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Epigenetic mechanisms in stress and adaptation

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Review: Epigenetic mechanisms in stress and adaptation

Abstract

Epigenetic mechanisms are processes at the level of the chromatin that control the expression of genes but their role in neuro-immuno-endocrine communication is poorly understood. This review focuses on epigenetic modifications induced by a range of stressors, both physical and psychological, and examines how these variations can affect the biological activity of cells. It is clear that epigenetic modifications are critical in explaining how environmental factors, which have no effect on the DNA sequence, can have such profound, long-lasting influences on both physiology and behavior. A signaling pathway involving activation of MEK-ERK1/2, MSK1, and Elk-1 signaling molecules has been identified in the hippocampus which results in the phospho-acetylation of histone H3 and modification of gene expression including up-regulation of immediate early genes such as c-Fos. This pathway can be induced by a range of challenging experiences including forced swimming, Morris water maze learning, fear conditioning and exposure to the radial maze. Glucocorticoid (GC) hormones, released as part of the stress response and acting via glucocorticoid receptors (GRs), enhance signaling through the ERK1/2/MSK1-Elk-1 pathway and thereby increase the impact on epigenetic and gene expression mechanisms. The role of synergetic interactions between these pathways in adaptive responses to stress and learning and memory paradigms is discussed, in addition we speculate on their potential role in immune function.

Introduction to stress

The nervous, endocrine and immune systems engage in intense communication throughout the mammalian physiological system via neuronal, hormonal and immunological networks respectively. These systems are widely integrated with each other and allow the organism to adapt to environmental changes and challenges. Stress has been defined as 'a threat, real or implied, to the maintenance of a narrow range of vital homeostatic parameters necessary for survival' (McEwen, 2000). Stress induces a number of biological responses which enables the organism to adapt to the challenge and increase its likelihood of survival. Immediate activation of the sympathetic nervous system releases adrenaline from stores in the adrenal medulla, preparing the organism for a fight or flight response. In addition, stress increases levels of neurotransmitters such as serotonin (5-HT) and noradrenaline (NA) in specific brain regions (i.e. hypothalamus, hippocampus) (Linthorst et al., 1995a; 1996; Linthorst et al., 2002). 5-HT and NA act at the hypothalamus to activate the hypothalamic-pituitary-adrenal (HPA) axis resulting in the release of GCs from the adrenal cortex into the circulation (Carrasco and Van de Kar, 2003). It is hypothesised that the rise in extracellular 5-HT in the hippocampus supports the animal's risk assessment of the nature of the stressor so it can make appropriate adaptations (Linthorst and Reul, 2008).

Animal models for investigating the effects of acute stress

Researchers investigating the effects of stress *in vivo* have developed a number of behavioral tests designed to model neuropsychiatric disorders, for review see Nestler and Hyman (2010). In many stress studies animals are exposed to stressors for a limited amount of time and subsequent changes in physiology and behavior determined. Stressors are generally described as either physical or psychological but in reality most stressors present both types of stress and therefore their 'classification' is based on which biological system (physical or

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psychological) is affected the most. Physical stressors, such as a hot or cold environment, have a direct effect on the body, disturbing homeostatic processes and influencing the physiological state of an organism. In contrast, psychological stressors involve higher brain areas, such as the neocortex, amygdala and hippocampus, to process and/or interpret information about the threat. Consequently, in case of psychological stress, the threat may be implied but not necessarily experienced (Herman, 2003).

Both physical and psychological stress activate the HPA axis leading to downstream adrenocorticotropic hormone (ACTH) release from the anterior pituitary and subsequent release of GCs (cortisol in humans, corticosterone in rodents) from the adrenal cortex into the circulation. GCs were so named after their effects on glucose metabolism were discovered. Circulating GCs, however, have equally important roles in modulating the immune response and in cognitive processes such as memory formation (see next section). Despite both physical and psychological stressors activating the same central HPA pathway, a number of studies have provided evidence that the body responds to the different types of stress in distinct ways (Dayas et al., 2001; Yuen et al., 2009). Furthermore, because of its nature, psychologically stressful events lead to profound, long-term changes in behavior.

Role of memory formation in the stress response

Memory formation is the storage of information which can be recalled in the future. The processes involved in the formation of memories have been the focus of numerous studies spanning over 30 years but a definitive pathway is still being elucidated. Biochemical pathways leading to memory formation can be examined through the use of behavioral tests such as the Morris water maze, Pavlovian fear conditioning, radial maze and others - a summary of the most common behavioral tests for stress/learning and memory studies are outlined in Table 1. All of these tests have a 'stressful' component which must be taken into

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account when interpreting experimental findings. Indeed in recent years there has been considerable overlap between investigations into stress responses and learning and memory processes, with signaling pathways emerging that are common to both areas. Long term responses to stress include the formation of episodic memories which increase the chance of avoiding the stressful challenge in the future or improve the ability of the organism to cope with any subsequent repeat exposure to the stressor (Reul and Chandramohan, 2007; Reul et al., 2009) This is, in part, due to the strong consolidation of memories by the induction of stress hormones such as GCs, however, the exact mechanism for this consolidation remains unclear (Oitzl and de Kloet, 1992; Cordero and Sandi, 1998; De Quervain et al., 2009; Sandi, 2011).

Epigenetics

As discussed in the first review of this series (Mathews and Janusek, 2011), epigenetic modifications change the expression of genes without altering the DNA sequence. These modifications can occur on the DNA itself to influence gene expression by changing the accessibility of genes to transcription/translation factors. Alternatively, other distinct epigenetic modifications can occur on the highly conserved tails of histone proteins, responsible for organizing DNA into chromatin, thereby affecting chromatin assembly and organization of DNA.

According to the National Institute of Health, epigenetic changes can be 'heritable changes in gene activity and expression (in the progeny of cells or of individuals) and also stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable'. The latter aspect explains how environmental influences and life experiences can result in differences in gene expression and downstream biological changes, even after the initial

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influence has disappeared. The different types of epigenetic modifications influencing gene expression are reviewed extensively by Kouzarides (2007) and Mathews and Janusek (2010). This review focuses on DNA methylation, histone methylation, phosphorylation and acetylation and as such, only these epigenetic modifications will be discussed.

DNA modifications

DNA methylation occurs when a methyl group is added to one of the pyrimidine bases of DNA. Methylation occurs on the 5th carbon of cytosine residues (5mC) situated adjacent to a guanine residue (CpG site). Parts of DNA sequences with a high concentration of CpG residues are referred to as CpG 'islands' and generally located at the start of the gene sequence within the promoter region. The methylation of DNA within the promoter region of a gene may be why this modification has such a profound effect on gene expression, generally silencing the expression of the respective gene (Illingworth and Bird, 2009).

