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Stress, epigenetic control of gene expression and memory formation

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Abstract

Making memories of a stressful life event is essential for an organism's survival as it allows it to adapt and respond in a more appropriate manner should the situation occur again. However, it may be envisaged that extremely stressful events can lead to formation of traumatic memories that are detrimental to the organism and lead to psychiatric disorders such as post-traumatic stress disorder (PTSD). The neurotransmitter glutamate and the ERK MAPK signaling pathway play a principal role in learning and memory. Glucocorticoid hormones acting via the glucocorticoid receptor have been shown to strengthen the consolidation of memories of stressful events. The ERK MAPK signaling pathway and glucocorticoid receptor-mediated actions have recently been shown to drive epigenetic modifications and conformational changes in the chromatin, stimulating the expression of neuroplasticity-related genes involved in stress-related learning and memory processes. The main epigenetic regulatory mechanisms are histone modifications and DNA (de-)methylation. Recently, studies have demonstrated that these processes are acting together in concert to regulate gene expression required for memory consolidation.

This review explores the role of stress in learning and memory paradigms and the participating signaling pathways and epigenetic mechanisms and the enzymes that control these modifications during the consolidation process of memory formation.

Introduction

Stressful events induce physiological and behavioral responses, generally referred to as the 'stress response', that are essential in order to cope with the challenge. The brain coordinates these responses and acts to consolidate memories of the events through processes involving the neocortex and the limbic system (e.g. amygdala and hippocampus). Memories of stressful events are particularly strong and can last for a lifetime. This allows the organism to respond better if a similar circumstance arises in the future. Memories are established by the emotional and contextual aspects of the stressful event which require processing by the amygdala and hippocampus respectively. Hormones such as glucocorticoids enhance the formation of memories indicating that the stress response and learning and memory processes are highly integrated systems (De Kloet et al., 1999;Smeets et al., 2009). It has been known for quite some time that these cognitive processes require changes in gene expression in neuronal populations of the hippocampus and other brain regions. Furthermore, since the 1990s considerable information has become available regarding the intracellular signaling mechanisms which allow communications between the extracellular milieu and the nuclear compartment (Adams and Sweatt, 2002). The intracellular signaling pathways have been thought to steer the required changes in gene expression following a challenge or learning event but it was unclear how exactly this was brought about. During the last decade evidence has been accumulating that the functional state of genes is dictated by the conformation of chromatin, which can be altered by chromatin modifying and remodeling proteins (Kouzarides, 2007). These epigenetic changes involving covalent modifications of the DNA and histone proteins are presently recognized to represent a principal interface between intracellular signaling pathways and gene expression. Currently the molecular mechanisms underlying how signaling molecules interact with chromatin-modifying enzymes to precipitate changes in gene expression are being investigated. Recently, evidence has revealed a novel role for epigenetics mechanisms underlying changes in learning and memory are currently a prime subject of investigation in neuroscience. This review

addresses some of these new developments within the scope of the stress and learning memory field.

Stress

The stress response can be elicited by a number of stimuli that are referred to as stressors which can be physical and/or psychological in nature (Chrousos, 2009). A physical stressor involves relatively little cognitive interpretation of the event and usually results in an immediate and automatic response, while psychological challenges require the animal's ability to assess situations and make decisions through cognitive evaluations. Stress hormones such as adrenaline and nor-adrenaline, secreted from the adrenal medulla and sympathetic nerve terminals, respectively, facilitate the 'fight or flight' response. This reaction is essential to cope with a stressor immediately and in an appropriate manner in order to increase the chances of survival. Stress responses also involve the neuroendocrine systems in particular the hypothalamic-pituitary-adrenal (HPA) axis which is activated by limbic and ascending brainstem and pontine pathways as well as others. During a stressful event there is a significant increase in the activity of the HPA axis via stimulation of parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) (De Kloet et al., 2005;De Kloet and Reul, 1987;Herman et al., 2003;Linthorst and Reul, 2008;Schobitz et al., 1994). These neurons project to the median eminence and release neuropeptides such as corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) into the portal vessel system. This leads to secretion of adrenocorticotropin-releasing hormone (ACTH) from the anterior pituitary into the blood stream causing the secretion of glucocorticoids from the adrenal cortex. Closing the neuroendocrine loop, glucocorticoids exert negative feedback on secretion-driving mechanisms via GRs in the anterior pituitary and PVN (De Kloet et al., 1987;Reul and De Kloet, 1985). Glucocorticoids also act on GRs in other parts of the brain (e.g. hippocampus, amygdala, prefrontal cortex, brainstem and pontine nuclei) some of which provide afferent control on PVN activity (Herman et al., 2003). Glucocorticoid

hormones are classically known for their role in maintaining and regaining homeostasis after a challenge. For instance, they increase gluconeogenesis to support recovery after a stressful event. Glucocorticoids have been shown to enhance the consolidation of memories following stressful events suggesting a mechanistic role in stress-mediated memory formation. Although this action of glucocorticoid hormones has been known since the 1980s, it is (De Kloet et al., 1988;Oitzl and De Kloet, 1992) only recently that considerable steps have been made to elucidate the underlying molecular mechanisms involved as well as the neuroanatomical site of action (see below).

Is it stress or is it learning and memory?

Before discussing the signaling and epigenetic mechanisms associated with stress and learning and memory models, there is an issue regarding these models we think is important to address. It needs to be considered that typical stress tests such as the forced swim test have (long-term) behavioral consequences that strongly suggest a cognitive component in this paradigm (see below). Conversely, frequently used behavioral paradigms such as fear conditioning and the Morris water maze (MWM) paradigms test cognitive performance but because of their aversive aspects (e.g. electric foot shock, forced swimming) they are without doubt stressful. In all behavioral paradigms, i.e. the forced swim test, fear conditioning and MWM learning, an action of glucocorticoid hormones via the GR is of principal importance for the consolidation of the learned behavioral response (Oitzl and De Kloet, 1992;Roozendaal et al., 2006b;Sandi et al., 1997). Clearly, this glucocorticoid dependence underlines the presence of a stress component in these behavioral tests. Furthermore, in addition to the shared glucocorticoid hormone dependency other pathways are commonly activated in all of these paradigms as discussed later.

