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Effect of Hippocampal CREB Deletion on Models of Anxiety, Depression, and Antidepressant Response

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Abstract
Depression is the most common psychiatric disorder, yet current antidepressants are inadequate as they require weeks of treatment to alleviate symptoms. The mechanism by which the immediate effects of antidepressants cause later behavioral improvements remains unknown, focusing research on downstream signaling events triggered by antidepressant administration. Expression and activity of the transcription factor CREB are increased by antidepressant drugs, and CREB targets include genes known to be involved in antidepressant response. However, the effects of manipulating CREB depend on the brain region examined, with initial studies showing an antidepressant role for CREB in the hippocampus. To investigate the hippocampal-specific importance of CREB in depression-related behaviors and response to antidepressant drugs, we used CrebloxP/loxP mice, in which CREB deletion could be induced by injection of an adeno-associated virus expressing Cre recombinase. Robust and specific deletion of CREB protein throughout the hippocampus was achieved via viral injection to this region in adult mice. Acute response to antidepressants in the forced swim test, a common behavioral assay for antidepressant efficacy, was unaffected by hippocampal CREB deletion. In an assay sensitive to chronic antidepressant response, the novelty-induced hypophagia (NIH) paradigm, hippocampal CREB deletion did not alter response to chronic antidepressant treatment. However, mice with hippocampal deletion of CREB also responded to acute antidepressant treatment in the NIH, an accelerated response to antidepressants, as control mice responded only to chronic, but not acute, treatment in this paradigm. Additionally, loss of CREB from the hippocampus increased hippocampal neurogenesis, which may be related to the accelerated response to antidepressants in the NIH in these mice. These results mimic the phenotype of a constitutive knockout of CREB, suggesting that the phenotype does not result from developmental loss of CREB. The CREB-family protein CREM was upregulated following deletion of CREB, demonstrating that regulation within this family is highly dynamic. CREM may functionally compensate for the lack of CREB by maintaining or increasing expression of CREB target genes, including Bdnf and Bcl-2. These genes are known to regulate cell survival and differentiation, and may contribute to the observed increase in hippocampal neurogenesis, although further study is necessary to confirm this hypothesis. This work indicates that CREB family proteins are important regulators of hippocampal neurogenesis and behaviors associated with antidepressant response. However, behavioral results suggest that CREB in the hippocampus may not be necessary for the behavioral response to antidepressants, challenging a previous study that suggested increased CREB activity was sufficient to produce an antidepressant behavioral response. This mouse model of specific loss of CREB function will be useful in dissecting the role of CREB in specific brain regions, potentially resolving this discrepancy. Overall, future study of the role of CREB and its targets in the downstream mechanisms of antidepressant response may contribute to the development of novel therapeutics.

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EFFECT OF HIPPOCAMPAL CREB DELETION ON MODELS OF ANXIETY, DEPRESSION, AND ANTIDEPRESSANT RESPONSE

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To my parents, for inspiring me to start this process,

and to David, without whom I could never have finished it.

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ABSTRACT

EFFECT OF HIPPOCAMPAL CREB DELETION ON MODELS OF ANXIETY, DEPRESSION, AND ANTIDEPRESSANT RESPONSE

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Depression is the most common psychiatric disorder, yet current antidepressants are inadequate as they require weeks of treatment to alleviate symptoms. The mechanism by which the immediate effects of antidepressants cause later behavioral improvements remains unknown, focusing research on downstream signaling events triggered by antidepressant administration. Expression and activity of the transcription factor CREB are increased by antidepressant drugs, and CREB targets include genes known to be involved in antidepressant response. However, the effects of manipulating CREB depend on the brain region examined, with initial studies showing an antidepressant role for CREB in the hippocampus. To investigate the hippocampal-specific importance of CREB in depression-related behaviors and response to antidepressant drugs, we used $Creb^{loxP/loxP}$ mice, in which CREB deletion could be induced by injection of an adeno-associated virus expressing Cre recombinase. Robust and specific deletion of CREB protein throughout the hippocampus was achieved via viral injection to this region in adult mice. Acute response to antidepressants in the forced swim test, a common behavioral assay for antidepressant efficacy, was unaffected by hippocampal CREB deletion. In an assay sensitive to chronic antidepressant response, the novelty-induced

