris-buffered saline (TBS) for phospho-antibodies], then incubated with primary antibody overnight at 4°C, followed by incubation with the appropriate secondary antibody for 2 h at room temperature. Primary and secondary antibodies were dissolved in the same blocking buffer. Band intensity was determined and quantified using an Odyssey IR scanner (LI-COR Biosciences). The blots were then stripped and re-probed with antibody against total histone H3 (Levenson et al., 2004) or total mitogen-activated protein kinase (MAPK). Levels of acH3K14, acH2B, acH4, pH3S10 and 3meH3K27 were normalized to total histone H3 levels and pERK1/2 levels were normalized to total MAPK levels for each sample (Patterson et al., 2001; Chwang et al., 2006).

**Antibodies**

The primary antibodies and their dilutions are: acetylated histone H3 at lysine 14 (acH3K14; 1:1,000; Millipore), acetylated histone H2B (acH2B; 1:2,000; Millipore), acetylated histone H4 (acH4; 1:3,000; Millipore), phosphorylated histone H3 at serine 10 (pH3S10; 1:1,000; Millipore), tri-methylated histone H3 at lysine 27 (3meH3K27; 1:2,000; Millipore), total H3 (1:2,000; Millipore), phospho-p44/42 MAPK (1:2,000; Cell Signaling Technology, Leiden, Netherlands) and p44/42 MAPK (1:2,000; Cell Signaling Technology). The secondary antibodies were goat anti-rabbit (1:25,000; LI-COR Biosciences) and donkey anti-mouse (1:20,000; LI-COR Biosciences).

**Statistics**

Data are expressed as mean ± SEM. The discrimination index and total object exploration time were analyzed with unpaired t-tests. One-sample t-tests were used to determine whether the discrimination index was different from zero (i.e., chance level) and thus whether memory was expressed. Normalized histone PTM and pERK1/2 data are expressed as the percentage of the mean of the saline-treated home cage control group and analyzed with two-way ANOVAs with training
RESULTS
Posttraining Norepinephrine Administration into the BLA Enhances the Consolidation of Object Recognition Memory

We first examined the effect of norepinephrine administration into the BLA after object recognition training on memory consolidation. For this, rats were trained on the object recognition task for 3 min and immediately after the training trial given bilateral infusions of norepinephrine (1.0 µg in 0.2 µl) or saline into the BLA. To determine whether animals exhibit a long-term memory for the object seen during the training trial, rats were given a 24-h retention test in which one object was familiar and the other object was novel. If the animal generates a long-term memory for the familiar object, it will spend significantly more time exploring the novel object during the retention test. Figure 1A shows a schematic diagram of the experimental design.

Moreover, one-sample t-test indicated that norepinephrine-treated rats exhibited a significant exploration preference for the novel object ($t_{8} = 10.78; p < 0.0001$). The total time spent exploring the two objects during the retention test trial did not differ between groups ($p = 0.31$; Figure 1D), indicating that the drug infusion did not induce a general change in the rats’ incentive to explore the objects.

Figure 1 shows a representative photomicrograph of an infusion needle tip terminating within the BLA as well as the histological analyses of the infusion needle tip placements of all rats included in the analysis.

Norepinephrine treatment immediately after the training trial enhanced long-term memory for the familiar object. As shown in Figure 1B, the total time spent exploring the two identical objects during the 3-min training trial, before drug treatment, did not differ between groups ($p = 0.65$). Figure 1C shows the discrimination index during the 24-h retention test trial. One-sample t-test revealed that the discrimination index of saline-treated control rats did not differ significantly from zero (i.e., chance level; $t_{7} = -0.16; p = 0.88$), indicating that they did not show any evidence of retention of the training. Norepinephrine administration into the BLA immediately after object recognition training significantly enhanced the discrimination index ($p < 0.01$).

Figure 1. Norepinephrine administration into the BLA enhances the consolidation of object recognition memory. (A) Diagram of the experimental procedure. Rats were trained for 3 min on an object recognition task followed immediately by bilateral intra-BLA infusions of norepinephrine (NE; 1.0 µg in 0.2 µl; $n = 9$) or saline (Sal; $n = 8$). (B) Total exploration time during the 3-min training trial, before drug treatment, did not differ between groups. (C) Norepinephrine significantly increased the discrimination index on the 24-h retention test. (D) Total exploration time during the retention test did not differ between groups. Data are shown as mean ± SEM. **$p < 0.01$.

Figure 2. Histological analyses. (A) Representative photomicrograph illustrating placement of a cannula and needle tip terminating in the BLA and a diagram representing the different nuclei of the BLA, the lateral nucleus (L), basal nucleus (B), and accessory basal nucleus (AB), and central nucleus (CEA). Arrow points to needle tip. (B) The location of needle tips within the BLA of all rats included in the analysis for the behavioral study. (C) The location of needle tips within the BLA of all rats included in the analysis for the molecular study.
A memory-enhancing dose of norepinephrine administered into the BLA after object recognition training reduces histone acetylation levels in the IC

To determine whether this memory-enhancing dose of norepinephrine administered into the BLA after object recognition training triggers changes in the chromatin state in the IC, we examined changes in the following histone markers: acetylation of histone H3 at lysine 14 (acH3K14), acetylation of histone H2B (acH2B) and acetylation of histone H4 (acH4), as well as phosphorylation of histone H3 at serine 10 (pH3S10) and tri-methylation of histone H3 at lysine 27 (3meH3K27). Some rats received norepinephrine (1.0 µg in 0.2 µl) or saline into the BLA immediately after 3 min of object recognition training. Other groups of rats received the same drug infusions without training. For both groups, changes in histone markers in the IC were assessed 1 h after drug treatment. Changes in histone PTMs, normalized to total histone H3 levels, are shown as percentage (mean ± SEM) relative to saline-treated home cage control rats. We also investigated whether the norepinephrine administration altered pERK1/2 levels in the IC 1 h after training. Changes in pERK1/2 levels, normalized to total MAPK levels, are also shown as percentage of the saline-treated home cage control rats. Figure 2C shows histological analysis of injection needle tip placement in the BLA of all rats included in the analysis for the molecular experiments. Total exploration time of the two identical objects during the 3-min training trial, before drug treatment, did not differ between groups (p = 0.19).

Our findings indicate that this memory-enhancing dose of norepinephrine administered into the BLA after the training experience induced a global reduction in histone acetylation, whereas it did not alter the phosphorylation or methylation state of the histone molecules. pERK1/2 levels also remained unchanged. Norepinephrine administered into the BLA of home cage control animals did not induce any changes in histone PTMs or pERK1/2 levels. As shown in Figure 3A, two-way ANOVA for acH3K14 levels indicated no main norepinephrine (F_{1,41} = 1.10; N.S.) or training effect (F_{1,41} = 0.19; N.S.), but a significant interaction between both factors (F_{1,41} = 7.38; p < 0.01). Norepinephrine infused into the BLA of home cage control rats did not change acH3K14 levels in the IC 1 h later when compared to non-trained saline control rats (p = 0.67), indicating that the norepinephrine administration alone is insufficient to alter acH3K14 levels within the IC. The 3-min object recognition training session by itself, which is not sufficient to induce long-term memory, also did not significantly change acH3K14 levels when compared to home cage control rats (p = 0.14). However, norepinephrine infusions into the BLA after object recognition training significantly reduced acH3K14 levels in the IC when compared to saline-treated trained rats (p < 0.01) as well as when compared to norepinephrine-treated home cage control rats (p = 0.05). Thus, these findings indicate that norepinephrine selectively decreased acH3K14 levels in the IC in the context of object recognition training.

As shown in Figure 3B, two-way ANOVA for acH2B levels indicated a significant norepinephrine effect (F_{1,41} = 4.70; p < 0.05), no main training effect (F_{1,41} = 0.01; N.S.), but a significant interaction between both factors (F_{1,41} = 5.22; p < 0.05). Highly comparable to the effect on acH3K14, norepinephrine infused into the BLA of home cage control rats did not change acH2B levels within the IC (p = 0.93). The object recognition training alone also did not significantly alter acH2B levels (p = 0.12). However, posttraining administration of norepinephrine into the BLA after object recognition training significantly reduced acH2B levels in the IC when compared to saline-treated trained rats (p < 0.01).

As shown in Figure 3C, two-way ANOVA for acH4 levels indicated an almost significant norepinephrine effect (F_{1,41} = 3.72; p = 0.06) whereas the object recognition training did not have any effect (F_{1,41} = 0.08; N.S.). Most importantly, there was a significant interaction effect between norepinephrine treatment and training (F_{1,41} = 7.53; p < 0.01). Intra-BLA norepinephrine administration to non-trained control rats did not change acH4 levels in the IC (p = 0.53). In contrast, norepinephrine infused into the BLA after object recognition training significantly reduced acH4 levels when compared to saline-treated trained rats (p = 0.01) or norepinephrine-treated home cage control rats (p = 0.05).

Figure 3D shows pH3S10 levels. Two-way ANOVA revealed no norepinephrine (F_{1,41} = 0.47; N.S.), training (F_{1,41} = 0.02; N.S.) or interaction effect (F_{1,41} = 0.10; N.S.). Thus, these findings indicate that the norepinephrine administration did not change pH3S10 levels in either trained or non-trained animals.

Figure 3E shows that 3meH3K27 levels also remained unchanged. Two-way ANOVA revealed no significant norepinephrine (F_{1,41} = 0.53; N.S.), training (F_{1,41} = 0.30; N.S.) or interaction effect (F_{1,41} = 0.67; N.S.). Thus, these findings indicate that the norepinephrine administration did not change 3meH3K27 levels in either trained or non-trained animals.

As shown in Figure 3F, two-way ANOVA for pERK1/2 levels revealed no norepinephrine (F_{1,41} = 1.42; N.S.) or training effect (F_{1,41} = 0.05; N.S.), or interaction between the two factors (F_{1,41} = 0.40; N.S.). Thus, these findings indicate that the norepinephrine administration also did not change pERK1/2 levels in either trained or non-trained animals.
the IC. In this experiment we trained rats on an object recognition task and created an “arousal-like” situation by administering norepinephrine into the BLA after the training trial. Consistent with earlier findings (Roozendaal et al., 2008), this norepinephrine infusion enhanced the consolidation of object recognition memory. To determine whether the “arousal-like situation” triggers chromatin alterations in the IC, we assessed a battery of histone PTMs known to be involved in neural plasticity and memory formation (Levenson et al., 2004; Chwang et al., 2006; Fischer et al., 2007; Koshibu et al., 2009, 2011; Bousiges et al., 2010; Graff et al., 2012). We observed that this memory-enhancing dose of norepinephrine reduced the acetylation levels of H3K14 as well as that of H2B and H4 in the IC 1 h after object recognition training. These effects were specific to trained rats, as norepinephrine infusions into the BLA of home cage control animals did not induce any changes in these histone markers. pH3S10 and 3meH3K27 levels were not altered by either the norepinephrine infusion or object recognition training alone.