DNA methylation can occur by two type of enzymes; methyl-transferases *de novo,* which set-up methylation marks during early development and are critical for the survival of organisms, and maintenance methyl-transferases which are essential for maintaining the methylation code (Wu and Zhang, 2010). The importance of demethylation has been investigated to a greater extent in recent years as it has become apparent that DNA methylation status can change quite rapidly (Wu and Zhang, 2010). The exact biochemical processes involved in DNA demethylation remains unclear but there are a number of mechanisms currently being discussed including enzymatic removal, deamination and base excision repair, nucleotide excision repair, oxidative demethylation and radical S-adenosylmethionine (SAM)-based demethylation (for review see Wu and Zhang (2010)).

In addition to methylation and demethylation, mammalian DNA can by hydroxylated (oxidation of CH group to form COH) at the carbon 5 position of methylated cytosine residues (Penn et al., 1972) but the induction and function of this hydroxy-methylation (5hmC) modification is unknown. More recent studies (Tahiliani et al., 2009; Zhang et al., 2010) have identified 5-methylcytosine (5mC) hydroxylases like ten-eleven translocation proteins (TET1, TET2 and TET3) as enzymes responsible for hydroxylation of methylcytosine but the function of this mark still remains unclear. It has been proposed that 5hmC facilitates passive demethylation (promoting gene transcription) by preventing DNMT's maintaining the methylation status of DNA, however, this is yet to be confirmed (Tahiliani et al., 2009). Hydroxy-methylation of DNA recruits 5hmC-specific factors and prevent the association of some 5mC-specific enzymes/transcription factors in DNA methylation assays and cancer cell lines, indicating distinct roles for the two types of cytosine modification in gene expression (Valinluck and Sowers, 2007; Tahiliani et al., 2009; Ko et al., 2010).

Histone modifications

Histone proteins are responsible for organizing DNA into chromatin which can exist in a densely-packed 'closed' configuration or in a loose, 'open' state, which is required for gene transcription. There are four core histone proteins, H2A, H2B, H3 and H4, each with a highly conserved N-terminal tail which can be modified at certain sites by a range of specific enzymes to influence the structure and function of the chromatin.

Histone methylation occurs on either arginine or lysine residues in histones H3 and H4. The enzymes which add (methyl-transferases) or remove (demethylases) methyl groups are specific to the residue on which they act, either lysine or arginine, and can also be specific for

the location or site of the residue within the histone tails. For example, mixed lineage leukemia proteins (MLLs) are methyl-transferases which act almost exclusively to methylate lysine residue 4 of histone H3 (Cosgrove and Patel, 2010). The function of the methylation mark depends on the site on which it occurs; lysine methylation of H3K4 (lysine 4 of histone 3) is associated with transcriptionally active chromatin and gene transcription whereas methylation on H3K9 is associated with gene silencing (Kouzarides, 2007; Akbarian and Huang, 2009). The consequence of methylation is also dependant on how many methyl groups are added to each residue; lysine residues hold one, two or three methyl groups and are classified as mono, di or trimethylated respectively, whereas arginine residues can only hold up to two methyl groups (Kouzarides, 2007). The function of methylated H3K20 varies greatly with methylation status, mono-, di- and trimethylation of this site has been associated with gene transcription, gene repair and gene silencing respectively (Balakrishnan and Milavetz, 2010). The consequence of epigenetic marks is further complicated by the interaction of different modifications on each other; for example, protein arginine methyl-transferase (PRMT) methylation of H3R2 prevents methylation of H3K4 by MLL and therefore inhibits subsequent gene expression *in vitro* (Hyllus et al., 2007).

Histone phosphorylation occurs when a phosphate group is added to an amino acid residue (usually serine) in the highly conserved histone tail by specific kinases and can be removed by specific phosphatases (dephosphorylation) (Thomson et al., 1999b; 1999a; Clayton et al., 2000; Soloaga et al., 2003). The phosphorylation status of chromatin is determined by the net activity of kinases and phosphatases, influenced by signaling cascades. Phosphorylation of certain residues is often associated with other epigenetic marks, for example if histone H3 in dentate gyrus neurons is phosphorylated at serine 10 then it is also generally acetylated at lysine 14 (Chandramohan et al., 2007). This co-modification is associated with the opening of

previously condensed chromatin (Cheung et al., 2000; Clayton et al., 2000; Nowak and Corces, 2000) .

Histone acetylation occurs when an acetyl group ($COCH_3$) is added to a lysine residue by histone acetyl-transferases (HATs). Once again these enzymes act at distinct residues/sites within the N-terminal tail of histone proteins. Given the neutralization of the negative lysine charge by the addition of a positively charged acetyl group, histone acetylation is the modification most likely to decondense chromatin and expose previously silent genes for transcription (Kouzarides, 2007). This epigenetic mark is therefore almost exclusively associated with active gene transcription for numerous genes studied. Acetyl groups are removed by histone deacetylases (HDACs).

Some epigenetic marks such as methylation of DNA can be passed on to subsequent generations through mitotic and meiotic cell division (Nakayama et al., 2000; Schreiber and Bernstein, 2002; Champagne, 2008). This is an interesting property which is not shared by alternative mechanisms of altering gene expression (i.e. the influence of transcription factors). Although changes in the epigenetic profile of DNA were traditionally thought to be hereditary, recent studies indicate these changes can be transient (Ng et al., 2009). Epigenetic modifications are therefore a set of biochemical tools which control the expression of genes and are themselves controlled by signaling mechanisms and provide another level at which the environment can impact on gene expression (Figure 1).

Role of epigenetic mechanisms in the long-term impact of early life stress

The epigenetic profile of rats is profoundly influenced by the maternal behavior they experienced during the first week of life (Weaver et al., 2004). Rats which received high

levels of maternal care (HMC rats, maternal care defined as licking, grooming and arched-back nursing) during the first week of life were less anxious, less sensitive to stress in adult life and had higher levels of GR expression in the hippocampus compared with rats experiencing low levels of maternal care as pups (LMC rats).