In the forced swim test rodents (rat, mouse) are placed into a tank containing water, usually at 25^{0}C, from which they cannot escape (Linthorst et al., 2008). The test consists of an initial swim of 15

minutes, which is followed by a second swim of 5 minutes 24 hours later. When initially exposed to the water the animal will swim and attempt to escape from the tank. After several minutes the animal displays increasingly immobile behavior, where they float in the water in an attempt to conserve energy. In the 24 hour re-test, the animal demonstrates an initial attempt to escape but will much more quickly adopt the typical immobility or floating posture compared with the first swim. Historically the Forced swim test has been regarded as a measure of despair-like behavior in rodents (Lucki, 1997;Porsolt et al., 1977a;Porsolt et al., 1977b). The forced swim test is now gaining momentum as an accepted learning and memory paradigm for an adaptive behavioral response (Bilang-Bleuel et al., 2005;De Pablo et al., 1989;West, 1990). It is currently thought that the immobility response is a learned behavioral response because in the 24h re-test the animal will remember that escape from the tank is not possible and therefore quickly adopts the floating position to conserve energy. This notion is further supported by recent observations that the consolidation of the behavioral immobility response requires activation of intracellular signaling pathways (e.g. ERK MAPK signaling, see below) which previously have been shown to play a critical role in 'accepted' learning and memory paradigms such as fear conditioning and MWM learning (Blum et al., 1999;Chwang et al., 2006) . In the forced swim test, due to the re-test being performed after only 24 hours, it can be argued that memories are not long-term. However, our lab has recently shown that when a re-test is conducted after 4 weeks instead of 24h, the rats still adopt the immobility posture displayed in the 24 hour re-test, suggesting that even after 4 weeks the rats still remember the initial event, supporting the theory that the memories made in the forced swim test are long-term (Gutierrez-Mecinas M., Collins, A., and Reul, J.M.H.M., unpublished observations).

Signaling pathways in memory consolidation

The dentate gyrus is the gateway of the hippocampus and known to play a major role in pattern separation of incoming sensory information and memory formation. The dentate gyrus receives sensory information from the entorhinal cortex via the perforant pathway. After processing, the

dentate gyrus mainly passes the information on to pyramidal neurons in the CA3 area of the hippocampus where the information is further integrated and processed and subsequently passed on to the CA1 area, the main output region of the hippocampus. The granule neurons within the dentate gyrus are kept under a strong tonic inhibition by local GABAergic interneurons. The granule neurons are only excited by signals that are strong enough to overcome the GABAergic inhibition and therefore, these granule neurons show only a sparsely distributed activated response, i.e. in relatively few neurons, upon stimulation of the perforant path (Rolls and Kesner, 2006;Treves and Rolls, 1994). Using activation markers (phospho-acetylated histone H3 (H3S10p-K14ac), c-Fos; these markers will be addressed in depth later in this text) of dentate granule neurons we have been able to visualize the effects of GABAergic manipulations on stress-induced responses. Experiments using the partial GABA-A receptor reverse agonist FG-7142 (which evokes a reduction in GABAergic tone) resulted in an increase in baseline and novelty-induced levels of H3S10p-K14ac and c-Fos in the dentate gyrus. In contrast, the nonspecific benzodiazepine lorazepam blocked the novelty-induced epigenetic and gene expression changes in the dentate gyrus (Papadopoulos et al., 2010) . The observations correspond with a pivotal role of GABA in the control of dentate granule neuron excitability. Furthermore, the FG-7142-induced changes were abolished by the N-methyl-D-asparate receptor (NMDA-R) receptor antagonist MK-801 suggesting that GABA-A exerts its control over the granule neurons in a NMDA-R-dependent manner (Papadopoulos et al., 2010). The NMDA-R has been shown to be involved in neuronal plasticity processes in the hippocampus underlying memory formation (Fanselow and Kim, 1994). It has been shown in hippocampal neurons (in some studies focusing on hippocampal CA1 neurons) that glutamate-binding to NMDA-R opens the cation channel allowing the flow of Na^{+}/Ca^{2+} into the cell causing a rise in the intracellular Na^{+}/Ca^{2+} levels. The rise in Ca^{2+} within the neuron activates Ca^{2+}-calmodulin kinase II (CAMKII), adenylate cyclases 1 and 8 (ACy1, ACy8), protein kinase A (PKA) and the Ras/Raf/MEK/ERK signaling cascade (Atkins et al., 1998;Impey et al., 1999;Sweatt, 2004). Glucocorticoids have been shown to interact with the

noradrenergic system in the basolateral amygdala also resulting in an enhanced activation of the PKA pathway (Roozendaal et al., 2006a). It has been shown that after glutamate-induced Ca^{2+} influx or glucocorticoid binding to its receptor, CREB binding protein (CBP) is activated in cultured hippocampal and cortical neurons (Impey et al., 2002;Roozendaal et al., 2006a). CBP is a direct target of CAMKII becoming phosphorylated at S310 and as a consequence induces the expression of NMDA-R causing a positive feedback effect (Impey et al., 2002). Unlike glutamate, glucocorticoids can bind to a membrane bound or a cytoplasmic receptor and once activated both appear to have a role in memory formation. An experiment using a BSA:corticosterone conjugate (which cannot cross the cell membrane) that specifically activates membrane-associated GRs in the adrenal gland, resulted in the activation of the PKA pathway and phosphorylation of CREB (Roozendaal et al., 2010). In aggregate, these observations demonstrate that glutamate and glucocorticoid hormone share similar pathways and are closely linked in regards to memory formation. Therefore, they could have similar epigenetic effects downstream.