It has long been known that noradrenergic activation of the BLA mediates emotional arousal effects on the consolidation of long-term memory (McGaugh, 2000, 2004; McGaugh and Roozendaal, 2002). Extensive evidence indicates that such noradrenergic activation of the BLA enhances consolidation of different training experiences by facilitating time-dependent information storage processes in other brain regions, including the hippocampus, caudate nucleus and cortical areas (Packard et al., 1994; McGaugh, 2004; McIntyre et al., 2005). However, the molecular mechanism(s) underlying this BLA influence on information storage processes in its target regions remain to be elucidated. Our current finding that norepinephrine administration into the BLA after object recognition training induced a global reduction in histone acetylation, without altering the phosphorylation and methylation state, provides novel evidence indicating that arousal-associated BLA activity induces training-specific changes in histone PTMs in the IC (and likely other target areas). However, the pattern of histone PTM changes that we observed in the current study was rather unexpected. To date, most studies have linked a histone hyperacetylated state to decondensed chromatin structure with facilitated transcription, resulting in enhanced synaptic plasticity and long-term memory (Levenson et al., 2004; Chwang et al., 2006; Barrett and Wood, 2008; Gupta et al., 2010; Roozendaal et al., 2010). Previously, we found that inducing a hyperacetylated state within the IC by local posttraining administration of the HDAC inhibitor NaB enhanced object recognition memory (Roozendaal et al., 2010). Findings in the literature show that the nature and extent of specific histone PTM marks can vary considerably, depending on the memory task, brain region as well as other experimental parameters. For example, hyperacetylation of H3K14, but not H4, was observed in the CA1 subregion of the hippocampus 1 h after contextual fear conditioning (Levenson et al., 2004). On the other hand, spatial training in a water maze increased acetylation of H2B and H4 in the hippocampus, but did not affect the acetylation of H3K14 (Bousiges et al., 2010). In another study, the hyperacetylation of H3K4 after contextual fear conditioning was associated with an increased phosphorylation of H3S10 (Chwang et al., 2006). Graff et al. (2012) have also shown the combined acetylation of H3K4 and phosphorylation of H3S10 in the hippocampus after object recognition training. In the present study, we observed that the norepinephrine administration into the BLA did not produce the expected hyperacetylation. In fact, acetylation levels of H3K14 as well as that of H2B and H4 were significantly reduced 1 h after the training experience and drug administration, whereas...
the phosphorylation of histone H3S10 and tri-methylation of H3K27 levels were not altered. The only case where such a decrease in histone acetylation was previously demonstrated was in chronic stress (Ferland and Schrader, 2011). One study investigating histone acetylation changes after chronic social defeat indicated a transient decrease, followed by a persistent increase, in acH3K14 levels in the nucleus accumbens (Covington et al., 2009). The persistent increase in acetylation was associated with a reduction in histone deacetylase 2 enzyme activity (Covington et al., 2011). Our findings further indicate that the noradrenaline infusion did not alter pERK1/2 activity in the IC 1 h later. ERK1/2 activation has been demonstrated to be critical for histone H3 acetylation and phosphorylation in contextual fear conditioning in the CA1 region of hippocampus (Levenson et al., 2004; Chwang et al., 2006) as well as in stress conditions such as forced swimming that induces phospho-acetylation of H3 (Chandramohan et al., 2008; Gutiérrez-Mecinas et al., 2011; Mifsud et al., 2011). Although we cannot exclude that this 1-h time point may have not been optimal to capture the peak of the pERK1/2 response (Kobayashi et al., 2010), previous studies reported that phosphorylation of ERK1/2 was found in the IC 2–6 h after novel taste learning (Swank and Sweatt, 2001) or 1 h after contextual fear conditioning in the CA1 area of the hippocampus (Levenson et al., 2004).

Although it is currently unknown how a more extensive object recognition training experience, resulting in good 24-h memory, would affect histone PTMs and gene transcription in the IC, our observation that a memory-enhancing dose of noradrenaline reduced histone acetylation levels, and possibly consequent changes in transcriptional activity, within the IC is rather puzzling and does not seem to concur with prior evidence that direct administration of a protein-synthesis inhibitor into the IC impairs long-term memory of object recognition training (Baldéras et al., 2008). Moreover, in a previous study we found that systemic administration of the stress hormone corticosterone increased acH3K14 levels in the IC 1 h following training on an object recognition task and enhanced the consolidation of object recognition memory. These findings indicate that although both systemic corticosterone and intra-BLA administration of noradrenaline have the same behavioral outcome, i.e., an enhancement of object recognition memory, they were associated with opposite effects on histone acetylation within the IC. It is plausible that these differential molecular effects may be due to the different routes of drug administration: The systemically administered corticosterone could act directly in the IC to induce chromatin remodeling, whereas intra-BLA administration of noradrenaline must induce chromatin remodeling in the IC indirectly via neural pathways and network changes. It is not unlikely that noradrenaline administration into the BLA induces rapid changes in network properties, which might be associated with fast changes in histone acetylation. As histone acetylation–deacetylation is a highly dynamic process (e.g., NaB administration to a mammalian non-neuronal cell culture induced a fast hyperacetylation of core histones (t1/2 = 3–7 min) (Davie, 2003)), it is possible that the noradrenaline administration after object recognition training first induced a rapid increase in histone acetylation within the IC followed by deacetylation at 1 h. On the other hand, it should be noted that the exact role of the IC as part of the broader emotional learning and memory network is largely unknown and might be associated with decreased neural activity. For example, we showed in a previous study that systemic administration of a memory-enhancing dose of corticosterone after inhibitory avoidance training resulted in a rapid decrease in the number of pERK1/2-positive pyramidal neurons within the IC (Fornari et al., 2012b). Furthermore, a recent functional magnetic resonance imaging study in humans showed that the combined oral administration of cortisol and the noradrenergic stimulant yohimbine shortly before the encoding of emotionally arousing pictures shifted brain activation patterns and 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 when assessed 1 week later. It is possible that a reduced overall activity of the IC (and other frontal areas) during these conditions 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 an increased detection of novel or relevant stimuli and enhancing the consolidation of memory of arousing experiences (Menon and Uddin, 2010; van Stegeren et al., 2010).

Another intriguing possibility is that the primary role of BLA noradrenergic activation is to activate transcription factors and coactivators, which then could interact with chromatin modification mechanisms in its target regions. It is well established that the consequence of changes in histone PTMs on transcriptional activity depends on an intimate interaction with a large number of transcription factors and coactivators (Vescey et al., 2007). As indicated, in a previous study we demonstrated that direct administration of the HDAC inhibitor NaB into the IC enhanced the consolidation of object recognition memory (Roozendaal et al., 2010). However, co-administration of a glucocorticoid receptor (CR) antagonist or cAMP-dependent protein kinase (PKA) inhibitor completely abolished the effect of the HDAC inhibitor on memory enhancement. These findings indicate that inducing a histone hyperacetylated state via HDAC inhibition is not sufficient to enhance long-term memory. It is still necessary to have upstream arousal-associated signaling via GR and PKA activity. Presumably, these signaling events are triggering steps necessary to activate transcription factors and coactivators such as cAMP response-element binding (CREB) protein and CREB-binding protein (CBP; Roozendaal et al., 2010). Recently, we found that the β-adrenoceptor antagonist propranolol administered into the BLA after object recognition training did not prevent the effect of systemic NaB administration on hyperacetylation of H3K14 in the IC (Beldjoud et al., unpublished observation). However, the propranolol completely abolished the NaB-induced memory enhancement. These findings are similar to those of another study (Blank et al., 2014) indicating that temporary inactivation of the BLA with muscimol blocks the enhancement of inhibitory avoidance memory induced by HDAC inhibitor infusions into the hippocampus. These findings suggest that BLA (noradrenergic) activation might not directly alter histone acetylation mechanisms, but that it provides an additional obligatory factor, such as the activation of transcription factors and coactivators, that interacts with the chromatin remodeling changes in regulating gene transcription and neural plasticity. The currently observed reduction in acetylation levels should, in this case, not necessarily be interpreted as a direct effect of the BLA stimulation but might instead be an indirect consequence of feedback regulation mechanisms due to elevated transcription factor levels. In this perspective, mapping the genome-wide location of specific histone marks and transcription factors using chromatin immunoprecipitation (ChIP) will offer a more detailed understanding of the effect of noradrenergic activation of the BLA on chromatin modification mechanisms in influencing gene expression and may significantly contribute to our understanding of why emotionally arousing experiences are well remembered.
REFERENCES


A MEMORY-ENHANCING DOSE OF NOREPINEPHRINE ADMINISTERED INTO THE BASOLATERAL AMYGDALA AFTER OBJECT RECOGNITION TRAINING IS ASSOCIATED WITH GLUR2 AND PSD-95, BUT NOT PKMζ, EXPRESSION IN THE INSULAR CORTEX
ABSTRACT

Emotional memories are known to be vivid and long lasting. Extensive evidence indicates that noradrenergic activation of the basolateral complex of the amygdala (BLA) enhances the consolidation of memory of emotionally arousing training by inducing neural plasticity changes within its target regions. Post-training administration of norepinephrine into the BLA is also known to enhance memory consolidation of object recognition training. Some evidence indicates that noradrenergic activation of the BLA after object recognition training induces molecular changes in the insular cortex (IC) shortly after the training. However, little is known of whether the IC is involved in maintaining long-term synaptic plasticity changes for object recognition memory and emotional enhancement of this memory. In addressing this question, we investigated the long-term expression of the plasticity-related GluR2 subunit of the AMPA receptor, postsynaptic density protein 95 (PSD-95) as well as protein kinase Mζ (PKMζ) in the IC. Male Sprague–Dawley rats were trained on a 3-min object recognition task and immediately afterwards given bilateral intra-BLA infusions of saline or a memory-enhancing dose of norepinephrine (1.0 µg). When assessed 24-h after training, we found that the expression of GluR2 as well as PSD-95 was increased in IC synaptoneurosome fractions of animals given intra-BLA norepinephrine infusions, whereas PKMζ expression levels in IC cytosolic fractions were not changed. Moreover, we found that expression levels of GluR2 and PSD-95 were highly correlated. Norepinephrine infusions into the BLA of non-trained control rats did not affect the expression of GluR2 and PSD-95 in the IC. These findings indicate that BLA noradrenergic activity induces long-term training-associated changes in synaptic plasticity in the IC, which might be necessary for the behavioral expression of object recognition memory.