hypophagia (NIH) paradigm, hippocampal CREB deletion did not alter response to chronic antidepressant treatment. However, mice with hippocampal deletion of CREB also responded to acute antidepressant treatment in the NIH, an accelerated response to antidepressants, as control mice responded only to chronic, but not acute, treatment in this paradigm. Additionally, loss of CREB from the hippocampus increased hippocampal neurogenesis, which may be related to the accelerated response to antidepressants in the NIH in these mice. These results mimic the phenotype of a constitutive knockout of CREB, suggesting that the phenotype does not result from developmental loss of CREB. The CREB-family protein CREM was upregulated following deletion of CREB, demonstrating that regulation within this family is highly dynamic. CREM may functionally compensate for the lack of CREB by maintaining or increasing expression of CREB target genes, including *Bdnf* and *Bcl-2*. These genes are known to regulate cell survival and differentiation, and may contribute to the observed increase in hippocampal neurogenesis, although further study is necessary to confirm this hypothesis. This work indicates that CREB family proteins are important regulators of hippocampal neurogenesis and behaviors associated with antidepressant response. However, behavioral results suggest that CREB in the hippocampus may not be necessary for the behavioral response to antidepressants, challenging a previous study that suggested increased CREB activity was sufficient to produce an antidepressant behavioral response. This mouse model of specific loss of CREB function will be useful in dissecting the role of CREB in specific brain regions, potentially resolving this discrepancy. Overall, future study of the role of CREB and its targets in the downstream mechanisms of antidepressant response may contribute to the development of novel therapeutics.

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Chapter 1: Introduction and Overview

Depression is the most common psychiatric disorder, with a lifetime prevalence of nearly 20%, and is among the ten leading causes of morbidity and mortality worldwide (McKenna et al., 2005, Gonzalez et al., 2010). The economic costs of this disorder are enormous, estimated to be tens of billions of dollars every year in the US alone (Donohue and Pincus, 2007). Current antidepressant (AD) drugs perform only modestly better than placebo, and only patients with the severest depression show clinically significant improvement following treatment (Kirsch et al., 2008). Overall, fewer than 50% of patients experience full remission as a result of initial drug treatment, cognitive or psycho-therapy, or a combination of the two (Nelson, 1999). A delay of weeks to months is seen before AD drugs improve symptoms of depression (Fava and Kendler, 2000). The need for faster-acting and more efficacious AD drugs is widely acknowledged, yet no fundamentally new drugs have been developed in the last 50 years. "Novel" drugs developed recently exert their acute effects through the same basic mechanism as first-generation AD, namely elevation of synaptic levels of monoamines. The need for new AD drugs will be fulfilled only when our understanding of the disease pathology or the long-term mechanisms of AD action is improved.

While the immediate effect of antidepressant drugs is to increase levels of synaptic monoamines, particularly serotonin (5HT) and norepinephrine (NE), what remains unknown is how these immediate changes translate into the therapeutic effects that are seen only after long-term treatment. The molecular pathways linking the immediate effects, i.e. increased activation of monoamine receptors, may involve G-

protein signaling downstream of G-protein-coupled receptors for 5HT and NE. Indeed, antidepressants have been shown to increase coupling of $G\alpha_s$ (which is coupled to 5HT4, 5HT6, 5HT7, D1, D5, and adrenergic β receptors), as well as to increase activity of adenylate cyclase, which leads to increased levels of cyclic AMP (cAMP) and increased protein kinase A (PKA) activity (Nestler et al., 1989). Activity of other kinases, such as the calcium-calmodulin-dependent kinases (CamK) is also increased following long-term treatment with antidepressants (Popoli et al., 2000). Increases in intracellular calcium may result from activation of other monoamine receptors, which are coupled to $G\alpha_q$, such as 5HT2 and noradrenergic α1 receptors, through activation of phospholipase C (PLC) and release of internal calcium stores. Activation of the 5HT1A receptor, which is coupled to $G\alpha_i$ and therefore has an inhibitory effect on PKA signaling, may also lead to release of internal calcium stores. However, activity of 5HT1A receptors has also been shown to decrease following antidepressant administration, suggesting that these receptors may become desensitized by increased levels of synaptic 5HT (Green et al., 1986). The transcription factor CREB (cyclic-AMP response element binding protein) provides a point of convergence for these and other signaling pathways, as it is activated through phosphorylation by a number of kinases, including PKA, CamKs, and ribosomal S6 kinases (RSKs), which are in turn activated by a number of stimuli including growth factors, hormones, stress, and synaptic activity (Figure 1) (for a comprehensive review, see (Lonze and Ginty, 2002).