Analysis of the methylation state of the GR promoter during the development of HMC or LMC pups revealed changes in the methylation status of a specific cytosine residue (site 16 on exon 1_7), which corresponded to a specific region in the GR promoter containing a consensus sequence for a transcription factor known as early growth response factor 1 (Egr-1). At the embryonic stage site 16/exon 1_7 is unmethylated but DNA methylation of this site occurs shortly after birth. In HMC pups demethylation of site 16/exon 1_7 occurs during the first week of life, whereas this site in LMC rats remains methylated. Since this site contains a consensus sequence for Egr-1, the association of Egr-1 protein with the GR promoter was analysed by chromatin immuno-precipitation assay (ChIP) and found to be 3-fold higher in HMC rats compared with LMC rats. ChIP analysis of GR also found higher levels of acetylation at histone H3 lysine 9 (H3K9), a marker for uncondensed, transcriptionally active chromatin (Weaver et al., 2004).

Prolonging histone acetylation by intracerebroventricular (i.c.v) administration of histone deacetylase (HDAC) inhibitor trichostatin A (TSA) in LMC rats supported a role for histone acetylation in the response by promoting a phenotype closely associated with that of HMC rats (Weaver et al., 2004). Likewise, when Egr-1 binding to the GR promoter was inhibited by DNA methylation at site 16/exon 1_7 the HMC phenotype (less anxious, less sensitive to stress and higher GR expression in hippocampus) disappeared indicating a key role for demethylation of DNA in this phenotype (Weaver et al., 2004). Later studies found that the

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level of maternal care experienced when pups by female rats determined what kind of mother they would themselves become as a consequence of epigenomic changes in a different gene, namely the estrogen receptor gene located in the medial preoptic area, highlighting the trans-generational effects of epigenetic modifications (Champagne, 2008). Generally it appears that HMC rats become HMC mothers and LMC rats become LMC mothers (Champagne, 2008).

A link has also been demonstrated between early life experiences and adult behavior in humans. Numerous clinical studies have shown a positive association between childhood trauma and increased risk of developing psychopathological disorders such as depression as an adult (Heim et al., 2008). Children experiencing abuse, neglect or other adverse experiences displayed a sensitized stress response, GC resistance, increased corticotropin-releasing factor (CRF) activity, immune activation and reduced hippocampal volume; all features which have been observed in some depressed patients as adults (Heim et al., 2008). Reduced GR mRNA expression in the hippocampus of suicide victims who had been abused as children was associated with epigenetic changes in the neuron-specific GR promoter (NR3C1) when compared with suicide victims with no history of childhood abuse (McGowan et al., 2009). There were also higher levels of DNA methylation in the hippocampal GR promoter in abuse victims compared with non-abuse controls; this pattern of methylation resulted in reduced binding of transcription factor Egr1 and decreased levels of Egr1-induced gene transcription when investigated *in vitro* (McGowan et al., 2009). Based on these studies, epigenetic modifications play a key role in mediating the effect of early life experiences on responses to stress in adulthood.

Epigenetics: stress studies and signaling pathways

Our recent studies have investigated the effect of acute stressors on epigenetic modifications in brains of adult rats. The primary stressor used in our studies is the forced swim test and we are particularly interested in the subsequent immobility response displayed by the rats in the forced swim retest (24 h after initial test). There is controversy surrounding the interpretation of the animal's immobility response. Originally the immobility response was viewed as a 'depressive' behavior because it was thought that the animal had given up and showed despair (Porsolt et al., 1977; Lucki, 1997). In addition, the effects of some antidepressant drugs to lower the immobility response seemed to support this notion. On the other hand, acute administration of many antidepressants evokes arousal through central release of serotonin, noradrenaline and/dopamine which may provide an alternative explanation for the reduced immobility behavior. Furthermore, the effect of three administrations of antidepressant drug in the Porsolt forced swim test design is hard to reconcile with the clinical effects of antidepressants which take at least 3 weeks of treatment to emerge. Our group and others feel that the immobility response is an adaptive response to the knowledge that they cannot escape making it more appropriate to conserve energy to maximize the chance of survival (De Pablo et al., 1989; West, 1990; Korte, 2001; Bilang-Bleuel et al., 2005; Nestler and Hyman, 2010). The rapid adoption of immobility behavior in the retest supports the idea that the subject remembers the initial test and conserves its energy by floating. Further support of immobility being an adaptive response comes from forcing the rats to swim in water at different temperatures. Normally the rats are swum at 25^0C, however, when rats are swam at 19^0C rats they show increased struggling and reduced immobility during the retest, possibly because of the extreme loss of body temperature ($\sim 12^0$C). In contrast, rats that swam at 35^0C showed high levels of immobility and very little struggling (Linthorst et al., 2008). This experiment underscores the immobility behavior being an

adaptive response to the combined physical and psychological stress. The observed ability of the rats to recall the initial experience, as shown by the increase in immobility in the retest, lasts at least 4 weeks from initial test (Gutièrrez-Mechinas, Collins, & Reul; unpublished observations). Once again this finding supports a role of learning and memory processes in the adaptive immobility behavior shown and refutes the depression hypothesis.

Immunofluorescent staining confirmed epigenetic modifications were taking place in distinct brain areas with positive staining for phosphorylation of serine 10 and acetylation of lysine 14 in histone H3. Under baseline conditions very low levels of H3 phospho-acetylation were found in the dentate gyrus, neocortex, amygdala and striatum (Bilang-Bleuel et al., 2005; Chandramohan et al., 2007). On exposure to forced swim stress there was a marked increase in phospho-acetylation of histone H3, specifically in the dentate gyrus, which did not occur after exposure to purely physical stresses such as cold environment and ether exposure. This finding indicates that the psychological component of the stress may be responsible for the observed increase in H3 phospho-acetylation (Bilang-Bleuel et al., 2005).

N-methyl-D-apartate (NMDA) receptor blockade, using receptor antagonist MK-801, prevented the forced swim induced increase in H3S10p-K14ac positive neurons in the dentate gyrus of rats and also reduced the associated immobility behavior in the 1-day retest (Chandramohan et al., 2008). Neurons in the dentate gyrus are under tonic inhibitory control by gamma amino-butyric acid (GABA)-ergic interneurons. FG-7142 (β-carboline-3-carboxylic acid N-methylamide), a GABA-A receptor partial inverse agonist attenuated the GABAergic control of granule neurons in the dentate gyrus, resulting in a rise in the number of H3S10p-K14ac-positive neurons in this hippocampal structure (Papadopoulos et al., 2010). The proportion of granule neurons in the dentate gyrus that overcome the inhibitory

regulation is dependent on the strength of the stimulus. Most psychological stressors (predator exposure, novel environment etc.) activate <5% of dentate gyrus granular neurons from inhibitory control. In contrast, much stronger stimuli such as electroconvulsive shock or injection of depolarizing drugs can overcome virtually all inhibitory GABAergic control resulting in activation of all dentate granule neurons (Green and Vincent, 1987; Crosio et al., 2003).