INTRODUCTION

Emotionally arousing experiences are typically well remembered (for review, McGaugh, 2000). Exposure to a stressful or emotionally arousing training experience results in the release of the stress hormones epinephrine and glucocorticoids from the adrenal glands. It is well established that such stress hormones converge onto the noradrenergic system of the basolateral complex of the amygdala (BLA), which is highly implicated in regulating stress and emotional arousal effects on memory (McGaugh, 2000; McGaugh et al., 2002; McGaugh, 2004). Despite the evidence that BLA activity modulates memory consolidation in different brain areas that are engaged at the time of memory consolidation (McGaugh et al., 2002), little is known about the molecular mechanisms underlying such memory modulation. Previously, it has been reported that the β-adrenoceptor agonist clenbuterol administered into the BLA immediately after training on an inhibitory avoidance task induces a rapid increase in the levels of activity-regulated cytoskeletal (Arc) protein in the dorsal hippocampus (McIntyre et al., 2005). A more recent study has extended these findings to object recognition memory, showing that increased Arc expression in hippocampal synapses was observed only in the context of a highly arousing training condition (McReynolds et al., 2014). Recently, we have shown that norepinephrine infusions into the BLA modulate the consolidation of object recognition memory (Roozendaal et al., 2008). Moreover, local infusion of a memory-enhancing dose of norepinephrine into the BLA after object recognition training altered the expression pattern of histone post-translational modifications (PTMs) in the insular cortex (IC), a brain region involved in recognition memory (Balderas et al., 2008; Roozendaal et al., 2010) 1-h after training (Beldjoud et al., 2015). In a separate experiment we found that the histone deacetylase (HDAC) inhibitor sodium butyrate (NaB) administered systemically after object recognition training also induces changes in histone PTMs in the IC 1-h after training. This treatment also resulted in memory enhancement. When noradrenergic activity within the BLA was blocked with the β-adrenoceptor antagonist propranolol the NaB effects on histone PTMs in the IC also prevented memory enhancement (Chapter 3). Taken together, these findings reinforce the notion that BLA activity facilitates memory consolidation by its modulatory influence on synaptic plasticity in different brain regions at the time of memory consolidation.

Long-term memory is thought to rely on persistent changes in synaptic strength (Kandel, 2001). This phenomenon is associated with molecular changes at the synapse. GluR2 is the most abundant subunit of the AMPA receptor (AMPARs) in the adult brain and together with GluR1 plays a critical role in synaptic plasticity and memory (Mead and Stephens, 2003; Migues et al., 2010). GluR2 subunits maintain the increased synaptic strength (Sutton et al., 2006). For example, overexpression of GluR2 has been shown to promote spine formation (Passafaro et al., 2003; Saglietti et al., 2007). Other findings suggest that the postsynaptic density protein PSD-95 may also have an important role in synapse maturation and function (El-Husseini et al., 2000; Schnell et al., 2002; Béïque and Andrade, 2003). Furthermore, PSD-95 is involved in AMPARs delivery during experience-driven plasticity in the barrel cortex (Ehrlich and Malinow, 2004), indicating a close relationship between these two synaptic plasticity proteins.

Recently, it has been shown that viral transfection of IC neurons with protein kinase M ζ (PKMζ) enhanced long-term memory of conditioned taste aversion even when tested 16 days later, whereas induction of the dominant negative PKMζ disrupted that memory (Shema et al., 2011).
indicating that PKMζ expression might play a major role in maintaining long-lasting memories. PKMζ was shown to maintain memory by regulating GluR2-dependent AMPAR trafficking. Indeed, inactivating PKMζ in the amygdala impaired fear memory in rats which was correlated with a decrease in postsynaptic GluR2 expression (Migues et al., 2010). It was also indicated that the induction of hippocampal long-term potentiation increases the synthesis of PKMζ from its mRNA (Hernandez et al., 2003; Kelly et al., 2007). Interestingly, Shao et al. (2012) showed that overexpression of PKMζ in hippocampal neurons increased PSD-95 expression, spine size, and postsynaptic expression of GluR2, and that application of the PKMζ inhibitor ZIP prevented the increase in PSD-95 expression (Shao et al., 2012). In auditory memory, Migues et al. (2010) demonstrated that PKMζ maintains long-term memory by regulating the trafficking of GluR2-containing AMPARs. Another study indicated that monkeys with better recognition memory displayed a greater proportion of dendritic spines coexpressing GluR2 and PKMζ (Hara et al., 2012).

In the present study, we investigated whether these different synaptic-plasticity proteins, known to sustain long-term memory, play a role in the maintenance of the long-term object recognition memory observed after BLA noradrenergic activation. We used an object recognition task for which we previously showed that post-training infusion of norepinephrine into the BLA enhanced 24-h memory retention (Roozendaal et al., 2008; Beldjoud et al., 2015) and addressed the question of whether this memory-enhancing dose of norepinephrine administered into the BLA after object recognition training altered the long-term expression of these plasticity-related proteins in the IC. Rats were sacrificed 24-h after the training and brain tissue of the anterior IC was collected. The expression levels of GluR2, PSD-95 in synaptoneurosome fractions and of PKMζ in cytosolic fractions were investigated.

**MATERIAL AND METHODS**

**Subjects**

Male adult Sprague-Dawley rats (280–320 g at 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 12-h:12-h light:dark cycle (lights on: 7:00 – 19:00 h) with ad libitum access to food and water. Training was performed during the light phase of the cycle between 10:00 and 15:00 h. 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.

**Surgery**

Rats, adapted to the vivarium for 1 week, were anesthetized with a subcutaneous injection of ketamine (37.5 mg/kg of body weight; Alfalan) and dexmedetomidine (0.25 mg/kg; Orion) and received the non-steroidal analgesic carprofen (4 mg/kg; Pfizer). Oxygen (35%) mixed with ketamine (37.5 mg/kg of body weight; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion) and that application of the PKMζ inhibitor ZIP prevented the increase in PSD-95 expression (Shao et al., 2012). In auditory memory, Migues et al. (2010) demonstrated that PKMζ maintains long-term memory by regulating the trafficking of GluR2-containing AMPARs. Another study indicated that monkeys with better recognition memory displayed a greater proportion of dendritic spines coexpressing GluR2 and PKMζ (Hara et al., 2012).

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**Object Recognition Apparatus and Training Procedure**

The experimental apparatus was a gray open-field box (in cm: 40x × 40d × 40h) with the floor covered with sawdust and placed in a dimly illuminated room. The objects to be discriminated were transparent glass vials (3.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). On the training trial, each rat was placed individually in the training apparatus at the opposite end from the objects and was allowed to explore two identical objects (A1 and A2) for 3-min, which by itself is insufficient to induce long-lasting memory of the objects (Okuda et al., 2004; Bermudez-Rattoni et al., 2005; Roozendaal et al., 2006, 2008; Beldjoud et al., 2015). Rats’ behaviour was recorded with a video camera positioned above the experimental apparatus. Videos were analyzed off-line by a trained observer blind to treatment condition. The total time spent exploring the two objects during the training trial was taken as a measure of object exploration. Rats showing a total exploration time of <10 s on the training trial were removed from analyses, because previous findings indicated that such rats do not acquire the task (Okuda et al., 2004). To avoid the presence of olfactory cues, the sawdust was stirred and the objects were cleaned with 70% ethanol after each animal.

**Drug Administration**

Norepinephrine (1.0 µg; Sigma-Aldrich) was dissolved in saline and administered into the BLA immediately after the object recognition training trial (Roozendaal et al., 2008; Beldjoud et al., 2015). Other rats received intra-BLA infusions of norepinephrine (1.0 µg) or saline without training. Bilateral infusions of drug or an equivalent volume of saline were administered into the BLA via 30-gauge injection needles connected to 10-µl Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 2.0 mm beyond the cannula tips and a 0.2-µl injection volume per hemisphere was infused over a period of 30 s by an automated syringe pump (Stoelting Co., Dublin, Ireland). The injection needles were retained within the cannulae for an additional 20 s 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 drug infusions into the adjacent central amygdala do not affect memory consolidation (Roozendaal and McGaugh, 1996, 1997). Drug solutions were freshly prepared before each experiment.

**Cannula Placement Verification and Insular Cortex Tissue Collection**

Twenty-four hours after training and drug treatment (or drug treatment to non-trained control rats), rats were deeply anesthetized with an overdose of pentobarbital (100 mg/kg, ip). Within 90 s after...
the pentobarbital injection, the rats were decapitated, the brains rapidly removed and flash frozen by submersion for 2 min in a beaker filled with pre-cooled isopentane on dry ice. Flash-frozen brains were stored at -80°C until tissue processing. The anterior part of the brain was cut on a cryostat into 350-µm-thick coronal slices for IC tissue collection. The rest of the brain, containing the BLA, was immersed in 4% formaldehyde for at least 3 days, and then transferred to a 30% sucrose solution for cryoprotection. Coronal sections of 50 µm were cut on a cryostat, collected on gelatin-coated slides, and fixed in 100% acetone before staining with cresyl violet. The sections were examined under a light microscope and determination of the location of injection needle tips in the BLA was made according to the atlas plates of Paxinos and Watson (2007) blind to drug treatment condition. Rats with injection needle tip placements outside the BLA or with extensive tissue damage at the injection needle site were removed from analyses.

**Cytosolic and Synaptoneurosome Preparation**

IC tissue was dissected from frozen 350-µm-thick coronal slices using a 1.25 mm brain puncher (Stoelting). Six punches from the anterior IC were collected from 3 consecutive slices (approximate range of coordinates: AP, +2.7 to +0.3 mm; ML, ±4.0 to 6.0 mm; DV, 3.0 to 7.0 mm). The crossing of the rhinal fissure and the medial cerebral artery was used as a reference point.

Synaptoneurosome isolation was performed with some adaptations that also permit the collection of the cytosolic fraction. The cytosolic fraction was obtained according to Shema et al. (2011). All procedures were performed on ice, and all solutions and centrifugations were chilled to 4°C prior to use. Tissue punches were homogenized with a pestle, 25 strokes, in 100 ml of homogenization buffer solution (in mM: NaCl, 124; KCl, 5; CaCl<sub>2</sub>, 2 H<sub>2</sub>O, 0.1; MgCl<sub>2</sub>, 6 H<sub>2</sub>O, 3.2; NaHCO<sub>3</sub>, 26; glucose, 10; pH 7.4, containing 1 complete Mini protease inhibitor cocktail tablet (Roche), and 1 complete Plos STOP phosphatase inhibitor cocktail tablet (Roche). The homogenate was centrifuged at 3,000 x g for 5 min to produce a supernatant (S1) and pellet (P1). The supernatant (S1) was centrifuged again at 100,000 x g for 30 min to produce a supernatant (S2, cytosolic fraction) and pellet (P2, particulate fraction).