GRs and NMDA-Rs are essential for epigenetic regulation of gene expression in the dentate gyrus

A number of studies have shown that activation of GRs in the hippocampus play an important role in adaptive behavioral responses including memory formation in a number of stressful learning paradigms in rodents such as Morris water maze learning, contextual fear conditioning and the forced swim test. (Bachmann et al., 2005;Bilang-Bleuel et al., 2005;Chandramohan et al., 2007;De Kloet et al., 1988;Oitzl and De Kloet, 1992;Revest et al., 2005;Sandi et al., 1997;Veldhuis et al., 1985). Furthermore, it has been found that specifically GR activation in the dentate gyrus is required immediately after the initial forced swim test in order for the animal to display the enhanced immobility behavior in the re-test (Bilang-Bleuel et al., 2005;De Kloet et al., 1988). Thus, GR activation in dentate neurons is required during the consolidation phase of memory formation to allow expression of the immobility response at future occasions. In contrast, less stimulating

information appears to not require glucocorticoids in order for memory consolidation to take place (Buchanan and Lovallo, 2001). Intracellular GR once bound glucocorticoid hormone, it dimerizes and is translocated into the nucleus where it can act as a transcription factor by binding to glucocorticoid response elements (GREs) in the promoter region of glucocorticoid-responsive genes. This is the classical mechanism of GRs but these hormone receptors can also exert effects via interaction with various signaling molecules including transcription factors, immediate-early gene products and chromatin-modifying enzymes. We discovered a novel non-genomic mechanism of GR action when we investigated the signaling pathways involved in the formation of the combinatorial histone H3 phospho-acetylation marks and subsequent c-Fos induction. Since GRs do not have any intrinsic protein kinase or acetyl-transferase activity we postulated several years ago that GRs may exert their action indirectly by influencing the activity of the NMDA receptor-activated ERK MAPK pathway. We found that indeed in addition to GRs concurrent activation of NMDA-Rs were required for the formation of the combinatorial histone H3 phospho-acetylation mark and c-Fos induction in the dentate gyrus. Pre-treatment of rats with the GR antagonists RU486 or ORG34517, or the NMDA receptor antagonist MK801 resulted in a strong attenuation of the novelty stress and forced swim stress induced phospho-acetylation of histone H3 and c-Fos in dentate gyrus granule neurons (Bilang-Bleuel et al., 2005;Chandramohan et al., 2007). Combined administration of RU486 and MK801 completely blocked the novelty stress-induced responses (Chandramohan et al., 2007). Furthermore, the sole activation of GRs through a single injection of corticosterone had no effect on dentate gyrus H3S10p-K14ac and c-Fos (Chandramohan et al., 2007).Moreover, blockade of GRs and NMDA-Rs also resulted in an impairment of the forced swimming-induced immobility response in the re-test (Bilang-Bleuel et al., 2005;Chandramohan et al., 2008;Reul et al., 2009). Thus, the stress-induced formation of H3S10p-K14ac and c-Fos in the dentate gyrus (and memory formation associated with the stressful event (Chandramohan et al., 2008) depend on concurrent activation of

GRs and NMDA-Rs. Recent follow-up experiments have shown that GRs act through a rapid interaction with NMDA-R-activated ERK1/2 and other down-stream signaling partners (see below).

There is receptor specificity in the glucocorticoid dependence of the forced swimming-induced immobility response because blockage of the other glucocorticoid-binding receptor, i.e. the mineralocorticoid receptor (Reul and De Kloet, 1985), showed no effect on the immobility response thereby illustrating the specificity of the GR-medicated pathway involved (Bilang-Bleuel et al., 2005;Veldhuis et al., 1985) Thus, both GR- and NMDA-R-activated pathways seem to be essential in the consolidation of memory formation of psychologically stressful events such as forced swimming and they also appear to converge on similar epigenetic mechanisms, i.e. the S10 phosphorylation and K14 acetylation in histone H3 (see below). Whether concurrent activation of GRs and NMDA-Rs in dentate granule neurons is also required for (contextual) fear conditioning and Morris water maze learning has, to our knowledge, never been studied in detail.

Histones and their role in epigenetics

In the previous text we already touched on epigenetic mechanisms but since this concerns a rather new field in neuroscience it makes sense to address some of the basics. The core histones H2A, H2B, H3 and H4 have approximately 150 base pairs (bp) of DNA wrapped around a complex of 2 copies of each making up the universal units of chromatin known as the nucleosomes. The core histone's N-termini protrude from the nucleosome and are sites of reversible post-translational modifications (PTMs) such as acetylation, methylation and phosphorylation (Fig 1). Replacement of the canonical histones with variants such as H2A.Z for the variant H2A also takes place and can alter gene expression. The PTMs at the histone molecules are thought to act as docking sites for chromatin remodeling complexes and chromatin-interacting proteins such as heterochromatin-binding protein-1 (HP-1) or (other) histone modifying enzymes. There has also been the suggestion of a 'histone code' whereby specific histone modification patterns located on one or more of the N-terminal tails