Once the inhibitory control is overcome by excitatory inputs (mainly glutamatergic), glutamate binds to NMDA receptors resulting in a rise in intracellular calcium (Ca^{2+}) concentration. Increased intracellular Ca^{2+} levels trigger a number of biochemical signaling cascades including the activation of adenylate cyclase (and subsequent protein kinase A (PKA) activation), Ca^{2+}-calmodulin kinase II (CAMKII) and the mitogen-activated protein kinase (MAPK) cascade leading to activation of the extracellular-regulated protein kinases 1 and 2 (ERK1/2). Inhibition of ERK1/2 activation, using an inhibitor (i.e. SL-327) of the upstream MAPK ERK kinase (MEK), abolished phospho-acetylation of histone H3 and the subsequent immobility response in response to forced swim stress (Chandramohan et al., 2008). ERK1/2 targets a number of downstream kinases including mitogen and stress-activated kinases 1 and 2 (MSK1/2). MSK1/2 double knock-out mice subjected to the same forced swim protocol (although initial test was for 10 min as opposed to 15 min) showed no increase in phospho-acetylation of histone H3 and the immobility response was also blocked compared with control mice (Chandramohan et al., 2008). This study shows that blocking ERK1/2 and MSK signaling prevents forced swim induced epigenetic changes and subsequent immobility behavior during the retest, indicating a key role for these signaling molecules in the adaptive behavioral response to stress.

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Once activated, MSK can phosphorylate residues on histone tails including S10 which may explain its critical role in inducing epigenetic modifications (Figure 2), however, MSK has no known acetylase activity (Hauge and Frödin, 2006; Arthur, 2008; Drobic et al., 2010). MSK can also phosphorylate cAMP responsive element (CRE) binding protein (CREB) which after dimerization can bind to CRE sites in the promoters of many cAMP- and Ca^{2+}-responsive genes thereby stimulating gene expression (Figure 2). Furthermore, CREB can recruit a number of histone modifying enzymes to the chromatin including p300 and/or CREB binding protein (CBP), both of which have histone acetyl-transferase (HAT) activity and have been shown to potentiate long term memory formation (Vecsey et al., 2007). Despite the promising role of CREB in promoting stress-induced epigenetic modifications, investigations into its activation in response to forced swim stress revealed widespread activation throughout the dentate gyrus, the rest of the hippocampus and many other brain areas (Bilang-Bleuel et al., 2002). The widespread phosphorylation of CREB in dentate gyrus neurons after psychological challenges such as forced swimming presents a conundrum which will be addressed further below.

An alternative downstream target of ERK activation is E twenty-six (ETS)-domain protein (Elk-1), a transcription factor which can bind to Elk binding sites in serum response elements of gene promoters and recruit HATs such as p300 (Li et al., 2003). Immunohistochemical studies identified pElk-1 expression in the same neurons that were positive for H3S10p-K14ac, pERK1/2 and pMSK1 confirming a potential role for this signaling molecule in acetylation of H3 (Gutierrez-Mecinas et al., 2009).

Expression of the immediate early gene *c-fos* is commonly used as a marker for neuronal activation as it is often expressed when neurons become activated (Hoffman et al., 1993). c-

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Fos is a transcription factor which acts in conjunction with other proteins to induce transcription of a wide variety of genes. Expression of c-Fos in the dentate gyrus was increased after exposure to forced swim stress and immunohistochemical staining demonstrated that c-Fos was expressed in specific H3S10p-K14ac, pERK1/2, and pMSK1 positive granule neurons within the dentate gyrus (Chandramohan et al., 2007; 2008; Gutierrez-Mecinas et al., 2009). More recent studies have provided evidence for the phospho-acetylation of histone H3 tails within the *c-fos* promoter by ChIP and real time polymerase chain reaction (qPCR) analysis, finding an enhanced enrichment of H3S10p-K14ac in the *c-fos* promoter after exposure to forced swim stress (Gutierrez-Mecinas et al., 2009; Trollope and Reul, unpublished results). It therefore seems likely that c-Fos expression is induced by phospho-acetylation of histone H3 and may be the link between biochemical signaling pathway activation and changes in target gene expression.

In view of the strong transactivation potential of pCREB regarding c-fos gene transcription (Berkowitz et al., 1989; Boutillier et al., 1992; Herdegen and Leah, 1998), it is obvious that the forced swimming-induced sparse induction pattern of c-Fos in dentate granule neurons in the face of a virtually ubiquitous activation of CREB in these neurons is a striking conundrum. Basically, the question is why is c-Fos only sparsely induced in dentate neurons when pCREB is generated in all neurons? Our recent signaling and epigenetic studies may provide an answer to this question. We have shown that the induction of c-Fos in dentate neurons requires the activation of GRs in conjunction with the NMDA/ERK1/2/MSK1-Elk-1 signaling pathway which results in the phosphorylation and acetylation of histone H3 tails. This combinatorial histone mark is known to be involved in the opening of condensed, inactive chromatin rendering it available for transcription (Cheung et al., 2000; Clayton et al., 2000; Nowak and Corces, 2000). As transcription factors like pCREB require an open

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chromatin structure in order to transactivate gene promoters, the sparse induction pattern of c-Fos in dentate neurons is most likely the result of an open chromatin configuration of this gene evoked by the phospho-acetylation of histone H3 specifically in these neurons. In dentate neurons in which the ERK MAPK pathway is not activated and dual histone marks do not evolve, the chromatin containing the *c-fos* gene will remain condensed and inactive and any generated pCREB will not be able to transactivate the *c-fos* gene promoter. Thus, it seems that in dentate granule neurons GR and NMDA-ERK-MAPK driven histone modifications and subsequent chromatin remodeling is a prerequisite for the transactivation potential of transcription factors like CREB. In other parts of the brain c-Fos induction does not require histone H3 phospho-acetylation and thus in these areas the gene seems to be in an open chromatin configuration. Accordingly, in areas such as the neocortex, a widespread CREB activation is matched by a widespread c-Fos induction (Bilang-Bleuel et al., 2002). The details of our concept are outlined in Figure 2.