Synaptoneurosome fractions were prepared from the P1 pellet following the McReynolds et al. (2010) protocol. The P1 pellet was resuspended in 100 µl of homogenization buffer for 15 min, with frequent vortexing. The total volume was brought to 500 µl by adding homogenization buffer. The homogenate was backfilled into a 1-ml syringe, then filtered through three layers of 100-µm nylon mesh inside a 13-mm syringe filter holder (Millipore). The filtered solution was backfilled again into a 1-ml syringe and filtered through a 5-mm pore nitrocellulose filter Durapore<sup>®</sup> membrane (Millipore) inside a 13-mm syringe filter holder. The final filtered solution was centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in 50 µl of cold homogenization buffer. Protein concentration was estimated using a colorimetric assay kit (DC protein assay kit, Bio-Rad).

**Western Blotting**

Equal protein concentrations were run on a discontinuous polyacrylamide gel consisting of a 10% or 7.5% acrylamide resolving, depending on the size of the protein to be identified, and a 4% acrylamide stacking gel. The gel was then blotted onto a polyvinylidene difluoride (PVDF) membrane for immunoblotting (Millipore). Membranes were blocked for 1-h in Li-cor blocking buffer (Li-cor), diluted in phosphate-buffered saline (PBS) 1:3, then incubated overnight at 4°C in primary antibodies followed by incubation in the secondary antibody for 1-h at room temperature. Primary and secondary antibodies were dissolved in the same blocking buffer. For synaptoneurosome molecule investigation, the membrane was washed at 75 kDa based on the molecular weight markers. The upper part was probed first in GluR2 antibody, and then stripped and re-probed in PSD-95 antibody. The lower part was incubated in β-actin antibody. For PKM<sub>ζ</sub> antibody, then stripped and re-probed in anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody. To confirm the separation of synapses from cell somas in the synaptoneurosome preparation, blots were also probed with the primary antibody for the nuclear protein total histone H3 (TH3). Band intensity was determined and quantified using an Odyssey IR scanner (Li-cor Biosciences). The signal integrative intensity was normalized to GAPDH for PKM<sub>ζ</sub> (Shema et al., 2011) or β-actin for synaptoneurosome molecules (McReynolds et al., 2010). The ratio was then expressed as percentage relative to the corresponding saline control group and conditions were compared.

**Antibodies**

The primary antibodies and their dilution were as follows: AMPAR GluR2 subunit antibody; anti-GluR2 (mouse; 1:2,000, Millipore), post-synaptic density-95 antibody; anti-PSD-95 (rabbit; 1:2,000, Millipore), protein kinase M zeta (PKM<sub>ζ</sub>) antibody which recognizes PKM<sub>ζ</sub> at 50-55 kDa (rabbit; 1:2,000, Cell Signaling), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody: anti–GAPDH (rabbit; 1:2,000, Cell Signaling), anti-β-actin (mouse; 1:2,000, Abcam); Total histone H3 (rabbit; 1:2,000, Millipore). The secondary antibodies used were goat anti-rabbit (1:25,000; Li-cor) and donkey anti-mouse (1:20,000, Li-cor).

**Statistical analysis**

Data are expressed as mean ± SEM. The percentages of GluR2, PSD-95 and PKM<sub>ζ</sub> were compared using a Student’s t-test to make pair-wise comparisons between drug groups. A probability level of < 0.05 was considered statistically significant. Pearson correlations were used to evaluate the relationship between expression levels of GluR2 and PSD-95. The number of animals per group is shown in the figure legend.

**RESULTS**

A diagram of the experimental procedure concerning the object recognition training and drug administration into the BLA is shown in Figure 1A. Animals were trained on an object recognition memory task for 3-min and afterwards received a bilateral infusions of a memory-enhancing dose of norepinephrine (1.0 µg) or saline into the BLA. Other rats received infusions of norepinephrine or saline without training. Animals were returned to their home cage until they were sacrificed 24-h after the training.

As shown in Figure 1B, total exploration time during the 3-min training trial, before drug treatment, indicated no difference between the two trained groups (t<sub>p</sub> = 0.47, p = 0.64).
Figure 2 shows a representative infusion needle tip terminating within the BLA and the location of infusion needle tips of all rats included in the final analyses. The synaptoneurosome fractions as well as cytosolic fractions were isolated from the anterior IC and immunoreactivity of the plasticity-related proteins GluR2, PSD-95 and PKM-ζ were revealed by Western blot.

**Figure 1.** Experimental procedure. (A) Diagram of the behavioral protocol and tissue collection. Rats were trained on an object recognition task for 3 min, followed immediately by bilateral administration of norepinephrine (NE, 1.0 µg in 0.2 µl) or saline (Sal) into the BLA. Other rats received infusions of norepinephrine or saline without training. Brain tissue was collected 24-h after the training session. (B) Representative diagram of punch location in the IC. (C) Total exploration time (in s) of the two identical objects during the 3-min training trial, before drug treatment, did not differ between groups. N=11-15 per group.

**Figure 2.** Histological analyses. (A) Representative photomicrograph illustrating placement of a cannula and needle tip terminating in the BLA. Arrow points to needle tip. (B) Diagram representing the different nuclei of the BLA, the lateral nucleus (L), basal nucleus (B) and accessory basal nucleus (AB), and central amygdala (CEA). (C) The location of needle tips within the BLA of all rats included in the analysis.

**Effect of post-training infusions of norepinephrine into the BLA on GluR2 expression in the IC 24-h after training**

As shown in Figure 3A, Western blot analysis of synaptoneurosome fractions from the anterior IC indicated an increased GluR2 protein expression 24-h after training in animals that received intra-BLA infusions of norepinephrine compared to their corresponding controls that received saline ($t_{25} = -3.03, p < 0.01$). The same dose of norepinephrine infused into the BLA of non-trained control rats did not affect GluR2 protein expression in IC synaptoneurosome fractions ($t_{12} = -0.53, p = 0.61$) (Figure 3B).

**Figure 3.** Effect of noradrenergic activation of the BLA on GluR2 protein expression, normalized to β-actin levels, in IC synaptoneurosome fractions 24-h after object recognition training. (A) A memory-enhancing dose of norepinephrine (NE; 1.0 µg in 0.2 µl) infused bilaterally into the BLA significantly increased GluR2 protein expression in IC synaptoneurosome fractions 24-h after object recognition training ($** p < 0.01; n =11-15 rats per group). (B) The same dose of norepinephrine (1.0 µg in 0.2 µl) administered into the BLA of non-trained animals, did not affect GluR2 protein expression in IC synaptoneurosome fractions (n = 7 rats per group). Data are presented as mean ± SEM. Representative Western blots from IC synaptoneurosome fractions are shown for each condition.
Effect of post-training infusions of norepinephrine into the BLA on PSD-95 expression in the IC 24-h after training

As shown in Figure 4A, Western blot analysis of synaptoneurosome fractions from the anterior IC also revealed enhanced PSD-95 protein expression 24-h after training in animals that received intra-BLA infusions of a memory-enhancing dose of norepinephrine (1.0 µg) when compared to controls that received saline ($t_{25} = -2.83; p < 0.01$). The same dose of norepinephrine (1.0 µg) infused into the BLA of non-trained control rats did not affect PSD-95 protein expression in IC synaptoneurosome fractions ($t_{25} = -0.95, p = 0.36$) (Figure 4B).

Figure 4. Western blot quantification of the effect of noradrenergic activation of the BLA on PSD-95 protein expression normalized to β-actin levels, in IC synaptoneurosome fractions 24-h after object recognition training. (A) Norepinephrine (NE; 1.0 µg in 0.2 µl) infused bilaterally into the BLA significantly increased PSD-95 expression in IC synaptoneurosome fractions 24-h after object recognition training ($**p < 0.01; n = 11-15$ rats per group). (B) The same dose of norepinephrine (1.0 µg) did not affect PSD-95 protein expression in IC synaptoneurosome fractions taken from animals that were not trained ($n = 7$ per group). Data are presented as mean ± SEM. Representative Western blots from IC synaptoneurosome fractions from animals that received either saline or norepinephrine post-training into the BLA after object recognition training or without training.

Relationship between GluR2 and PSD-95 expression in synaptoneurosome fraction of the IC

We examined the relationship between expression levels of GluR2 and PSD-95 in synaptoneurosome fraction of the anterior IC of individual animals 24-h after object recognition training and immediate intra-BLA infusions of norepinephrine (1.0 µg in 0.2 µl) or saline. As shown in Figure 5, there was a significant positive relationship between the expression levels of these two plasticity-related proteins (Pearson correlation: $r = 0.80, p < 0.001$).

To verify the good separation of synapses from cell somas in the synaptoneurosome preparation, synaptoneurosome and whole IC homogenate samples were immunobloted for the membrane-specific PSD-95 protein as well as for the nuclear-specific protein total histone H3 (TH3). The immune blot showed that enriched synaptoneurosome fractions displayed higher expression levels of PSD-95 than the whole IC homogenate, indicating an enrichment of synaptic tissue in the synaptoneurosome preparation (Figure 6A). In contrast, TH3 expression was present in the whole IC homogenate but not in synaptoneurosome fractions which indicates of a good separation of synapses from the cell somas in the synaptoneurosome preparation (Figure 6B).

Figure 5. Expression levels of GluR2 and PSD-95 in IC synaptoneurosome fractions of individual animals 24-h after object recognition training and post-training intra-BLA infusions of norepinephrine or saline (Pearson correlation: $r = 0.80, p < 0.001$; $N = 26$ rats).

Figure 6. Validation of the synaptoneurosome preparation. (A) Blot for PSD-95 shows higher PSD-95 protein levels in the synaptoneurosome (SN) fraction when compared to whole IC homogenate (WH). (B) Western blot for the nuclear protein total histone H3 (TH3). TH3 was present in the whole IC homogenate but not in synaptoneurosome fractions, indicating a good separation of synapses from the cell somas in the synaptoneurosome preparation.
Effect of post-training infusions of norepinephrine into the BLA on PKM-ζ expression in the IC 24-h after training

PKMζ levels were determined in cytosolic fractions of the IC. As shown in Figure 7, the memory-enhancing dose of norepinephrine (1.0 µg) infused post-training into the BLA did not change the expression of PKM-ζ protein in the IC 24-h later \( (t_{12} = 1.032, p = 0.31) \).

![Graph showing PKMζ expression levels](image)

**Figure 7.** Western blot quantification of the effect of norepinephrine infused into the BLA on PKM-ζ protein expression normalized to GAPDH levels, in IC cytosolic fractions 24-h after object recognition training. Post-training infusion into the BLA of a memory-enhancing dose of norepinephrine (NE; 1.0 µg) after object recognition training did not change PKM-ζ protein expression in the IC 24-h later \( (n = 9-13) \). Data are presented as mean + SEM. Representative examples of Western blots from IC cytosolic fractions from animals that received either saline or norepinephrine infusions into the BLA are shown.