can form recruitment sites for specific remodeling complexes and can orchestrate gene expression and other cell functions such as chromosome condensation as a preparation for cell division (Fig 1.(Jenuwein and Allis, 2001;Strahl and Allis, 2000)). Chromatin also contains the less well-known histone H1s (also termed the 'linker histone') and if located on the nucleosomal dyad this structure is known as the chromatosome (Noll and Kornberg, 1977). The role of the linker histone is much less understood and its role in chromatin regulation which was once thought to be solely condensing the chromatin and silencing genes is currently the subject of further investigations (Trollope et al., 2010). Histone variants and PTMs largely dictate the conformation of the chromatin and thereby exert a strong influence on gene expression. Acetylation of histone H3 and H4 is associated with an 'open' chromatin conformation (Fig. 1) making the DNA accessible to protein remodeling complexes, as shown by an increase in sensitivity to DNase 1 digestion at active genes (Hebbes et al., 1994). Histone acetyl-transferases (HATs) and histone de-acetylases (HDACs) are by far the most studied enzymes in regards to histone PTMs however; they are not exclusive to histones as they can also modify other non-histone proteins such as p300/CREB-associated factor (PCAF) which can acetylate P53 and GATA1, and HDAC1 which deacetylates P53. In fear conditioning studies it was shown that levels of both H3K9me2 (i.e. histone H3 carrying 2 methyl-groups at lysine 9 (i.e. K9) and H3K4me3 are increased in the hippocampus. Interestingly, treatment of animals with sodium butyrate, a HDAC inhibitor, resulted in significant increase in H3K4me3 and a decrease in H3K9me2 marks demonstrating a clear link between acetylation and methylation in histones (Gupta et al., 2010). Mice carrying a single-allele deletion of MLL (i.e. mixed lineage leukemia), a well-known histone methyl-transferase for H3K4 methylation, showed impaired memory consolidation in the long-term contextual fear conditioning paradigm (Gupta et al., 2010). Histone methylation is rather complicated and its effect on chromatin structure and gene expression depends on the number of methylation marks on a lysine residue and the location of the marked lysine residue; it can be an active or a repressive mark for gene expression. H3K4me3 is regarded as an active mark (Akbarian

and Huang, 2009) while H3K9me3 is a repressive mark (Sabbattini et al., 2007). The H3K9me2 mark is mainly regulated by the histone methyl-transferase (HMT) EHMT1 (Euchromatic histone-lysine N-methyltransferase 1) and is present at transcriptionally silent regions (Tachibana et al., 2002). The presence of this mark acts as a docking platform for the chromo-domain of heterochromatin protein 1 (HP1) (Bannister et al., 2001). In turn HP1 associates with other proteins such as the HDAC Suv39h1 in yeast, which can remove acetylation marks from histones, allowing remodeling and condensation of the chromatin leading to transcriptional repression (Hiragami and Festenstein, 2005). The phosphorylation of histones is regulated by kinases and can be associated with two distinct cellular functions. The combination of the Ser10 phospho-mark with acetylation at lys14 in histone H3 has been found to be associated with the opening of condensed chromatin and the induction of expression from previously silent genes (Clayton et al., 2000). Histone H3 phosphorylated at Ser28 and Ser10 is associated with chromosome condensation during the metaphase of mitosis. In mammals dual phosphorylation of H3 at serine 28 and 10 is thought to be brought about by the Aurora B kinase (Goto et al., 2002) but can also be produced by mitogen- and stress-activated kinases 1 and 2 (MSK1/2; (Arthur and Cohen, 2000). The removal of the phospho-mark is carried out by phosphatases such as protein phosphatase 1 (PP1). Using novel object recognition and MWM paradigms, PP1 transgenic mice carrying an inducible neuron-specific inhibitor of PP1 known as NIPP (nuclear inhibitor of PP1) were shown to display an enhanced long-term memory as compared to the control animals (Koshibu et al., 2009). The authors went on to show that this was due to differences in distinct PTMs in the hippocampus. The dual PTM H3S10p-K14ac, was increased at the promoter of CREB after learning tests in control mice while the PP1 knockout animals showed an even greater increase in H3S10p-K14ac at this promoter compared to the control mice. Differences in other PTMs were observed as well (Koshibu et al., 2009). These epigenetic changes were associated with a significant increase in CREB expression as well as long term memory (LTM) in PP1-deficient mice compared to control mice. Similar results were later found

in the amygdala suggesting that PP1 could be a universal negative regulator of memory consolidation (Koshibu et al., 2009;Koshibu et al., 2011). Histone H3 is by far the most studied histone in terms of its PTMs and particularly the dual modification Ser10p-K14ac has received considerable attention with regard to its role in learning and memory (see below). The combinatorial mark is thought to be associated with the local opening of chromatin allowing the expression of a specific set of otherwise silenced genes (Clayton et al., 2000) such as *c-fos* in dentate gyrus neurons (Chandramohan et al., 2007;Reul and Chandramohan, 2007;Reul et al., 2009). The enzymes that regulate these processes have been partly elucidated and will be discussed later.

DNA methylation and its role in epigenetics

Another major epigenetic process is DNA methylation, which involves the covalent addition of a methyl group to the 5-position of the nucleotide cytosine (5-methylcytosine; 5meC) within the DNA structure by enzymes known as DNA methyl-transferases (DNMTs). In mammals DNA methylation mainly occurs at CpG rich sites in the DNA. When the promoter region of a gene is methylated it is generally considered that the gene is silenced, possibly because the methyl groups prevent proteins (e.g. transcription factors) binding to their cognate recognition sites in gene promoter regions or the methyl groups attract the binding of repressive proteins such as MBDs (methyl-CpG-binding domain proteins). DNA methylation is not an absolute necessity for a gene to be repressed e.g. the human alpha-globin gene is unmethylated regardless of its transcriptional status (Bird et al., 1987). DNA methylation has always been thought of as a relatively stable mark; however, evidence is accumulating that the mark is much more dynamic than first thought. The first enzyme shown to be able to remove this modification was MBD2 (Bird, 2002); this process however requires a huge amount of energy in order to break the carbon-carbon bond. There is another mechanism that has been proposed which does not involve removal of the methyl group from the cytosine but results in the 5-methyl-cytosine being excised and replaced with an unmodified cytosine (Tahiliani et al.,