The hippocampus expresses high levels of GR and is responsible for co-ordination of neuroendocrine and behavioral responses to stress making it a prime site for studies of the stress response *in vivo* (Reul and de Kloet, 1985; De Kloet and Reul, 1987; De Kloet et al., 2005). In the early 1980s it was observed that the behavioral immobility response, as displayed in the forced swim re-test, is strongly dependent on glucocorticoid hormones secreted during the initial forced swim challenge and acting via GRs (Veldhuis and De Kloet, 1983; Jefferys et al., 1983). Follow-up work demonstrated that specifically GRs in the dentate gyrus are crucial for the forced swimming induced behavioral immobility response (De Kloet et al., 1988). Using the selective receptor antagonists RU38486 and ORG 34517 we examined whether there was a relationship between the histone H3 phospho-acetylation response and the behavioral immobility response following forced swimming. The forced

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swimming induced increases in both H3S10p-K14ac positive neurons in the dentate gyrus and immobility behavior in the 24-h re-test were dependent on GR activation. Thus, in recent years a picture has emerged indicating that the induction of H3S10p-K14ac and c-Fos in dentate granule neurons as well as the consolidation of the behavioral immobility response require signaling through both the GR as well as the NMDA-ERK-MAPK pathways (Bilang-Bleuel et al., 2005; Chandramohan et al., 2007; Reul and Chandramohan, 2007; Reul et al., 2009). Blockade of either pathway lead to abrogated epigenetic, gene expression and behavioral responses. Recent work suggests that the interaction of signaling pathways is not occurring at the genomic level but rather through direct cross talk between participating signaling molecules at the protein-protein level. After forced swimming, GR activation enhances the downstream activation of the ERK1/2 dependent kinases MSK1 and Elk-1 resulting in enhanced responses in histone H3 phospho-acetylation and c-Fos induction in dentate granule neurons (Gutierrez Mecinas et al., 2009). This is a novel non-genomic mechanism of GC hormone action.

A serendipitous finding emerging from the above studies found a decrease in hyperacetylation of histone H4, another epigenetic modification, following forced swim stress at multiple locations within the brain (neocortex, hippocampus etc) (Trollope and Reul, unpublished results). In contrast, the stress-induced increase in H3 phospho-acetylation (H3S10p-K14ac) and c-Fos was specifically located to the dentate gyrus, with the majority of positive neurons situated amongst the mature neurons of the middle and outer aspects of the granular cell layer in the dorsal blade of the dentate gyrus (Bilang-Bleuel et al., 2005; Chandramohan et al., 2007; 2008). Since young, immature neurons of the dentate gyrus are located in the inner aspect of the granular cell layer, close to the subgranular zone, this finding indicates a high degree of neuroanatomical specificity with regard to this molecular

response to stress and highlights how the accessibility of a gene may vary between cell populations and tissues depending on chromatin conformation.

Epigenetics: role in learning and memory

Adaptive and coping behaviors such as immobility in the forced swim retest and freezing in the fear conditioning retest are dependent on successful memory formation during initial training/tests. GR activation during the consolidation phase of the forced swim test is required for the normal expression of behavioral immobility in the retest. Studies in learning and memory tests such as contextual fear conditioning and Morris water maze learning have identified a similar dependence on GR at a critical stage in memory consolidation hinting that these tests may all share a central pathway for memory formation (Oitzl and de Kloet, 1992; Cordero and Sandi, 1998; Revest et al., 2005). Histone modification and c-Fos induction have also been shown to occur in response to a number of learning and memory paradigms *in vivo* (Chwang et al. 2007). In these studies, ERK1/2 inhibition (by the specific MEK inhibitor U0126) and MSK1 knockout mice were used in fear conditioning and Morris water maze tests to identify an ERK1/2-MSK1-H3S10p-K14ac dependent signaling pathway in the mouse hippocampus. This pathway was responsible for the adaptive behavior learnt and displayed in these tests; blocking this pathway blocked the learned behavior (Chwang et al., 2007). This ERK1/2-MSK1-H3S10p-K14ac pathway identified by Chwang et al. (2007) is analogous to the one we identified in response to forced swim and novelty stress indicating that a common pathway is activated by a range of stressful learning paradigms.

Memory formation plays a key role in both stress responses and learning paradigms, and epigenetic modifications are critical in the response to a variety of stressors such as forced swim, Morris water maze and fear conditioning (Chwang et al., 2007; Chandramohan et al.,

2008). Taken together, these observations indicate that epigenetic modifications underlying changes in gene expression may be contributing to the process of memory formation and/or retrieval alongside previously identified modulators of learning and memory processes.

Application of the signaling pathways to the chromatin during immune stress

The focus of this review has been the role of epigenetic modifications in psychological stress responses and memory formation but there is evidence in the literature of similar pathways playing a role in the epigenetic response to immune stress. This may not be surprising since the immune system is highly integrated with the neuronal and endocrine systems and interacts significantly with key modulators of both systems (Besedovsky and Rey, 2007).

GCs, released after HPA axis activation, modulate the expression of a broad range of cytokines (Wiegers and Reul, 1998). The traditional view is that GCs generally inhibit proinflammatory cytokine expression but in other studies GC release increases the expression of receptors for these proinflammatory cytokines (Wiegers and Reul, 1998; Liberman et al., 2007). This apparent conundrum may be explained if the role of GCs is to 'optimise the course of a biological response' and regulate the balance between pro- and anti-inflammatory cytokine expression (Wiegers and Reul, 1998; Elenkov and Chrousos, 2002). One mechanism by which GCs may optimise the biological response was proposed by Wiegers and Reul (1998). They used the example of T-cell proliferation to demonstrate that the action of GCs to reduce cytokine expression (in this case IL2) is not necessary paralleled by a reduction in biological response. Indeed, the fact that GCs reduced the time of the peak expression of IL2 receptor (IL-2R) by two days actually resulted in a faster, superior biological response compared with the response in the absence of GCs (Wiegers et al., 1995; 2001). This GC-induced optimisation was possible because it is the receptor expression, and not cytokine

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availability, which is the limiting factor in this phase of the response (Wiegers and Reul, 1998). They showed that CD4 levels were increased during GC-induced rises in T-cell proliferation (Wiegers et al., 2000). Furthermore, these GC-induced responses were desensitized if the rats had experienced high circulating GC levels for an extended period of time (Sterzer et al., 2004).