**DISCUSSION**

The main finding of this experiment is that norepinephrine administered into the BLA after object recognition training induces long-term changes in the expression of plasticity-related proteins in the IC. This dose of norepinephrine has previously been shown to be sufficient for inducing long-term enhancement of object recognition memory (Roozendaal et al., 2008; Beldjoud et al., 2015) and here we show that this norepinephrine-induced memory enhancement is associated with increased synaptic expression of the GluR2-AMPAR subunit as well as PSD-95 in the IC 24-h after the training. This increase in expression of GluR2 and PSD-95 was observed only in animals that received the norepinephrine infusions after object recognition training and not in animals that received the same drug treatment without the training experience, indicating that norepinephrine by itself does not alter expression levels of GluR2 or PSD-95. Further, we observed that norepinephrine infusions into the BLA did not increase the expression of PKMζ in the IC after object recognition training.

Extensive evidence indicates that noradrenergic activation of the BLA enhances memory consolidation of emotionally arousing training experiences by inducing neural plasticity changes in its efferent brain regions (McGaugh, 2000). Previously, we reported that norepinephrine infused into the BLA after object recognition induced neural plasticity in the IC (Beldjoud et al., 2015), which is associated with increased synaptic expression of the GluR2-AMPAR subunit as well as PSD-95 in the IC.

However, it is unclear whether such BLA noradrenergic activity can induce long-term changes in efferent brain regions that might maintain long-term memory. Our finding that norepinephrine infused into the BLA immediately after object recognition training resulted in an enhanced expression of GluR2 protein in IC synaptoneurosome fractions 24-h after training is of a particular interest considering evidence from other brain regions that GluR2 subunits maintain synaptic strength (Sutton et al., 2006). Although, this did not address the question as to whether such higher GluR2 expression levels in IC synaptoneurosome fractions are due to de novo synthesis or increased delivery of existing protein and whether this resulted in an increased number of functional AMPARs, our findings are also consistent with other evidence that overexpression of GluR2 subunits has been reported to promote spine formation (Passafaro et al., 2003; Saglietti et al., 2007), a process that is thought to mediate at least some forms of memory formation as indicated in Aplysia (Bailey and Kandel, 2008). Moreover, another study reported that mice trained on a stressful water-maze spatial task, which resulted in robust memory, had an increased GluR2 AMPARs expression in hippocampus synaptoneurosome fractions, which was not the case for mice trained under a less efficient mildly stressful condition whether immediately or 45 min post-training (Conboy and Sandi, 2010).

In this experiment, the same dose of norepinephrine infused into the BLA after object recognition training also enhanced the expression of PSD-95 within the IC, an effect that was not observed in control animals that did not experience the training. Interesting findings from El Husseini (2000) and others (Schnell et al., 2002; Béïque and Andrade, 2003) have associated PSD-95 expression with an increase in the number and size of dendritic spines and they reported a role for PSD-95 in synapse stabilization and plasticity. PSD-95 and AMPARs seem to be functionally linked, as PSD-95 enhances synaptic transmission by adding AMPA receptors to synapses (Ehrlich and Malinow, 2004). Yudowski and colleagues (2013) showed that acute inactivation of PSD-95 resulted in an approximate 80% decrease in GluR2 surface expression in hippocampal neurons, indicating that PSD-95 is necessary to maintain AMPAR stability at the synapse. Our data confirm the relationship between PSD-95 and GluR2 AMPAR as indicated by a strong positive correlation between the expression of these two proteins in individual animals.

In our study the memory-enhancing dose of norepinephrine infused into the BLA did not induce any change in the expression of PKMζ 24-h after the training. This results may be useful relative to an ongoing debate as to the role of PKMζ in synaptic plasticity. PKMζ is an atypical isoform of protein kinase C (PKC) that has been implicated in long-term maintenance of neuronal plasticity and memory. Indeed, it has been shown that LTP induction increases the synthesis of the PKMζ from its mRNA (Muslimov et al., 2004; Kelly et al., 2007). Work from Shema and al. (2011) indicated that viral transfection of IC neurons by microinjection of PKMζ, which produced an overexpression of PKMζ, enhanced long-term taste aversion memory (16-day-old) whereas transfection with a dominant negative mutation of PKMζ disturbed that memory. In an object location memory task, (Migues et al., 2010) found that inactivating PKMζ with the PKMζ inhibitor ZIP in the dorsal hippocampus abolishes 1-day and 6-day-old object location memory. However, more recent studies using a genetic approach that generates mice that lack both protein kinase C-ζ (PKC-ζ) and PKMζ...
showed that these mice did not show deficits in learning or memory in several types of memory tasks when compared with wild-type mice controls (Lee et al., 2013). In a separate experiment transgenic mice lacking PKC-ζ and PKM-ζ showed normal LTP as well as normal learning and memory performance (Volk et al., 2013), suggesting that PKM-ζ might not be necessarily required for the maintenance and expression of long-term memory. The debate of PKM-ζ involvement in memory persistence is still open.

In summary, our present findings indicate that noradrenergic activity within the BLA after object recognition training triggers long-term and training-specific changes in the expression of plasticity-related proteins in the IC. Future investigations will be necessary to determine whether such plasticity-related changes take part in the maintenance of emotional arousal-induced enhancement of object recognition memory.

REFERENCES


chapter 7
GENERAL DISCUSSION
GENERAL DISCUSSION

The series of experiments presented in this thesis is integrated into a large framework that investigates the mechanisms by which arousal-induced noradrenergic activation within the basolateral complex of the amygdala (BLA) facilitates the consolidation of memory in its many target regions (McGaugh, 2000; McGaugh and Roozendaal, 2002; Roozendaal and McGaugh, 2011).

While the general characteristics of this postulate have been largely and extensively explored, it remains that the neural and molecular mechanisms underlying this memory facilitation remain largely unknown.

Throughout the chapters of this thesis, I investigated the molecular basis of the facilitatory effects of BLA activity on memory consolidation and maintenance in the target brain regions involved. Emerging evidence indicates that changes in gene expression are an excellent way to investigate how the brain expresses its plasticity (Flavell and Greenberg, 2008). This refers to epigenetic changes that include histone posttranslational modifications (PTMs) and DNA methylation and today it is becoming increasingly evident that histone PTMs act as a code in the regulatory mechanism of gene regulation (Fischle et al., 2003; Bannister and Kouzarides, 2011). Thus, the current studies examined whether noradrenergic activation of the BLA regulates memory consolidation by enabling chromatin modification in such target regions.

Extraction, identification and quantification of histones from small quantities of specific brain tissue

Because current histone isolation protocols require large amounts of tissue, which limits their application for analyzing small tissue samples from a specific brain region, I developed an improved method, which I described step by step in Chapter 2. This allows reproducible and reliable results for histone PTM identification from small quantities of tissue and quantification without losing anatomical precision. I used this method with success in the following chapters.

The method is an adaptation of several well-established histone-isolation methods (Levenson et al. 2004; Shechter et al. 2007; Leuba and Zlatanova 2009; Rumbaugh and Miller 2011) with the advantage of being adapted for the purpose of working with very small amounts of tissue (1 mm³ total volume of tissue), using the Palkovits’ punch technique (Palkovits, 1973).

One aspect of this method is that protein assay estimation is not possible because the final obtained after TCA precipitation yields a very low amount of usable protein. In this chapter, I also present tips and trouble shooting facilitating the concentration of samples and antigens for reliable and reproducible results. I also present data that demonstrates the validity of our approach.

The method has been tested on the CA1 region of the hippocampus as well as on the insular cortex (IC). With the CA1 region we used a 0.75 mm puncher tool in order to improve accuracy and the results are shown in figure 5 of Chapter 2. The method has been also used on different subregions of the medial prefrontal cortex (mPFC) (using a 1 mm puncher tool) with the same satisfactory results.

Chromatin remodeling in the insular cortex associated with the consolidation of object recognition memory requires basolateral amygdala noradrenergic activity

In Chapter 3, I addressed the question whether BLA noradrenergic activity triggers epigenetic changes in its target brain regions, which could explain the long-lasting memory of a single experimental event that is observed after BLA activation (for review, see McGaugh, 2000). I was inspired by previous work from Roozendaal et al. (2010) that reported that administration of the histone deacetylase (HDAC) inhibitor sodium butyrate (NaB) into the IC after object recognition training enhanced long-term memory of a training protocol, which by itself is not sufficient to induce long-term memory of the object (Roozendaal et al., 2008). The IC was targeted in this study because of its demonstrated importance for object recognition memory (for review Bermudez-Rattoni et al., 2005; Balderas et al., 2008). Therefore, in this study I boosted memory of object recognition training by a systemic administration of a memory-enhancing dose of NaB immediately after the training experience, while simultaneously interfering with noradrenergic BLA activity. To do this, noradrenergic activity of the BLA was either maintained intact (control groups) or blocked by a post-training infusion of the β-adrenoceptor antagonist propranolol.

Interesting data emerged from both the behavioral and the molecular study. NaB injected post-training enhanced object recognition memory as well as object location memory (data not shown) when tested 24 h later. The main behavioral finding was that noradrenergic inactivation of the BLA prevented the memory-facilitation effect of the HDAC inhibitor NaB. These findings indicate that the memory-enhancing effect of NaB administration requires concurrent noradrenergic activation within the BLA. These findings are generally consistent with those of Roozendaal and al. (2008) who
demonstrated that norepinephrine infused into the BLA after a 3-min object recognition training procedure enhanced long-term memory, whereas propranolol infused into the BLA after a more extended 10-min training trial impaired memory consolidation of this training.

In the second part of this chapter I examined how NaB administration altered different histone PTMs within the IC and whether these effects also depend on noradrenergic activity within the BLA. Therefore, I used Western blot to examine different histone PTMs. I decided not only to examine acetylation marks at different histone molecules (H3 and H2B) but also whether the NaB might induce secondary changes on the phosphorylation of histone H3 at serine 10 and tri-methylation of histone H3 at lysine 27. Several findings have indicated that these histone PTMs are important in regulating memory consolidation (Korzus et al., 2004; Levenson et al., 2004; Fischer et al., 2007; Gupta et al., 2010; Graff and Tsai, 2013) as well as in mediating stress or emotional arousal effects on neural plasticity (Xie et al., 2013).