2009). This process is part of the DNA repair pathway. More recently, yet another mechanism has been proposed whereby 5-methyl-cytosine is converted into 5-hydroxy-methyl -cytosine by the Ten eleven translocation protein 1 (TET1) enzyme which is thought to posses hydroxylase activity. It is thought that while 5-methyl-cytosine marks are often present in promoter regions of silenced genes, the 5-hydroxy-methyl -cytosine marks may be present at active genes and therefore it seems not to be essential to remove the 5-methyl-cytosine mark for gene transcription but simply to hydroxylate it (Tahiliani et al., 2009). However, bisulfite sequencing does not discriminate between these two marks and therefore can impede the interpretation of these data. Interestingly, HP1 stimulates DNMT1 activity specifically in an EHMT1-mediated histone methylation-dependent manner demonstrating that EHMT1 can be multifunctional in gene repression directly through histone modifications or indirectly through promoting DNA methylation indicating the complexity and cooperation that exists between regulatory complexes (Smallwood et al., 2007). Gupta and co-workers showed that after fear conditioning training in rats there was an increase in the H3K4me3 mark in the gene promoter of *egr1* (early growth response factor 1) in the hippocampus. Interestingly this mark coincided with an increase in DNA methylation of the *egr1* gene promoter along with an increase in mRNA expression of the gene. These data suggests that a gene can be actively transcribed whilst its DNA is methylated. Brain-derived neurotrophic factor (*bdnf*) gene expression is also increased after fear conditioning and also shows an increase in the H3K4me3 mark; however this gene displays lower levels of DNA methylation. These data show that active genes involved in memory consolidation can show either an increase or a decrease in DNA methylation. Direct evidence for a role of DNA methylation in memory formation is still under investigation. It may very well be possible that any role of DNA methylation may be gene-dependent.

Histone PTMs in learning and memory

It is clear that in learning paradigms signaling pathways lead to the activation of transcription factors and chromatin remodeling proteins, changing in the functionality of the chromatin through PTMs of histones and/or DNA methylation changes that participate in differential gene expression responses and memory formation. The research centered on the combinatorial H3S10p-K14ac modifications has provided substantial insight into how signaling pathways and epigenetic mechanisms directed by these pathways may play a role in memory consolidation in different learning paradigms. It is now 10 years ago that *in vitro* work showed that the H3S10p-K14ac mark was involved in the induction of immediate-early genes such *c-jun* and *c-fos* (Clayton et al., 2000). It has also been shown *in vivo* that this combinatorial mark is located in granule neurons of the dentate gyrus of the rat and mouse hippocampus. In other parts of the brain such as the neocortex, amygdala, striatum and olfactory bulb, this mark is only present in very few neurons. After a psychologically stressful event like forced swimming, novelty or predator exposure there is a significant increase in the number of neurons expressing this active mark and this is happening uniquely in the dentate gyrus (Bilang-Bleuel et al., 2005;Chandramohan et al., 2008;Chandramohan et al., 2007). The mark is present transiently. It appears as early as 15min after psychological stress, peaks between 60 and 120 minutes and returns to –very low– baseline levels at approximately 4 hours after the stressful challenge (Chandramohan et al., 2008;Chandramohan et al., 2007). The induction of c-Fos occurs virtually in parallel with the formation of H3S10p-K14ac (Chandramohan et al., 2008;Chandramohan et al., 2007). The same phenomenon was observed after other stressful paradigms such as fear conditioning and Morris water maze (Chandramohan and Reul, unpublished data). Subsequent co-localization experiments using double immuno-fluorescence demonstrated for the first time that in the dentate gyrus, H3S10p-K14ac is associated with neurons that express c-Fos. Moreover, recently we have also shown using a chromatin immuno-precipitation assay (ChIP) and Real Time PCR that the dual modification is indeed located at the promoter of the *c-fos* gene in the hippocampus and that this *c-fos*-associated mark increased after forced swim stress (AF Trollope and JMHM Reul, unpublished

observations). The requirement of this dual mark for *c-fos* induction has also been displayed in animals that have been subjected to electroconvulsive shock treatment, a technique well known to induce an exaggerated c-Fos induction in the hippocampus (Tsankova et al., 2004). What is especially intriguing about this dual modification is that in another major brain tissue, the neocortex, which is well-known to express c-Fos after forced swimming (Bilang-Bleuel et al., 2000) required different epigenetic marks compared to those in the hippocampus. After forced swimming, the neocortex appeared not to present the H3S10p-K14ac modifications at the *c-fos* promoter; the degree of acetylation of H3 remained unchanged whereas the acetylation of H4 decreased (AF Trollope and JMHM Reul, unpublished observations). Therefore, recently we postulated that the *c-fos* gene in the dentate gyrus and neocortex are in a different state with the dentate gyrus gene being in silenced state requiring the formation of H3S10p-K14ac to open (Chandramohan et al., 2007;Reul et al., 2009). Furthermore, these observations indicate that different signaling pathways may possibly be involved in specific tissues and consequently alternative combinations of histone modifications and/or transcription factors may be recruited in order to induce the expression of c-Fos and other gene products. The decreased H4 acetylation at the *c-fos* promoter in the face of increased c-Fos expression after forced swimming was surprising however it has been shown that GRs can inhibit acetylation through inhibition of HATs and recruitment of HDACs to gene promoter sites (Ito et al., 2000). Whether this mechanism is also taking place at the *c-fos* gene in the neocortex and hippocampus is currently unknown. With regard to the hippocampus it is currently also unknown whether the decrease in H4 acetylation is taking place at those promoters showing increased H3S10p-K14ac marks or not. In terms of functional relevance it may be speculated that a decreased H4 acetylation may be involved in a decreased responsiveness of the *c-fos* gene to repeated stress.