Interleukin 4 (IL-4) is an anti-inflammatory cytokine produced by T-cells which acts in an autocrine manner to elicit a key role in the differentiation of T-cells. GCs regulate the expression of this cytokine indirectly by removing the inhibitory control of IL-4 by IL-12 (Elenkov and Chrousos, 2002). In addition to its role as an anti-inflammatory agent, IL-4 has a neuromodulatory function and its receptor is functionally expressed in the granular neurons of the dentate gyrus in adult rats (Nolan et al., 2005). Preventing IL-4 production by T-cells in meningeal spaces severely impairs performance in behavioral studies targeting learning and memory processes such as the Morris water maze (Derecki et al., 2010). Further investigations found a strong correlation between IL-4 production and upregulation of brain-derived neurotrophic factor (BDNF) mRNA and protein in the hippocampus. BDNF is associated with neural plasticity and is an important factor in the consolidation of memories (Schaaf et al., 2000). Since the hippocampus is one of the key sites for memory processing, it was hypothesised that meningeal T cell-derived IL-4 production is enhancing cognitive function via BDNF expression in the hippocampus (Takei et al., 2010). The signaling pathways activated by IL-4 which are responsible for the enhanced performance in learning and memory tasks is unknown, however, since BDNF expression has been accompanied by increased acetylation of histone H3 and H4 (Takei et al., 2010), and since IL-4 can phosphorylate MAPK and induce phospho-acetylation of H3 (H3S10p-K14ac) in T-cells

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(Kraus et al., 2010), a similar, epigenetic based mechanism to that proposed to occur in response to psychological stress (Reul and Chandramohan, 2007) is promising.

Role of epigenetics in psychiatric and neurological disorders

Although epigenetic modifications are beginning to emerge as having a role in a range of diseases this review will focus on just a couple, namely post traumatic stress disorder (PTSD) and multiple sclerosis.

Post traumatic stress disorder (PTSD)

Since the 1980's, PTSD has been recognised as a pathological anxiety disorder, which can develop after exposure to a traumatic event (Yehuda and Bierer, 2009). Prior to this it was thought that once the threat (stressor) was no longer present the symptomatic effects of stress would disappear. It seems clear now that a certain proportion of victims of trauma (rape, war situations) will develop PTSD-associated symptoms over the course of time, even after the stressful situation or time period has passed. Symptoms associated with PTSD include hyperarousal (alertness, possibly mediated by the release of noradrenaline), avoidance of stressful situations and the recall of traumatic memories (Nair and Singh Ajit, 2008). Physiological changes including a reduction in basal cortisol levels, an increase in corticotrophin releasing hormone (CRH) in cerebral spinal fluid and increased expression of GR in lymphocytes have been found in PTSD patients when compared with matched controls (Yamamoto et al., 2009; Yehuda and Bierer, 2009). Since all these changes are associated with the HPA axis this has lead to the notion that the pathology of PTSD is caused by aberrant function of the HPA axis (Yehuda and Bierer, 2009). PTSD has also been associated with abnormal immune function. Patients with PTSD show high levels of circulating inflammatory markers, lower natural killer cell activity and lower T-lymphocyte counts when

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compared with matched controls (Pace and Heim, 2011). It is unclear whether the changes in immune factors are a result of the aberrant HPA axis or initiate the sensitisation of the HPA axis since IL1 has been shown to elicit long-lasting changes in HPA axis function and vice versa (Schöbitz et al., 1994; Linthorst et al., 1994; 1995b; Labeur et al., 1995; Linthorst et al., 1995a; Reul et al., 1998; Linthorst et al., 1999; Schmidt et al., 2003).

Administration of FG-7142, a partial indirect GABA-A receptor inverse agonist which inhibits GABA function, can induce PTSD-like symptoms in humans whereas exposure to GABA-A agonists like alcohol during a traumatic experience reduce the likelihood of developing PTSD (Kalueff and Nutt, 1996; Evans and Lowry, 2007). These effects may be linked to epigenetic modifications since studies in rats have shown that lorazepam, a benzodiazepine indirect GABA-A receptor agonist, has strong anxiolytic properties and inhibits phospho-acetylation of histone H3 (H3S10p-K14ac) in response to stress (Papadopoulos et al., 2010). Treatment of rats with FG-7142 has the opposite effect, i.e. the animals show increased anxiety-related behavior and enhanced levels of H3S10p-K14ac-positive neurons in the dentate gyrus in response to a novel environment challenge (Reul and Nutt, 2008; Papadopoulos et al., 2010). It is thought that traumatic early life experiences increase the likelihood of developing PTSD in later life. Work of Meaney and colleagues has shown that the DNA methylation status is critical in linking early life experiences with adult responses to stress (Weaver et al., 2004). Demethylation of genes involved in immune function, such as toll-like receptor 1 and 3 (TLR1 & TLR3), interleukin 8 (IL8), lymphotoxin alpha (LTA) and killer cell lectin-like receptor subfamily G, member 1(KLRG1) have been observed to correlate strongly with the occurrence of PTSD following a traumatic event (Uddin et al., 2010). Exposure to single prolonged stress (SPS, see Table 1) provoked a phenotype in rats similar to PTSD and therefore these rats were used as an animal model of

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PTSD in subsequent studies (Yamamoto et al., 2009). When SPS/PTSD rats were assessed in fear conditioning tests there was increased acetylation of histone H3 and H4 in the promoter regions for exon I and IV of the BDNF gene in the hippocampus when compared with fear conditioned control rats (no exposure to SPS). The changes in histone acetylation induced by fear conditioning training in the animal model of PTSD was associated with increased freezing behavior during the subsequent retest (Takei et al., 2010). Taken together these studies indicate an emerging role for epigenetic modifications in the development and prevalence of PTSD.

Multiple sclerosis

Multiple sclerosis (MS) is a neuroimmunological disease which is characterised by demyelination of neurons in the central nervous system resulting in a number of detrimental symptoms including muscle weakness, cognitive impairment including deficits in memory formation and retrieval, fatigue, mood disorders and many others (Heesen et al., 2007). Studies on MS patients and the MS animal model (experimental autoimmune encephalomyelitis (EAE)) have shown an involvement of the HPA axis in the vulnerability for and progression of this autoimmune disease (Stefferl et al., 1999; 2001; Heesen et al., 2007). Levels of DNA methylation in the promoters of specific genes are lower in patients suffering from MS compared with healthy controls. One of these genes, peptidyl argininedeiminase 2, is overexpressed in MS and promotes the citrullination (conversion of arginine to citrulline) which alters how the protein is folded due to an increase in hydrophobicity causing a pathological phenotype (Mastronardi et al., 2007). Exploratory studies have also shown a surprising role for the histone deacetylase inhibitor TSA as a beneficial treatment for MS suggesting a loss of acetylation marks is also occurring during disease progression (Camelo et al., 2005).