The main molecular finding was that noradrenergic activity within the BLA controls chromatin remodeling in the IC during memory consolidation of object recognition training. NaB administered systemically post-training to animals with intact noradrenergic signaling within the BLA increased not only levels of AcH3K14, but it also increased phosphorylation levels at histone H3 at the serine 10 site (pH3S10) while it decreased 3MeH3K27 levels. I did not observe any significant effects of the NaB administration on AcH2B levels. These findings further support the view of histone crosstalk, where one modification can lead to subsequent modifications (Strahl and Allis, 2000; Fischle et al., 2003; Kouzarides, 2007; Izzo and Schneider, 2010; Bannister and Kouzarides, 2011). It is likely that these combined modifications might be required to induce plasticity and alter memory processes. In fact, hyperacetylation of H3K14, but not H4, was observed in the CA1 subregion of the hippocampus 1 h after contextual fear conditioning (Levenson et al., 2004). On the other hand, spatial training in a water maze increased acetylation of H2B and H4 in the hippocampus, but did not affect the acetylation of H3K14 (Bousiges et al., 2010). In another study, the hyperacetylation of H3K14 after contextual fear conditioning was associated with an increased phosphorylation of H3S10 (Chwang et al., 2006). Graff et al. (2012) have also shown the combined acetylation of H3K14 and phosphorylation of H3S10 in the hippocampus after object recognition training.

As indicated, when BLA noradrenergic activity was blocked, the enhancing effect of NaB on memory performance was abolished. At the molecular level, my findings showed that propranolol administration into the BLA did not prevent the NaB-induced increase in AcH3K14 levels, but did completely block the NaB effect on pH3S10 and 3MeH3K27. It therefore appears that a signal coming from the BLA is necessary for a subsequent effect of NaB on H3 phosphorylation and methylation. These findings indicate that noradrenergic activity within the BLA is a co-requirement to enable the effects of direct HDAC inhibition on chromatin modifications and object recognition memory. Several important conclusions can be drawn from this study: (i) BLA activity influences chromatin remodeling within the IC; (ii) a signal coming from the BLA is necessary to induce a subsequent action of NaB on pH3S10 and 3MeH3K27. Furthermore and of equal importance, the NaB injection was able to change AcH3K14 and pH3S10 levels in the IC only of animals that received the NaB injection post-training and not of home cage control rats that received the NaB injection without training. This finding indicates that the acetylation and phosphorylation changes observed are not the result of non-specific effects of NaB, such as the pain due to the intraperitoneal injection of hypertonic NaB, but due to the conjunction of NaB with either the encoding of new information by the training or the emotional arousal associated with the training experience.

Effect of histone deacetylase inhibition and noradrenergic suppression of the basolateral amygdala on histone methylation in prefrontal cortex subregions in the context of object recognition training

In Chapter 4, I further investigated whether the memory-enhancing effect of the HDAC inhibitor NaB depends on noradrenergic activity within the BLA. In this study I specifically investigated whether NaB administration after object recognition training induced histone PTM changes within the medial prefrontal cortex (mPFC) and whether these effects were blocked by concurrently inactivating noradrenergic signaling within the BLA. The mPFC is not only involved in higher cognitive functions such as executive control, but also appears to be critically involved in the regulation of memory consolidation (Akriv and Maroun, 2006; Roozendaal et al., 2009; Barseyan et al., 2010). However, most studies investigating the roles of the prelimbic (PL) and infralimbic (IL) subregions of the mPFC in memory have examined their effect on fear memory. The expression of conditioned fear memory involves a major role of the PL, rather than the IL, cortex (Sierra-Mercado et al., 2011). On the other hand, the IL cortex has been primarily implicated in the consolidation of fear extinction and inhibitory learning (Milad and Quirk, 2002; Laurent and Westbrook, 2009).

Some evidence indicates that the mPFC is also involved in regulating memory of object recognition training (Akriv and Maroun, 2006; Barseyan et al., unpublished observation), whereas other findings have shown that the mPFC and BLA critically interact in regulating memory consolidation for emotionally arousing training experiences (Roozendaal et al., 2009) as well as in influencing performance on other emotionally motivated tasks (Timms, 1977; Pérez-Jaranay and Vives, 1991; Garcia et al., 1999; Quirk and Gehlert, 2003; Roozendaal et al., 2004).

As I already showed in Chapter 3 that the administration of NaB not only altered the acetylation state of histone molecules but also induced secondary changes on other PTMs, in this study I sought to extend the range of investigated PTMs, so I focused on different methylation changes. In contrast to acetylation and phosphorylation, the role of methylation changes of histone molecules in relation to learning and memory is largely under-investigated. One prior study indicated that systemic NaB administration was able to regulate histone methylation in the hippocampus after a fear conditioning task (Gupta et al., 2010).

I investigated the effect of NaB administration after object recognition training on several histone methylation marks in both the PL and IL subregions of the mPFC, and determined whether these two brain regions show similar or different patterns of changes in our experimental conditions. In this study, as for the experiments reported in Chapter 3, NaB was injected immediately after the object recognition training with or without inactivation of BLA activity.

NaB administration did not significantly alter 3meH3K4, 3meH3K27 or 2meH3K9 levels in either the PL or IL cortex. Noradrenergic inactivation of the BLA activity increased dimethylation of histone H3 at lysine 9 (H3K9) within the IL cortex whereas it had no effect on the other methylation marks. No changes in methylation levels were observed in the PL cortex after BLA inactivation. This finding that NaB did not induce any clear changes in the methylation state of...
histone molecules in the mPFC was rather unexpected. We were unable to draw clear conclusions from this experiment on the role of IL and PL in object recognition memory. Further investigations would be needed to further investigate the effect of the HDAC inhibitor NaB injected directly into the IL or PL after object recognition. It appears that the dose of NaB administered after object recognition memory did not yield any clear histone modifications. One could raise the question whether NaB administration had an effect on histone acetylation processes within the mPFC. I investigated histone acetylation in both PL and IL twice using the same methods as with my other manipulations and did obtain any exploitable signal in any of the four group conditions on the blot (data not shown). One possible explanation is that although hyper-acetylation may have occurred after NaB treatment, it was not detectable in these regions in the conditions of this experiment. It is possible that hyper-acetylation in the mPFC occurs at a different scale or with a different time dynamic than what we observed in the IC. Another possibility is that NaB simply had no effect on histone acetylation in the mPFC. But before reaching any conclusion on this issue, a simple experiment (see below in the “future directions” section) could be to create better memory retention of the object by 10 min training and examine the changes of histone acetylation as well as other already investigated histone PTMs to see whether training that is adequate for expression of long-term memory triggers histone changes. This experiment could answer two questions: whether training triggers histone changes such as acetylation and others in the mPFC but also which of the two mPFC subregions (IL or PL) is involved in this type of memory consolidation. In order to directly examine the effect of NaB on these two brain regions we could then directly administer the HDAC inhibitor into either the IL or PL.

In these two chapters I showed that that blocking noradrenergic transmission within the BLA with propranolol prevented the effects of systemic NaB administration on some histone marks within the IC and, importantly, the enhancement of object recognition memory. Further, NaB administration did not significantly alter histone methylation levels in the PL or IL. However BLA propranolol infusion significantly increased levels of 2meH3K9 in the IL when BLA activity was blocked in control animals. These findings strongly suggest that BLA noradrenergic activation is required to modulate the consolidation of memory, at least in part, by altering the chromatin structure in its target regions.

Noradrenergic activation of the basolateral amygdala enhances object recognition memory and induces chromatin remodeling in the insular cortex

In Chapter 5, instead of reducing noradrenergic signalling from the BLA with propranolol, I employed the reciprocal approach and investigated whether an infusion of a memory-enhancing dose of norepinephrine into the BLA after object recognition training is sufficient to alter histone PTMs in the IC that are involved in brain plasticity. Extensive evidence indicates that noradrenergic activation of the BLA is critically involved in mediating emotional arousal effects on memory enhancement by influencing synaptic plasticity and information storage processes in other brain regions (Introini-Collision et al., 1991; Ferry et al., 1999; Hatfield and McGaugh, 1999; LaLumiere et al., 2003; Huff et al., 2005; Roozendaal et al., 2002, 2009; Barsegian et al., 2014). Noradrenergic activation of the BLA also enhances the consolidation of object recognition memory (Roozendaal et al., 2008).

The main finding of this experiment was that the “arousal-like” situation induced by norepinephrine administration into the BLA after object recognition training induced a global reduction in histone acetylation in the IC without significantly altering the phosphorylation and methylation state on histone H3. In fact, the acetylation of histone H3 at lysine 14 as well as H2B and H4 was decreased in the IC 1 h after object recognition training in the group that received local post-training norepinephrine administration into the BLA, whereas pH3S10 and 3meH3K27 levels remained unchanged.

Considering that most studies have shown that AcH3K4 is elevated 1 h after training (Chwang et al., 2006; Gupta et al., 2010; Roozendaal et al., 2010), these data were rather surprising, especially since activation of the noradrenergic system with the norepinephrine infusion had no observable effect on pH3S10, a histone mark that was important in our previous experiment. In that experiment pH3S10 increased when BLA activity was intact (and memory improved), but decreased when BLA activity was blocked (and memory was not improved).

The results concerning norepinephrine administration into the BLA on histone acetylation are also unexpected, considering that our group has previously shown that corticosterone administration (whose effects are thought to be mediated through BLA activation (Roozendaal and al, 2010), enhanced AcH3K14 in the IC and hippocampus when administered after the object recognition training experience (Roozendaal et al., 2010)). One possible explanation for this apparent contradiction is that these differential molecular effects may be a consequence of different routes of drug administration. The chromatin remodeling induced by systemically administered corticosterone could act directly on the IC via corticosterone receptors within this structure (Morimoto et al., 1996), whereas intra-BLA administration of norepinephrine may induce chromatin remodeling in the IC indirectly via neural pathways and network changes.

So, because of the highly dynamic process of histone acetylation-deacetylation (Davie, 2003) we suggest that a rapid and transient hyper-acetylation of H3K14 may have occurred within an extremely short timeframe following the hyper-activation of the noradrenergic system of the BLA. Further investigation of this issue (as outlined below the “future directions” section) could focus on monitoring of post-training histone acetylation dynamics in the IC at different time points during memory consolidation and their relation to their related enzyme activity (HAT and HDAC).

It is important to point out that in the experiments reported in chapter 3 the HDAC inhibitor prevented the deacetylation of histones, thus allowing measurement of hyperacetylation 1 h later. So, the question then is how other groups working on memory of stressful events such as fear conditioning or by corticosterone administration obtained hyper-acetylation? We must keep in mind that stress implies both norepinephrine and corticosterone acting on brain regions involved at the time of the training.