Modifying enzymes

During the last decade substantial information has been gathered regarding the identity of the signaling pathways, transcription factors and histone modifying enzymes (protein kinases, HATs) involved in the formation of the dual H3S10p-K14ac mark and indeed in what order these modifications seem to occur. After forced swimming there is a significant increase in CREB phosphorylation (pCREB) (Bilang-Bleuel et al., 2002), possibly by CAMKII and PKA albeit in different time domains (Impey et al., 1999). pCREB binds to the cAMP responsive element (CRE) in the promoter region of cAMP- and many Ca^{2+}- responsive genes such as *c-fos*. pCREB has been shown to recruit co-activators to the chromatin such as CBP and p300 to form the complex PCAF, a well-known HAT (Li et al., 2003;Schiltz et al., 1999). The importance of p300 in memory consolidation has been demonstrated through transgenic mice expressing a dominant negative form of p300 which results in impaired long-term recognition and contextual fear memory formation (Oliveira et al., 2007). The complex has also been shown to acetylate histone H3K14 *in vitro* (Schiltz et al., 1999). Therefore, potentially it is possible that PCAF is the HAT responsible for K14-acetylation in H3 as part of the H3S10p-K14ac dual mark. In a study on mutant CBP mice in which CBP's intrinsic acetyl-transferase activity had been removed but its native protein associations had been left intact, it was found that a loss of CBP's associated HAT activity entailed a significant decrease in c-Fos expression as compared to control mice (Korzus et al., 2004). However, the study focused on CA1 neurons of the hippocampus, thus neurons that do not express the dual H3S10p-K14ac mark in the *c-fos* gene promoter. Therefore, the study provides important knowledge about the role of histone H3 acetylation with regard to *c-fos* gene expression in general but not specifically with regard to the dual histone H3 marks.

As mentioned before the dual H3S10p-K14ac mark is specifically elevated in the dentate gyrus after psychological stressors such as forced swimming. However, in this hippocampal region the dual mark presents in sparsely distributed granule neurons. Thus, less than 5% of all granule neurons are

responding but their distribution is not at all random as H3S10p-K14ac-positive neurons are only observed among mature granule neurons (i.e. located in the upper two-thirds of the granular cell layer) in the dorsal blade of the dentate gyrus. We have observed this typical neuroanatomically specific response pattern after any challenge involving psychological stress such as novelty, forced swimming, MWM training and fear conditioning (Chandramohan et al., 2008;Chandramohan et al., 2007) Chandramohan and Reul, unpublished observations). In contrast, the phosphorylation of CREB after forced swimming occurs in nearly all dentate neurons (Bilang-Bleuel et al., 2002), suggesting that pCREB may have a more general role in these cells such as neuronal protection. Nevertheless, presently it cannot be excluded that in H3S10p-K14ac-expressing neurons the formation of pCREB via the recruitment of CBP/p300 may contribute to the acetylation step as part of histone H3 phospho-acetylation.

Signaling pathways

Recently, we made substantial steps towards elucidating the identity of the signaling molecules which are key to the establishment of the dual modification. Initial pharmacological studies using inhibitors and receptor antagonists as well as gene deletion studies pointed to a role of GRs, NMDA-Rs, MEK (MAPK ERK kinase) and MSK1 in the novelty and forced swimming-induced phosphorylation of S10 and the acetylation of K14 in histone H3 (Chandramohan et al., 2008;Chandramohan et al., 2007). Furthermore, it was shown that GR- and NMDA-R-mediated signaling was required concomitantly to generate the dual mark on histone H3. In aggregate, these observations led to the working hypothesis that H3S10p-K14ac is brought about by concomitant signaling via GR and the NMDA-R/MEK/ERK1/2 signaling pathways, (Chandramohan et al., 2007;Reul et al., 2009). Follow-up immuno-fluorescence studies showed that after forced swimming H3S10p-K14ac/c-Fos-positive dentate granule neurons were found to co-localize with phosphorylated ERK1/2 (pERK1/2, one of the main substrates of MEK) and phosphorylated MSK1 (pMSK1) (Gutierrez-Mecinas et al., 2009).

There was no expression of p-p38MAPK (another major MSK kinase) and pRSK1/2 (a MSK-like kinase) in dentate granule neurons after forced swimming underlining the specificity of the involved ERK MAPK signaling cascade (Gutierrez-Mecinas et al., 2009). In contrast to the sparse distribution of ERK MAPK signaling molecules, GRs are localized in all dentate granule neurons (Reul and De Kloet, 1986). Thus, based on these studies the S10 phosphorylation of histone H3 seemed to be the result of MSK1 activation following signaling through the NMDA-R/MEK/ERK1/2 cascade (Fig. 2). Nevertheless, this notion didn't clarify the acetylation of K14 of histone H3. However, we found co-localization of pERK1/2, pMSK1, H3S10p-K14ac and c-Fos with phosphorylated Elk-1 ((pElk-1, ETS domain protein-1)(Gutierrez-Mecinas et al., 2009)). It has been shown in vitro that the ERK MAPK signaling pathway can lead to the phosphorylation and subsequent activation of Elk-1 and CREB (Davis et al., 2000;Robertson et al., 1995;Yang et al., 2003). When pElk is bound to its binding site in the serum response element (SRE) of gene promoters (e.g. *c-fos)*, it can recruit HATs such as p300 to the chromatin which acetylates K14 in histone H3. Thus, in dentate granule neurons activation of ERK1/2 seems in addition to MSK1 phosphorylation lead to Elk-1 phosphorylation resulting in K14 acetylation in histone H3 (Fig. 2). In aggregate, we demonstrated that within the same dentate neuron the formation of pERK1/2 results in MSK1 activation and Elk-1 activation which in turn phosphorylate S10 and acetylate K14 in histone H3, respectively (Fig. 2; (Gutierrez-Mecinas et al., 2009). The order in which these marks are recruited onto the chromatin has been explored *in vitro*. It has been proposed that H3 Ser10p is a prerequisite for K14ac by the HAT Gcn5. Furthermore, it appears that in yeast H3 phosphorylation promotes the acetylation of K14 (Lo et al., 2000).

In addition to activation of the NMDA-R/MEK/ERK1/2/MSK1-Elk-1 pathway, histone H3 phospho-acetylation in dentate granule neurons after psychologically stressful events also required signaling through GRs in these neurons (Fig. 2). Recently, we discovered that, after forced swimming, activated GRs in dentate gyrus neurons rapidly (i.e. within 15 min) enhance the phosphorylation of MSK1 and Elk-1 thereby stimulating the phospho-acetylation of histone H3 downstream (M.