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Summary

Our studies so far have identified a unique role for two signaling pathways (i.e. the GR and ERK MAPK pathways), activated after exposure to specific stressors, which initiate epigenetic changes, modification of gene expression, memory formation and resulting in adaptive behavioral responses. Other research has described a role for these pathways in learning and memory paradigms such as the Morris water maze. Given the integration of the neuronal, endocrine and immune systems we speculate that these pathways, or components of them, play a role in other adaptive responses such as adaptive immunity and homeostasis. Although advances have been made to improve our understanding of the process surrounding the formation of long-term memories of significant (stressful) events in our lives there is still much ambiguity in the field. Epigenetic mechanisms and their role in memory formation may therefore be the molecular basis for the crossover between stress studies and learning paradigms as well as having a central role in hormonal and immunological adaptations.

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Legend to Figure 1.

Scheme representing the role of the epigenome in physiological and behavioral responses. Cells communicate by a number of chemical messengers which act at different locations to elicit a biological response. Generally, signaling systems exist whereby extracellular primary messengers (growth factors, cytokines, hormones etc) act via receptors at the cell surface or are transported or diffuse across the plasma membrane to induce activation of secondary messengers and/or transcription factors within the cytoplasm. Activated secondary messengers can have biochemical effects in the cytoplasm and/or are translocated into the nucleus to act on nuclear transcription factors or influence the epigenome or genome directly. The epigenome is regarded as the complex of changes, occurring at the chromatin level, which alter the expression of genes without changing the coding sequence of the genome. Changing the level of gene products, as a result of activation of signaling cascades, influences cell function and affects both the physiology and the behavior of the organism.

Legend to Figure 2.

Concept on the requirement of orchestrated signaling and epigenetic mechanisms in the induction of c-Fos in dentate granule neurons after a psychological challenge. A. Under baseline conditions the *c-fos* gene in an inactive state possibly involving methylation of the gene promoter DNA. B. Psychological stress causes the secretion of glucocorticoid hormone which act via GRs and the release of glutamate which acts on NMDARs to cause a rise in intracellular Ca^{2+} levels in sparsely distributed dentate granule neurons. Ca^{2+} influx activates CAMKII and the ERK MAPK cascade. We recently discovered that GRs seem to act like scaffolds facilitating the activation (i.e. phosphorylation) of MSK1 and Elk-1 by phosphorylated ERK1/2 (pERK1/2; Gutierrez-Mecinas et al., submitted). pMSK1 phosphorylates S10 residues in histone H3 tails which evokes the opening of the chromatin. If the *c-fos* gene promoter is methylated de-methylation (by DNA de-methylases (DDMs)) will take place at this stage or earlier. CAMKII and MSK1 and possibly other kinases (e.g. protein kinase A (PKA)) will phosphorylate CREB. pElk-1 will bind to the Elk-1 binding site in the serum response element (SRE) and recruit and phosphorylate the HAT p300. pCREB and GRs may also recruit HATs. The recruited HATs will acetylate the histone tails (including H3S10p rendering it into H3S10p-K14ac) and thereby stabilize the open chromatin configuration. This may also involve H3K4 methylation by HMTs but their activation/recruitment mechanisms are still unknown. Dimerized pCREB can now access the CRE within the *c-fos* promoter and transactivate gene transcription. The c-Fos response is a transient response as, over time, glucocorticoid hormones and glutamate diminish, Ca^{2+} levels plummet, protein phosphatase and HDAC activities rise and ultimately the *c-fos* gene will be inactivated, possibly involving DNA methylation of the gene promoter.

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Table 1. Behavioral tests commonly used to investigate responses to stress or learning and memory paradigms.

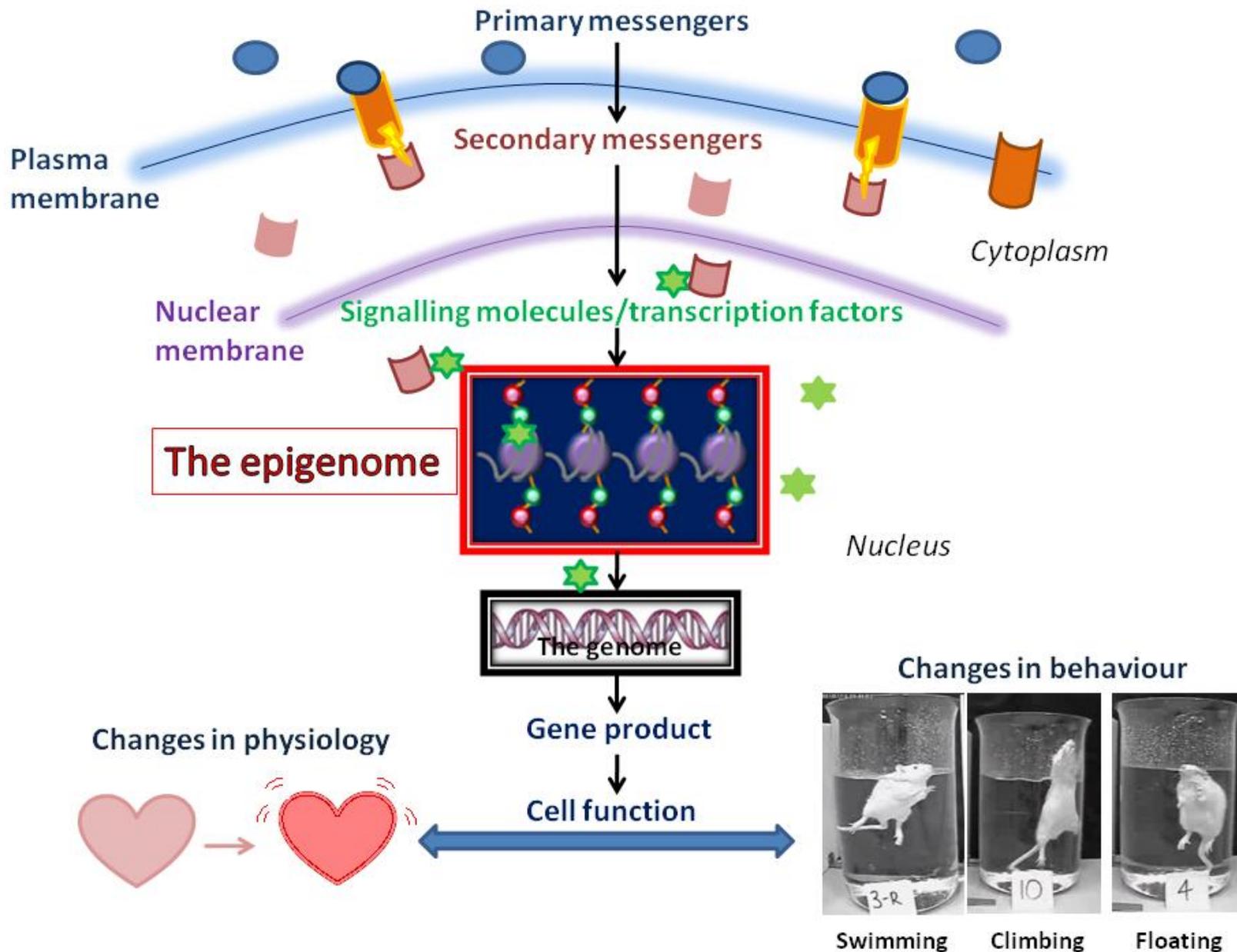| Behavioral test	General conditions	Adaptive behavior	Reward (if applicable)	References
Novel environment	Subjects are transferred to a new environment (usually for 30 min) and their behavior recorded.	Switch from exploratory to normal behavior	Identifying there is no threat	(Bassett et al., 1973)
Radial maze	8-arm maze which tests subjects memory by forcing them to make decisions about which arm to explore.	Finding food source	Food	(Olton and Samuelson, 1976)
Morris water maze	Subjects are trained (successive trials over consecutive days) to find an underwater platform and then the platform is removed to test their memory of its location.	Finding the platform	Escape from water	(Morris et al., 1982)
Forced swim test	Subjects are placed in are large beaker of water from which they cannot touch the bottom or escape and behavior monitored	Floating	Conservation of energy	(Porsolt et al., 1977$_a$; De Pablo et al., 1989; West, 1990; Korte,