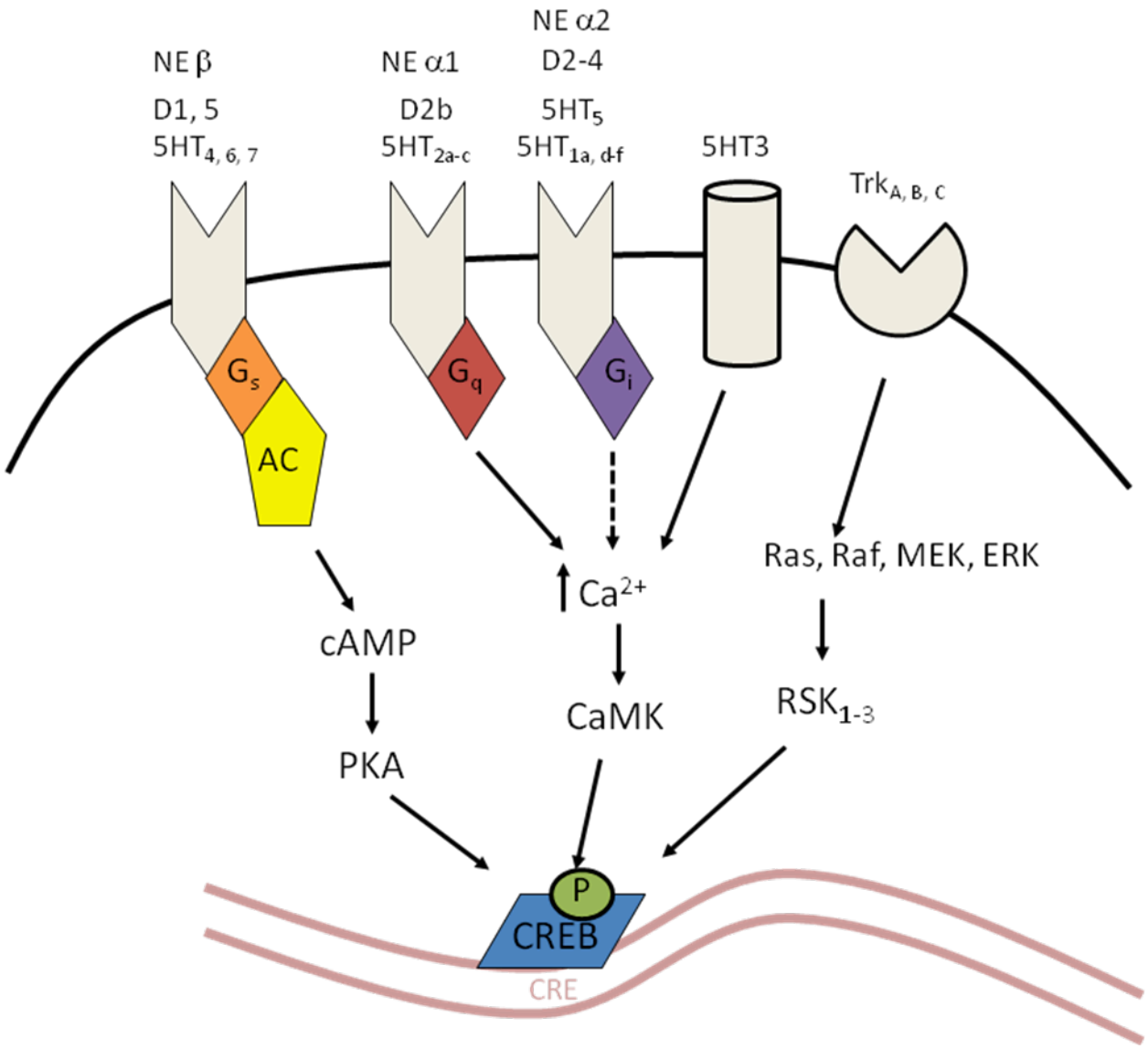

Figure 1. Regulation of cAMP response element binding protein (CREB) phosphorylation by antidepressant drugs. Clinically effective antidepressants elevate levels of norepinephrine (NE), serotonin (5HT), or dopamine (DA) levels immediately, as well as levels of neurotrophins after longer treatment. These neurotransmitters bind receptors on the membrane and activate second messenger pathways, including cAMP, Ca^{2+} and several kinases, including cAMP-dependent protein kinase (PKA), Ca2+-calmodulin-dependent kinase (CaMK), mitogen-activated protein kinase (MEK), extracellular signal-regulated protein kinase (ERK), and several forms of ribosomal S6 kinase (RSK_{1-3}). These kinases can phosphorylate protein substrates such as CREB, which binds to a cAMP response element (CRE) in DNA to regulate gene expression. Genes targeted by CREB might contribute to behavioral, endocrine, or cellular changes associated with chronic antidepressant treatment.

CREB structure and regulation

Since its discovery in 1987 by Montminy and colleagues, as the protein bound to the cyclic-AMP response element (CRE) in the somatostatin promoter (Montminy and Bilezikjian, 1987), much research has focused on CREB, as well as the mechanisms by which it is activated, and in turn, activates transcription of its target genes. The rat, mouse, and human forms of CREB demonstrate striking similarity, with a difference of only one to two amino acids (Hoeffler et al., 1988, Gonzalez et al., 1989, Cole et al., 1992), reflecting the importance of CREB for vital functions throughout evolution. Three activator forms of CREB have been described, CREBα, CREBΔ, and CREBβ, each consisting of 11 exons in mouse and human, with alternative splicing of one exon leading to each specific isoform (Blendy et al., 1996). CREBα and CREBΔ appear to be the predominant isoforms, with CREBβ expressed at much lower levels, although all are capable of responding to elevations in cAMP by increasing CRE-mediated transcription. Little is known about the function of several other isoforms of CREB, CREBαγ, CREBγ, CREBΩ, and CREBW. These isoforms lack the leucine zipper/DNA binding domain and nuclear translocation signal, and are expressed at high levels only in the testes, in contrast to the ubiquitous expression of the α, Δ, and β isoforms.