Gutierrez-Mecinas, A.F. Trollope, A. Collins, H. Morfeth, S. Hesketh, F. Kersante, J.M.H.M. Reul, submitted manuscript). This interaction of GR with the ERK MAPK signaling pathway is a novel non-genomic mechanism of glucocorticoid action in the brain.

DNA methylation and learning and memory

As mentioned, DNA methylation at gene promoters is thought to be associated with gene silencing. Evidence has been accumulating for a role of DNA methylation changes in the hippocampus in memory formation. After fear conditioning training in rats an up-regulation of hippocampal DNMT mRNA expression was found (Miller and Sweatt, 2007). Inhibition of DNMTs using 5-aza-deoxycytidine (5-AZA) and zebularine (zeb) in the CA1 region of the hippocampus resulted in a marked reduction of freezing behavior suggesting that the DNMTs are required for the consolidation of contextual memories (Miller and Sweatt, 2007). It was thought that the reduction in DNA methylation prevented the necessary silencing of the memory suppressor gene PP1. However, fear conditioning also resulted in a reduction in methylation at specific genes such as *reelin* resulting in an increased expression of this gene (Miller and Sweatt, 2007). These data demonstrate that DNA methylation is not a static process but it appears that it can be highly dynamic in the hippocampus during memory formation (Miller and Sweatt, 2007).

Epigenetic control of the DNA has been further complicated by the discovery of hydroxy-methylated cytosine residues (Tahiliani et al., 2009). This mark is thought to be associated with active genes. Whether the hydroxy-methyl-cytosine mark is part of the DNA de-methylation process and/or a DNA repair process is currently unclear. The role of the newly discovered mark in brain and behavior mechanisms also needs to be clarified.

Interdependence between PTMs of histones and DNA methylation

For considerable time it was thought that histone modifications and DNA methylation events are unrelated but based on various observations in recent years it is becoming increasingly clear that the two forms of epigenetic mechanisms do interface. It is presently still unresolved though how the mechanisms inter-relate, whether there is a dominance of one or the other, etc. It has been shown *in vitro* that active histone marks can prevent the spread of methylation of cytosine residues into gene promoter regions and that DNA methylation which is adjacent to a promoter does not always repress transcription of that gene (Brinkman et al., 2007). In the absence of DNA methylation at CpG islands there appears to be a dependence of the presence of H3K4me2/3 mark, in order to protect the DNA from methylation, suggesting that the histone K4 methylation status dictates subsequent DNA methylation (Ooi et al., 2007). Other *in vitro* evidence supports this idea by showing that repressive histone marks are a prerequisite for DNA methylation. The di-methylation of H4R3 by protein arginine methyltransferase (PRMT) recruits DNMT3a resulting in the methylation of adjacent CpG islands (Zhao, Rank, Tan, Li, Moritz, Simpson, Cerruti, Curtis, Patel, Allis, Cunningham, and Jane, 2009). As discussed earlier H3K9me2 formation by EHMT1 recruits HP1 to the chromatin causing DNA methylation in a DNMT1 dependent manner to silence the *Survivin* gene in cell culture (Smallwood et al., 2007). However, there is also evidence that histone modifications are a secondary event to DNA methylation, some DNMTs and MBDs (methyl-binding domains) recruit other complexes that posses HDAC activity (Bird, 2002). The order in which these events occur is not fully understood but as histone-modifying and –binding factors can form multimeric molecular 'machines' the various histone-based and DNA-based epigenetic modifications may be executed in a concerted action. How both epigenetic modifications interact *in vivo* is still unclear.

Epigenetics, early life events, and psychiatric and neurological disorders

There have been several studies by Meaney and co-workers showing a link between epigenetics and behavioral programming in offspring (Weaver et al., 2004) They showed that rat mothers who

displayed more and higher quality nurturing towards their pups, shown as increased pup licking (LG) and arched-back nursing (ABN), resulted in the pups displaying a significantly reduced level of DNA methylation along the egr-1 binding site in the GR promoter. This was associated with an increase in hippocampal GR expression while the opposite was found in pups whose mothers had shown low level LG and ABN. In a human study, compared to control subjects DNA methylation along the GR gene (NR3C1) was increased and GR expression was decreased in the hippocampus of victims of child abuse that had committed suicide (McGowan et al., 2009). These data impressively demonstrate that early life events can have an effect on epigenetic regulation of the GR gene and have implications regarding individual differences in the risk for psychopathology when adult. Other psychiatric conditions such as post-traumatic stress disorder (PTSD) have been shown to be linked to epigenetic changes in immune system genes such as the interleukin 8 (IL-8) gene (Segman et al., 2005). Blood samples taken from control individuals and PTSD patients were analyzed using microarrays for DNA methylation changes at CpG sites for over 14,000 genes. They found that immune system-associated genes were uniquely unmethylated in those with PTSD suggesting that these pathways are more active, e.g. the IL-8 gene is upregulated (Uddin et al., 2010). Epigenetic mechanisms are tightly regulated and if disturbed this can lead to psychiatric, neurological and developmental disorders such as for instance 9q subtelomeric deletion syndrome. 9q subtelomeric deletion syndrome occurs when the expression of the K9-HMT EHMT is reduced due to haplo-insufficiency. The classic phenotype of this disease is severe mental retardation, macroglossia, brachy(micro)cephaly, heart defects, a flat face with hypertelorism, synophrys, thickened lower lip and down-turned corners of the mouth (Kleefstra et al., 2006).