	(usually for 15 min). A second test, usually lasting 5 min is performed after a sufficient recovery period (usually 24 h) and again behavior is monitored.			2001; Bilang-Bleuel et al., 2005; Chandramohan et al., 2008; Nestler and Hyman, 2010)
Fear conditioning	Subjects are placed in a box and subjected to a short-lasting foot shock, sometimes preceded by a noise or light signal (conditioned cue). Trials are often repeated before allowing the subject time to recover (varies from hours to weeks). In the retest the subject is placed in a different environment and subjected to the cue but no shock (to test cue-dependent memory) or placed in the same box with no cue or shock (to test context-related	Freezing	No reward	(Rescorla, 1973; Davis and Astrachan, 1978)

	memory)			
Single prolonged stress	Subjects are exposed to 2 h restraint stress, followed immediately by forced swim test (usually 20 min), allowed to recover for a short period (~15 min) and then exposed to anaesthetic until unconsciousness is achieved.	Freezing	No reward	(Liberzon et al., 1997)
Immune/inflammatory stress	Can be experimentally induced by administration of bacterial endotoxins.	Sickness behavior	No reward	(Linthorst et al., 1995b)

[a] Please note, this interpretation of the immobility response during forced swim retest differs from our own.
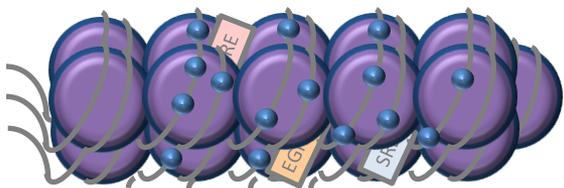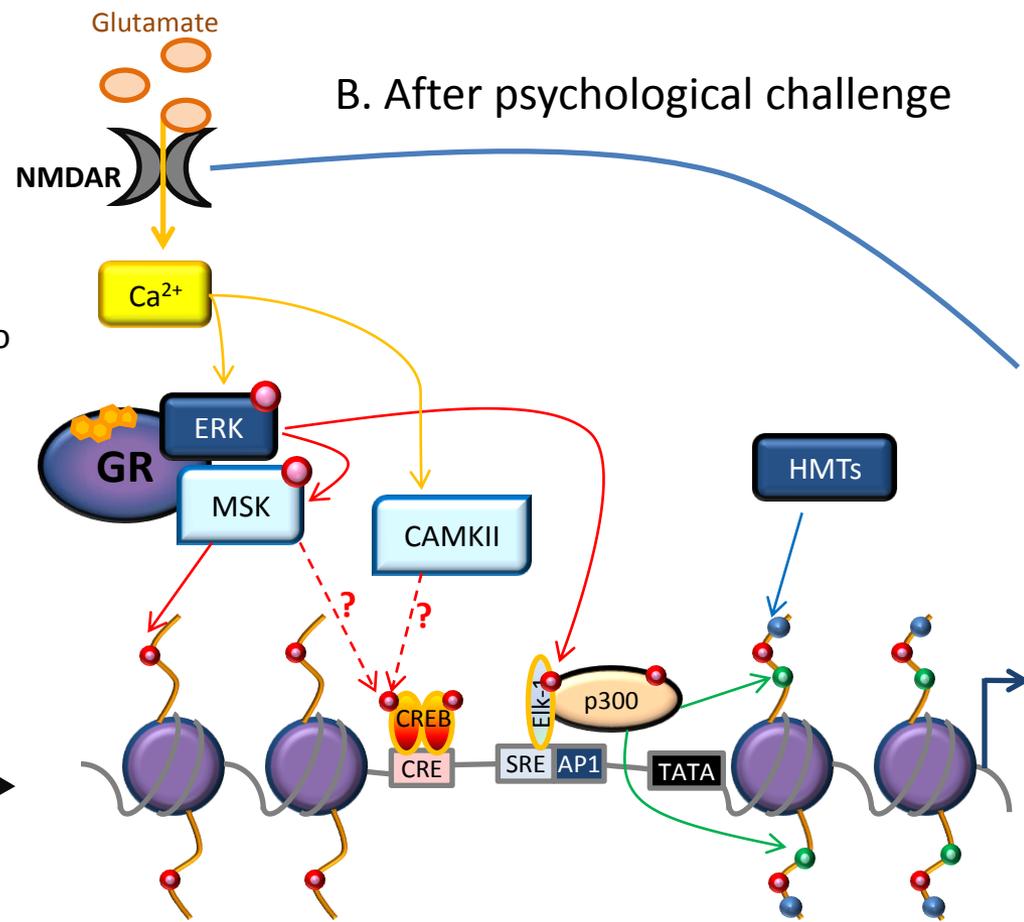
Primary messengers

Secondary messengers

Plasma membrane

Cytoplasm

Nuclear membrane

Signalling molecules/transcription factors

The epigenome

Nucleus

The genome

Changes in behaviour

Changes in physiology

Gene product

Cell function

Swimming   Climbing   Floating

A. Baseline conditions

B. After psychological challenge