In combination with the multiple isoforms of CREB, two other highly related proteins, cyclic-AMP response-element modulator (CREM) and the activating transcription factors (ATF-1, -2, -3 and -4) comprise the CREB/ATF family. Within this larger family, CREB, CREM, and ATF-1 comprise a subclass of proteins that can be phosphorylated by PKA (Gonzalez et al., 1989, Rehfuss et al., 1991, de Groot et al.,

1993). These three proteins can homo- or hetero-dimerize via leucine-zipper domains, and bind to CREs in this form (Yamamoto et al., 1988, Dwarki et al., 1990). DNA binding is due to the presence of a basic region of the protein encoded by the carboxy terminus, which is highly conserved within the family (Yun et al., 1990, Foulkes et al., 1991, Rehfuss et al., 1991). This flexibility in binding partners may contribute to the specificity of gene expression observed after CREB activation resulting from diverse signaling cascades, although exactly how, and whether, this occurs is presently unknown.

The expression of CREB family proteins differs from the nearly universal expression of CREB throughout tissues. CREM, and particularly one activating isoform, CREMτ, is expressed at high levels in the testes, with lower levels of expression in brain reflecting mainly the inducible cAMP early repressor (ICER), a transcription-repressing isoform (Foulkes et al., 1991, Laoide et al., 1993). ATF-1 is expressed highly in testes, muscle, fat, and also choroid plexus, at lower levels (Bleckmann et al., 2002).

CREB contains several serine residues (133, 142, and 143) which can be phosphorylated, of which serine 133 (Ser133) appears to be necessary for activation (Gonzalez and Montminy, 1989). Association with the CREB co-activator protein, CREB-binding protein (CBP), is favored when Ser133 on CREB is phosphorylated (Chrivia et al., 1993, Kwok et al., 1994). CBP promotes transcriptional activation by binding to transcriptional machinery, as well as through its histone acetyl-transferase activity. While Ser133 phosphorylation has been a convenient measure of CREB activity, phosphorylation at serines 142 and 143 also plays a role in regulating CREB (Parker et al., 1998, Gau et al., 2002, Kornhauser et al., 2002). CREB may maintain the

ability to activate transcription in the absence of phosphorylated Ser133 through interaction with other coactivators, at least in the pancreas (Conkright et al., 2003, Iourgenko et al., 2003, Screaton et al., 2004). Thus, while Ser133 phosphorylation remains the focus of most studies of CREB activation, it is important to keep in mind that it is not the only regulator of CREB function.