Further in our study we induced an “arousal-like” situation by directly infusing norepinephrine into the BLA. This method is known to produce a long-lasting memory in several memory tasks (for review, McGaugh, 2000). But most of all, this clearly confirms that for the same behavioral outcome and the same investigated brain region, memory consolidation of stressful events, which are superficially similar, may involve different molecular changes.
A memory-enhancing dose of norepinephrine administered into the basolateral amygdala after object recognition training is associated with GLUR2 and PSD-95, but not PKMζ, expression in the insular cortex

Previously, it has been reported that the β-adrenoceptor agonist clenbuterol administered into the BLA immediately after training on an inhibitory avoidance task induces a rapid increase in the levels of activity-regulated cytoskeletal (Arc) protein in the dorsal hippocampus (McIntyre et al., 2005). A more recent study has extended these findings to object recognition memory, showing that increased Arc expression in hippocampal synapses was observed only in the context of a highly arousing training condition (McReynolds et al., 2014). The BLA was shown to modulate the consolidation of object recognition memory (Roozendaal et al., 2008). Moreover, local infusion of a memory-enhancing dose of norepinephrine into the BLA after object recognition training altered the expression pattern of histone PTMs in the IC 1 h after training (Beldjoud et al., 2015). As discussed above in a separate experiment (Chapter 3), we found that the HDAC inhibitor NaB administered systemically after object recognition training also induces changes in histone PTMs in the IC 1 h after training. This treatment also resulted in memory enhancement. When noradrenergic activity within the BLA was blocked with the β-adrenoceptor antagonist propranolol the NaB effects on histone PTMs in the IC were prevented as well as its memory enhancement effect (Chapter 3). This set of results therefore suggests that whereas PTMs are frequently observed in contexts where memory is observed, these effects are not simple or systematic and therefore probably neither a necessary nor sufficient event for all types of memory.

In Chapter 6, I investigated whether different synaptic-plasticity proteins, known to sustain long-term memory, play a role in the maintenance of the long-term object recognition memory observed after the activation of noradrenergic system in the BLA with a memory-enhancing dose of norepinephrine.

The memory-enhancing dose I used has been previously shown to be efficient for the maintenance of long-term object recognition memory (Roozendaal et al., 2008; Beldjoud et al., 2015). In fact, while much is known about the effects of BLA noradrenergic activity on memory consolidation, much less is known about the molecular mechanisms that sustain the memory and its maintenance in the brain regions that are involved, at the time of consolidation, and which might then be required for retrieval. Therefore I aimed to investigate synaptic-related proteins, because of their important role in maintaining synaptic strength (Schnell et al., 2002; Béique and Andrade, 2003; Passafaro et al., 2003; Sutton et al., 2006; Saglietti et al., 2007). In addressing this question, I investigated the expression of plasticity-related proteins GLUR2, PSD95 as well PKMζ, in the IC, a brain region targeted by BLA and involved in long-term object recognition memory (Bermudez-Rattoni et al., 2005; Balderas et al., 2008; Roozendaal et al., 2008; Beldjoud et al., 2015).

I found that a memory-enhancing dose of norepinephrine administered into the BLA is associated with increased synaptic expression of GluR2-AMPA receptor subunit as well as PSD-95 in IC synaptoneurosome fractions 24 h after the training has occurred and that these two synaptic molecules where highly correlated. However, PKMζ expression levels remained unchanged with the same memory-enhancing dose of norepinephrine.

Our data clearly showed that noradrenergic activity within the BLA triggers the expression of plasticity-related proteins that sustain synaptic changes in the IC 24 h after the object recognition training had occurred or in other words, at the time when the retrieval of memory would normally be accessed.
Investigating histone modifications was the first step to prove the importance of BLA activity in chromatin remodeling in its target regions. There are many different ways to extend the findings of the present thesis to obtain a more precise understanding of the role of emotional arousal and BLA activity on histone PTMs in memory consolidation. Below I briefly indicate some of these possible future directions.

1) **Use of histone acetyl transferases (HATs)**

Regarding the HDAC experiment, reported in Chapter 3, further investigations could lead to better understanding of the role of BLA signals on pH3S10. One possible experiment could be to use local infusions of histone acetyl transferases (HAT), enzymes that directly stimulate the acetylation process of histone molecules, into the IC while blocking BLA activity.

2) **Interference with the histone acetyltransferase CREB-binding protein (CBP)**

Another approach would imply interfering with the CREB-binding protein (CBP) that has an intrinsic HAT activity. The histone acetyltransferase CBP mediates transcriptional activation by recruiting basal transcription machinery and acetylating histones. CBP is a critically important chromatin-modifying enzyme involved in regulating gene expression required for long-term plasticity and memory (Barrett and Wood, 2008).

3) **Use of stress-activated protein kinases**

One interesting experiment would be to investigate the modulatory effects after direct interference with phosphorylation of H3S10 by mitogen- and stress-activated protein kinases 1 and 2 (MSK1 and MSK2). MSK2 kinase all of which have been shown to play a role in the activation of mitogen-stimulated immediate-early response genes, such as c-fos and c-jun (Mahadevan et al., 1991; Thomson et al., 1999; Soloaga et al., 2003; Nowak and Corces, 2004). More details can be found in Sawicka and Seiser (2012). However, we need to keep in mind that these kinases have a broad range of actions on cell signaling and that a broad range of extracellular stimuli can activate them.

**Figure 1.** Chromatin remodeling provides a substrate for long-term changes in gene expression underlying memory of the object. Arousal induced by training activates the release of norepinephrine within the BLA (shown as a pink arrow), which is critical for enabling the modulation of consolidation. BLA activity modulates memory consolidation by influencing neuroplasticity in other brain regions such as the IC. In the condition where BLA activity is intact (left) both α- and β-adrenoceptors are active. NaB administration post-training induced an increase in AcH3K14 as well as pH3S10 and a decrease in 3meH3K27 in the IC and correlates with a memory of the objects. When β-adrenoceptors are blocked by propranolol (middle) the memory-enhancing effect of NaB is abolished. At the molecular level, propranolol did not block the NaB-induced increase in AcH3K14 levels, but it blocked the NaB effect on pH3S10 and 3meH3K27 levels. The hyper-activation by the norepinephrine infusion (right) into the BLA had a decreased effect on histone acetylation H3K14 as well as H2B and H4. Plasticity associated proteins (GluR2 and PSD-95) when assessed 24 h after training were increased.
4) Monitoring histone PTMs at various time points after training

A further development of our results showing that an “arousal like” situation with norepinephrine infusions into the BLA decreased histone H3 acetylation, would be to monitor the role of HAT and HDAC activity and/or H3 acetylation at different time points after training in conditions where BLA activity is boosted to induce a better memory performance. A limitation of this is that the two approaches (enzyme activity and histone isolation could not be done in the same animal because these two approaches require different technical approaches involved for acquiring the biological material from the trained animal).

5) Effects on natural, unboosted object recognition memory

To be able to see what is really happening in term of chromatin remodeling under a normal “low arousal” memory condition, one could compare the effect of 10 min object recognition training that is known to produce a good memory retention (Roozendaal and al., 2008). Thus comparing this with the 3 min object recognition plus noradrenergic BLA activation condition will provide an insight on what BLA activity is doing in terms of molecular changes in its target regions relative to memory acquired during a less arousing condition.

A complete clear experiment could be to reproduce that of one of Roozendaal and colleagues (2008) with a 10 min object recognition that results in a good retention of the training, and to block this memory by microinfusion of a β-blocker (propranolol) into the BLA. Another group could receive post-training norepinephrine into the BLA to boost the memory and as described above monitor HAT and HDAC activity with histone modification. This experiment would then address the question of the “pure” BLA effect on chromatin remodeling and explain the decrease of pH3S10 observed after BLA inactivation in chapter 3. However we have to know what we want exactly to target, since in chapter 3 there was also the systemic effect of NaB. It is important to point out that our work with NaB showed for the first time that BLA activity is required to enable the effect of NaB on memory consolidation.

6) Using different antagonists

The data of Chapter 5 (norepinephrine) appear to be in contradiction with the data from Chapter 3 (propranolol). There is in fact no real contradiction because they are not perfectly reciprocal, despite the fact they both show a clear involvement of BLA activity in modulating memory consolidation and in altering chromatin remodelling in BLA target brain regions.

In fact, in the “arousal-like” experiment (Chapter 5) the memory was boosted with a local infusion of norepinephrine into the BLA whereas in the propranolol experiment (Chapter 3) the memory was boosted with the HDAC inhibitor NaB. Further, local norepinephrine infusion into the BLA binds to different adrenoceptors such as the α1, α2 as well as β 1, 2 and 3 noradrenergic receptors. However, in the other experiment, the local infusion of β-adrenoceptor antagonist propranolol into the BLA blocks only β-adrenoceptors while α1- and α2-adrenoceptors can still be activated. It is incontestable that both α- and β-adrenoceptor play a major role in mediating the facilitatory effect of BLA activity on memory consolidation (Ferry et al., 1999). Therefore, it would be important to investigate whether manipulation of α-adrenoceptor activity within the BLA affects object recognition might also affect chromatin remodelling in its target regions and whether these effects differ from those observed after manipulation of β-adrenoceptor activity. Thus, interfering with these receptors by using specific receptor antagonists could provide a better understanding of the role of the BLA in mediating epigenetic changes in the IC and other brain regions.

7) Use of ChIP for a genome-wide analysis

To date, most studies investigate experience-driven epigenetic changes on candidate gene locations of specific histone marks related to gene expression as well as gene repression and transcription factors. Using chromatin immunoprecipitation (ChIP) allows comprehensive assessment of chromatin modifications across the genome and would offer a more detailed understanding of the effects of noradrenergic activation of the BLA and identify the fraction of the genome that leads to experience-driven chromatin remodeling. This could significantly contribute to our understanding of why emotionally arousing experiences are so well remembered. But beyond that, new technology such as mapping the genome using ChIPseq (Johnson et al., 2007; Blecher-Gonen et al., 2013) that is still in development will allow identification of the fraction of the genome related to epigenetic changes derived by experience-induced plasticity.

8) Study of the implication of miRNAs as epigenetic actors

Epigenetics is not restricted to histone PTMs, but also refers to DNA methylation and non-coding miRNAs and these two subfields of epigenetics in relation to experience-driven plasticity are still underdeveloped. While histone modification and DNA methylation will define which genes “could” be on or off, the downstream mechanism of non-coding miRNAs could “control” the regulation of transcription. In fact, the study of non-coding miRNAs called microRNA (miRNA) has added a complementary approach to investigate how gene expression is controlled. miRNAs can regulate protein formation by binding to mRNA after it is transcribed, and thereby preventing it from being translated into amino acids. miRNAs have been implicated in various aspects of dendritic remodeling and synaptic (Manakov et al., 2009; Scott et al., 2012; Wibrand et al., 2012). Thus, examining the role of miRNAs in regulating certain genes or proteins necessary for the formation and the maintenance of long-term memory after BLA noradrenergic activation could provide a better understanding on how genes control memory formation.

A first step would be to activate or inactivate the BLA after an object recognition memory and monitor miRNAs after object recognition memory. Because present scientific knowledge gives us no real information concerning the temporal dynamics of the miRNA but also which miRNA is expressed in a given memory tasks, testing a wide spectrum of time points relevant to the physiological and behavioral characteristics of a given memory task would be an essential first step in such an investigation.