Conclusion

Animals learn from stressful experiences and form memories of the event that may help them to deal better with future occurrences. The consolidation of these memories has been shown to

require distinct signaling and epigenetic mechanisms in neurons of the central nervous system. In recent years detailed knowledge of such mechanisms has become available regarding those occurring in dentate gyrus granule neurons in the context of the consolidation of stress-related memory formation. It was found that stressful learning events evoke the concomitant activation of GRs and NMDA-Rs and thereby in concert the activation of the downstream ERK MAPK signaling pathways targeting chromatin modifications. The signaling cascades lead to post-translational changes in histone molecules such as H3 resulting in the formation of the dual modification H3S10p-K14ac and subsequent induction of gene transcription of *c-fos* and other gene products specifically in granule neurons of the dentate gyrus. These events are critical for the consolidation of hippocampus-dependent memory in various behavioral paradigms. Evidence is also accumulating supporting a role of histone methylation and DNA methylation and de-methylation events in cognitive processes in the brain.

Thus, epigenetic mechanisms such as histone PTMs and DNA (de-)methylation play a principal role in steering gene expression processes underlying the consolidation of memory formation as part of the behavioral response to psychologically stressful events. It is also becoming increasingly clear that these mechanisms are working in concert and not independently. The understanding of these complex mechanisms is of great importance to resolve many neurological and psychiatric disorders in the future.

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Legends to the figures

Figure 1

(A) Schematic representation of ‘closed’ and ‘open’ chromatin. (B) Part of the N-terminal tail of histone H3 with some possible post-translational modifications. The orange rectangles denote the S10 phosphorylation and the K14 acetylation sites.

Figure 2

Schematic representation of the signaling and epigenetic pathways in granule neurons of the dentate gyrus thought to be involved in the consolidation process of memory formation after a psychologically stressful challenge. Activation of NMDA-Rs results in stimulation of the ERK MAPK signaling cascade. In conjunction with activated GRs this signaling cascade results in the activation of MSK1 and Elk-1 leading to the formation of the dual H3S10p-K14ac histone marks along the *c-fos* promoter and subsequently induction of gene transcription. Possibly, signaling via CREB may play a role as well. The induction of gene transcription is thought to be instrumental in the consolidation of memory formation in various stressful learning events such as forced swimming, Morris water maze learning and contextual fear conditioning.

Figure 1

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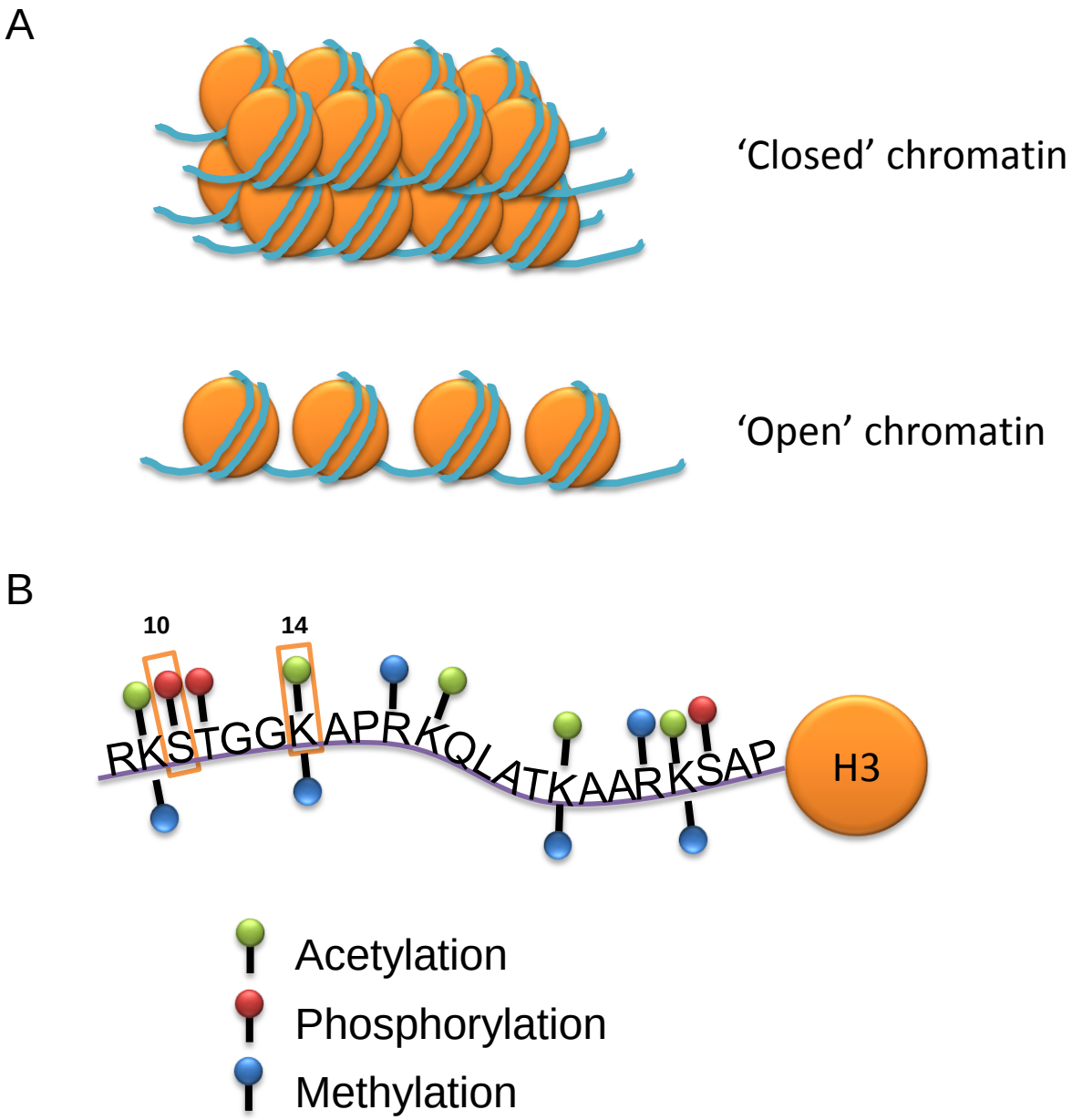

Figure 2 Revised

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Figure 2