Kinases capable of phosphorylating CREB can be found in numerous signaling pathways in neurons, downstream of G-protein coupled receptors, ionotropic and NMDA glutamate receptors, receptor tyrosine kinases, and voltage-gated calcium channels (Figure 1) (see (Lonze and Ginty, 2002) for a comprehensive review). Increased activation of $G\alpha_s$-coupled serotonin receptors (5HT4, 6 and 7) and noradrenergic β receptors from increased synaptic levels of these monoamines resulting from AD treatment, can lead to increased activity of adenylate cyclase, increasing levels of cAMP. Increased cAMP leads to activation of protein kinase A (PKA), which acts as a CREB kinase (Gonzalez and Montminy, 1989, Hagiwara et al., 1993). CREB can also be phosphorylated by calcium/calmodulin-dependent kinases (I, II, and IV) (Dash et al., 1991, Sheng et al., 1991, Sun et al., 1994). The increase in calcium necessary to activate such calcium-dependent kinases can be achieved through depolarization-induced calcium influx or release of internal stores of calcium. Thus, CREB can be phosphorylated as a result of depolarization, induced in the case of AD treatment by the ionotropic 5HT3 receptors, or activation of phosphatidylinositol turnover through the $G\alpha_q$-coupled $\alpha 1$ noradrenergic, dopamine 2b or 5HT2 receptors. Activation of 5HT1A receptors, which are coupled to $G\alpha_i$ may also lead to increases in intracellular calcium. In the long term,

increases in growth factor expression following antidepressant treatment can lead to activation of ribosomal S6 kinases (RSKs), which can also phosphorylate CREB, through the Ras/Raf/MEK/ERK pathway. Thus, CREB is activated by diverse signaling cascades, in particular those likely to be activated upon AD exposure.

Antidepressant drugs alter CREB expression and activity

The immediate effects of AD drugs on 5HT and NE signaling do not sufficiently explain the mechanism of action of these drugs, as their therapeutic benefits occur over a much longer time scale. CREB is a possible link between the immediate effects of these compounds and longer-term changes that underlie later improvements in mood. The ability of CREB to be activated by both cAMP and Ca^{2+} signaling cascades (see CREB Structure and Regulation), combined with its capability, as a transcription factor, to bring about long-term changes in gene expression, make it well-suited to play a significant role in transducing the effects of AD drugs. Indeed, many of the genes CREB is known to regulate, such as brain-derived neurotrophic factor (*Bdnf*) (Tao et al., 1998, Shieh and Ghosh, 1999, Tabuchi et al., 2002), *Bcl-2* (Riccio et al., 1999), and vascular endothelial growth factor (*Vegf*) (Braun et al., 2001, Impey et al., 2004), are important for neuroplasticity and cell survival, and have themselves been implicated in depression and AD response (for reviews, see (Nair and Vaidya, 2006, Tardito et al., 2006, Warner-Schmidt and Duman, 2008)). Based on its ability to be activated by converging signaling pathways, CREB appears capable of playing a role in the response to AD treatment, and indeed human post-mortem studies have shown that CREB is expressed at higher levels

in the brains of AD-treated patients, compared to untreated depressives (Dowlatshahi et al., 1998).

Alterations in expression

Numerous studies have examined AD-induced alterations in CREB expression in rodent brain at the level of mRNA, protein, as well as activating phosphorylation at Ser133. In one study, increased CREB mRNA was observed in the rat hippocampus after chronic (at least ten days) treatment with all AD tested: desipramine (DMI), imipramine (IMI), tranylcypromine (TCP), sertraline (SER), fluoxetine (FLX), and electroconvulsive shock (ECS) (Nibuya et al., 1996). An increase in CREB mRNA in the hippocampus was also observed in two other studies in mice and rats after chronic treatment with FLX (Blom et al., 2002, Tiraboschi et al., 2004). Reboxetine (RBX) also increased CREB mRNA in the hippocampus of rats (Tiraboschi et al., 2004). However, only one of these two studies confirmed the increase in CREB mRNA after chronic treatment with DMI found in the Nibuya, et. al. study (Blom et al., 2002). Additionally, a further study did not show an increase in CREB mRNA in the hippocampus after chronic treatment with either FLX or DMI (Laifenfeld et al., 2005).