9) Role of plasticity-related proteins in maintenance versus recall of memory

Regarding experience-driven protein plasticity as discussed in Chapter 6 future investigations will be necessary to see whether these plasticity-related proteins take part in the retrieval of object recognition memory or its maintenance. One experiment could be to see whether the expression
of GluR2 and/or PSD95 are necessary for retrieval in the condition where memory was boosted with an enhancing-dose of norepinephrine at the training time. Thus, interfering with the action of these two proteins could define their specific role in either memory retrieval or maintenance.

A proposed experiment could be to interfere with GluR2 and/or PSD95 using specific antagonists into the IC post-training or just before retrieval.

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Elke gebeurtenis die we als emotioneel ‘positief’ of ‘negatief’ ervaren leidt ertoe dat de amygdala (ook wel amandelkern genoemd) in de hersenen wordt geactiveerd. Zulke emotionele ervaringen worden in het algemeen beter onthouden dan alledaagse, niet emotionele gebeurtenissen, en het is nu bekend dat een emotionele ervaring leidt tot een verhoogde afgifte van de neurotransmitter noradrenaline in de basolaterale complex van de amygdala (BLA). Deze verhoogde BLA activiteit zorgt er dan vervolgens weer voor dat de informatieopslag in andere hersengebieden wordt versterkt zodat we ons deze gebeurtenis later goed kunnen herinneren. Echter, welke moleculaire processen er nu precies betrokken zijn bij deze geheugenverbetering voor emotionele ervaringen is grotendeels onbekend.

In de verschillende hoofdstukken van dit proefschrift heb ik onderzocht hoe een verhoogde BLA activiteit de moleculaire processen in andere hersengebieden kan aansturen en welke gevolgen dit heeft voor het verbeteren van het geheugen voor emotionele ervaringen. Recent onderzoek heeft aangetoond dat veranderingen in genexpressie, en dus de aanmaak van nieuwe eiwitten, hier nauw bij betrokken zijn. Om veranderingen in genexpressie te kunnen begrijpen is het belangrijk iets meer te weten over de structuur van het DNA. Het DNA zit als een klauwen opgerold rond vier verschillende histoneiwitten en gezamenlijk vormen zij het chromatine. Nucleosomen zijn de kralen in een chromatine keten, de plaatsen waar de DNA-streng zich om de histonen windt. Het uiteinde van ieder histonewit heeft een staart van aminozuurresten. Deze histonestaarten kunnen gemodificeerd worden (bijvoorbeeld door acetylering en methylering) (Engels: histone posttranslational modifications, PTMs), waardoor de associatie tussen DNA en histoneiwitten verandert en genexpressie gestimuleerd of juist geremd wordt. Het patroon van deze modificaties wordt de histoncode genoemd. Deze verschillende histon PTMs kunnen we onderzoeken en het is gebleken dat dit een goed beeld geeft van de mate van plasticiteit in diverse hersengebieden. In de experimenten zoals ze hier beschreven zijn, heb ik op verschillende manieren onderzocht of manipulatie van noradrenerge activiteit in de BLA de geheugenopslag voor een emotionele ervaring in andere hersengebieden beïnvloedt middels zulke veranderingen in histon PTMs.

In de introductie beschrijf ik het concept van emotioneel geheugen en de rol die de BLA speelt bij dit proces. Verder beschrijf ik wat er op dit moment bekend is over hoe histon PTMs en veranderingen in chromatinestructuur het leren en de opslag van deze informatie in het geheugen bevloeden. Zoals ik hierboven al schreef, noradrenerge activatie van de BLA speelt een cruciale rol bij de verbetering van het geheugen voor emotionele gebeurtenissen, en de BLA kan synaptische plasticiteit en geheugenvorming in andere hersengebieden reguleren. In dit proefschrift heb ik onderzocht hoe het manipuleren van noradrenerge activiteit in de BLA veranderingen in histon PTMs in andere hersengebieden kan beïnvloeden en welke rol dit speelt bij de verbetering van het geheugen voor emotionele gebeurtenissen. Omdat de expressie van langetermijngeheugen afhankelijk is van veranderingen in synaptische plasticiteit, heb ik verder onderzocht of het manipuleren van noradrenerge activiteit in de BLA vlak na een leertaak ook leidt tot een verandering in de expressie van bepaalde synaptische eiwitten 24 uur later.

In hoofdstuk 2 beschrijf ik een nieuwe methode voor het isoleren en identificeren van histoneiwitten uit hersenmateriaal. Deze methode is een verfijning van reeds bestaande methoden en is er speciaal op gericht om ook voldoende chromatine te kunnen isoleren uit
kleinere hersengebieden zodat het mogelijk wordt ook hier veranderingen in histon PTMs te kunnen onderzoeken.

In de volgende hoofdstukken heb ik deze methode gebruikt om te onderzoeken of BLA activatie na een leertaak leidt tot veranderingen in histon PTMs en hersenplasticiteit. In al deze experimenten heb ik gebruik gemaakt van een objectherkenningstaak bij ratten en gekeken naar plasticiteitsveranderingen in de insulaire cortex, dit omdat de insulaire cortex een belangrijke rol speelt bij het geheugen voor objectherkenning. Ik heb gebruik gemaakt van farmacologische manipulaties om de noradrenerge activiteit in de BLA te verhogen of juist te verlagen na het aanleren van deze objectherkenningstaak en dan met diverse moleculaire technieken onderzocht of dit leidt tot veranderingen in histon PTMs en synaptische plasticiteit in de insulaire cortex. Bij deze experimenten kon een rat 3 minutenlang twee gelijke objecten exploreren. Het is bekend dat 3 minuten onvoldoende is om langetermijngeheugen voor deze objecten te induceren. Dit stelde mij in de gelegenheid om dit geheugenspoor te boosten met een perifere injectie van de histondeaceylase-remmer natriumbutyraat (dit zorgt voor een verhoging van de acetyleringsgraad van histoneiwitten) of door directe toediening van noradrenaline in de BLA. Natriumbutyraat verbeterde het langetermijngeheugen voor deze leertaak (dit onderzocht ik door tijdens de testsessie een van de objecten te vervangen door een nieuw object), maar dit effect was tenietgedaan in ratten waar de noradrenerge neurotransmissie in de BLA was geblokkeerd middels lokale toediening van propranolol, een antagonist voor noradrenerge receptoren. Vervolgens heb ik onderzocht wat de gevolgen waren van toediening van natriumbutyraat op verschillende histon PTMs in de insulaire cortex en of propranolol toediening in de BLA ook deze moleculaire effecten van natriumbutyraat kon remmen. Natriumbutyraat leidde tot een verhoging van de acetylering en fosforylering van specifieke histoneiwitten maar verlaagde histonmethylering in de insulaire cortex gemeten 1 uur na de leertaak en farmacologische manipulatie. Gezamenlijk resulteerden deze veranderingen waarschijnlijk tot de verbetering van het geheugen. De remming van noradrenerge activiteit in de BLA blokkeerde niet alleen het effect van natriumbutyraat op het geheugen maar zorgde er ook voor dat een aantal van deze specifieke histon PTMs werd onderdrukt. Deze studie laat dus zien dat BLA activiteit betrokken is bij de regulatie van chromatinestructuur op het moment van geheugenvorming. In hoofdstuk 4 heb ik de effecten van perifere toediening van natriumbutyraat en gelijktijdige manipulatie van noradrenerge activiteit in de BLA onderzocht op histon PTMs in verschillende gebieden van de prefrontale cortex zoals de prelimbic, infralimbic and anterior cingulate cortex.

In hoofdstuk 5 heb ik onderzocht wat de effecten waren van het stimuleren van noradrenerge activiteit in de BLA op het geheugen voor een objectherkenningstaak en histon PTMs in de insulaire cortex. Ik vond dat noradrenaline toediening in de BLA het langetermijngeheugen voor het object verbeterde en dat dit gegezeld ging met een verlaagde acetylering van specifieke histoneiwitten in de insulaire cortex. Deze studie is interessant omdat het dus laat zien dat natriumbutyraat en noradrenaline alle twee een verbetering van het geheugen kunnen induceren, maar dat de moleculaire effecten van deze twee manipulaties op het niveau van histon PTMs van elkaar verschillen.

In hoofdstuk 6 heb ik vervolgens onderzocht wat de effecten zijn van toediening van noradrenaline in de BLA op genexpressie in de insulaire cortex. Ik vond dat ratten die noradrenaline kregen toegediend na de objectherkenningstaak een hogere expressie hadden van de plasticiteitseiwitten GLUR2 en PSD95 24 uur later, maar dat de expressie van PKM, een ander plasticiteitsmolecuul, onveranderd was gebleven.

Deze verschillende experimenten laten duidelijk zien dat veranderingen in histon PTMs en chromatinestructuur maar ook de expressie van diverse plasticiteitseiwitten in de insulaire cortex na een objectherkenningstaak nauwgezet gereguleerd worden door noradrenerge activiteit in de BLA en dat dit hersenmechanisme dus een belangrijke rol zou kunnen spelen bij de verbetering van het geheugen voor emotionele ervaringen.


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Beldjoud H, Messanvi F and Roozendaal B. Norepinephrine administration into the basolateral amygdala facilitates object recognition memory and is associated with GLUR2 and PSD-95, but not PKMζ, expression in the insular cortex (In preparation).


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CURRICULUM VITAE

Hassiba Beldjoud is an atypical PhD. She was born in Algiers (Algeria) on October 31st 1966 but lived in France until the age of nine. She started school only at the age of nine because of health reasons. Her primary language was French but she had to deal with the Arabic language at school when she returned to Algeria.

After her bachelors degree in Behavioral Biology, and because the Algerian biological faculties stopped their Masters degree programmes, Hassiba left the University and worked as a laboratory engineer for several years. Then, when the Masters programs were again re-opened, she left the comfortable life she had, to return to the University benches at the age of 37 driven by the thirst for knowledge to pursue masters and then a PhD in Neuroscience. Hassiba started her PhD research in Groningen in Benno Rozendaal’s lab, which then moved to Nijmegen.

Hassiba knows that she will always bear this atypical scientific background and that she will have to always find “atypical” ways to pursue her academic career but likes to say that “every road leads to Rome”. Her road will therefore remain atypical, but she WILL arrive.

Here is her path.

2012 – 2014: PhD student at Radboud University. Nijmegen. The Netherlands
1999 – 2009: Laboratory Engineer. Medical analyst in Algeria
1993 – 2003: My University career was then suspended because of a countrywide suspension of all post-graduate training for a 10-years period in Algeria. I left academia to work as a biologist engineer in a medical laboratory.