These discrepancies may have arisen for a number of reasons. While the dose of these drugs appears to have remained relatively consistent, different drug administration paradigms used could lead to differences in the effective dose and pharmacokinetics of the ADs. For example, in the Tiraboschi, et. al. study, which did not find an increase in CREB mRNA, DMI was administered to rats via subcutaneous minipump, whereas in the

two studies that did report increased CREB mRNA, DMI was administered by intraperitoneal (i.p.) injection to both mice and rats (Nibuya et al., 1996, Blom et al., 2002). However, the Laifenfeld, et. al. study, in which no difference in CREB mRNA was observed in the rat hippocampus after FLX or DMI also used an i.p. administration paradigm (Laifenfeld et al., 2005). This study, however, analyzed brains 24 hours following the last drug administration, a timepoint significantly later than the Nibuya, et. al. study (the timepoint of analysis was not specifically mentioned in the Blom, et. al. or Tiraboschi, et. al. studies). Finally, the method of quantifying expression may lead to such differences in results. Various methods were used in these studies, including RT-PCR (Tiraboschi et al., 2004, Laifenfeld et al., 2005), RNase protection assays (Blom et al., 2002), Northern blotting and *in situ* hybridization (Nibuya et al., 1996). Use of a different species (i.e. rat vs. mouse) may also lead to differences in results, although that does not appear to be the case here.

In addition to the hippocampus, another brain region often examined for changes in CREB expression is the frontal cortex (fctx). Increased CREB mRNA was observed in the fctx of rats following chronic treatment with RBX (Tiraboschi et al., 2004). However, no change in CREB mRNA was observed in the ctx of mice or rats following chronic treatment with DMI or FLX (Blom et al., 2002, Tiraboschi et al., 2004, Laifenfeld et al., 2005). Changes in CREB mRNA in this brain region appear to depend on which drug is used, but are more consistent across experimental variables such as those discussed above.

Changes in CREB expression following chronic antidepressant treatment have also been demonstrated at the protein level. In one study, increased CREB protein was observed in the hippocampi of rats after chronic treatment with DMI, as well as in the fctx after chronic treatment with either DMI or RBX (Tiraboschi et al., 2004). This study also examined chronic FLX treatment, but did not see changes in either of these brain regions, in contrast with another study, in which increased CREB protein was observed in the hippocampus following chronic FLX or ECS (Nibuya et al., 1996). Again, these studies differed in their drug administration paradigm (subcutaneous minipump vs. i.p. injection, respectively), which may explain the differences seen. Additionally, the Tiraboschi, et. al. study analyzed protein levels with western blots, whereas the Nibuya, et. al. study used immunohistochemistry, which is more sensitive and may have detected subtle changes that western blotting was not sensitive enough to detect. A study by Laifenfeld, et. al. (2005) did not report changes in CREB protein in the hippocampus or fctx of rats after chronic treatment with FLX or DMI, but this analysis was conducted at a much later timepoint (24 hours after the last drug administration) than the studies discussed above.

Because phosphorylation at Ser133 is known to be important in activating CREB, many of the same studies have investigated whether it is altered after chronic antidepressant treatment. An increase in phosphorylated CREB (pCREB) was observed in the hippocampus of rats after chronic treatment with FLX, and an increase in the fctx was observed after chronic treatment with FLX, DMI, and RBX (Tiraboschi et al., 2004). An additional study found similar effects of FLX to increase pCREB in the hippocampus

and cortex, but also the amygdala, hypothalamus, and thalamus of mice (Thome et al., 2000). However, the latter study did not observe an effect of DMI on pCREB levels, except in the hippocampus, where it was increased. In addition to the species difference, these studies differed in their methods (western blotting vs. immunohistochemistry, respectively). A third study reported increased pCREB in the fctx of rats after chronic treatment with FLX or DMI, but did not show changes in the hippocampus, potentially due to the later timepoint used (24 hours after the last administration) (Laifenfeld et al., 2005). Finally, one study reported a *decrease* in pCREB in the fctx of rats following chronic treatment with RBX or DMI (Manier et al., 2002). The design of this study seems consistent with those discussed above, although the time between the last drug administration and sacrifice of the animals is not explicitly stated.

Some details of the mechanism by which CREB expression and phosphorylation are increased following chronic AD treatment have emerged. The increase in expression of CREB after chronic antidepressant treatment appears to be reflective of increases in the α and Δ forms of CREB (Blom et al., 2002). Additionally, increased CREB phosphorylation following chronic AD treatment appears to depend on the activity of CamKIV and the MAP kinase pathway, rather than the cAMP/PKA pathway, at least in the case of treatment with FLX (Tiraboschi et al., 2004). Only chronic treatment with noradrenergic drugs (DMI and RBX) consistently increased PKA activity in both the fctx and hippocampus (Tiraboschi et al., 2004). Chronic treatment with all antidepressants tested in this study (FLX, DMI, and RBX) increased CamKIV activity in nuclear

fractions in the fctx, though did not change its activity in the hippocampus (Tiraboschi et al., 2004).

Alterations in activity

CREB activity is known to respond to stimuli on a rapid time-scale; expression of most genes induced in response to cAMP peaks at 30 minutes following a stimulus and returns to basal levels within four hours (Greenberg et al., 1985, Hagiwara et al., 1992). Increases in CREB activity have been demonstrated following chronic AD treatment. Using a "LacZ-reporter" mouse containing the gene encoding β-galactosidase under control of a promoter containing six CRE elements, increased CRE-mediated transcription was seen in the amygdala after chronic treatment with FLX, DMI and TCP (Thome et al., 2000). Additionally, FLX increased reporter gene expression in the cortex, hypothalamus, and thalamus, with non-significant increases in the dentate gyrus and CA3 regions of the hippocampus. TCP also increased reporter gene expression in the cortex, dentate gyrus, CA3, and hypothalamus.

A more indirect measure of CREB activity uses gel-shift assays to measure binding of CREB to CRE sites. Chronic treatment with FLX has been shown to increase CREB binding in the hippocampus and fctx (Nibuya et al., 1996, Frechilla et al., 1998), while DMI increased binding only in the fctx (Frechilla et al., 1998), and electroconvulsive-shock increased binding in the hippocampus (the only region that was examined in this study) (Nibuya et al., 1996). Additionally, the phospho-diesterase inhibitor rolipram, which increases levels of cAMP and thus stimulates CREB

phosphorylation, was shown to act in synergy with chronic IMI treatment to increase CREB binding in the hippocampus and fctx (Itoh et al., 2004). This increase in CREB binding was correlated with a behavioral response to the drug combination, a decrease in escape failures in a learned helplessness paradigm (see Modeling Behavior in Rodents). The effect of the drug combination on CREB binding and behavior was greater than that of either drug alone, suggesting that the increase in CREB phosphorylation and/or activity caused by rolipram may contribute to the antidepressant behavioral response.

CREB Targets

One reason for the observed discrepancies and complexities in the regulation of CREB activity and expression following AD treatment may be that the binding and transcriptional activation by CREB may differ at the promoters of specific target genes. Following a stimulus, CREB may act to increase expression of some of its targets, while decreasing expression of others, and these subsets of target genes likely change with different stimuli. Studies of CREB binding to specific promoters have begun to address this additional layer of complexity. In one study, CREB binding to DNA was shown to increase after electroconvulsive shock (ECS), a rat model of electroconvulsive therapy, which is a treatment used in the most refractory cases of depression (Tanis et al., 2008). ECS had previously been shown to increase CREB mRNA, protein, and binding to CREs (Nibuya et al., 1996), but in this study the specific promoters to which CREB bound were identified using chromatin immunoprecipitation followed by microarray analysis ("ChIP/chip"). Not only was CREB binding increased overall in the hippocampus and

frontal cortex, but the ratio of pCREB:CREB was increased. Again, regional differences in which promoters CREB occupied were observed between the hippocampus, fctx, and striatum. Although these numbers were small in comparison to the number of genes to which CREB binding increased following ECS, 1% of the promoters of CREB targets showed decreased binding, compared to 14-17% showing increased binding, suggesting that assessing global changes in CREB activity obscures the effects of AD treatment on some targets.

A second study, using a similar ChIP/chip approach examined pCREB binding in the nucleus accumbens (NAc) (Wilkinson et al., 2009). In this study, chronic treatment with IMI caused a reversal of changes in pCREB induced by chronic social defeat (CSD) stress, a model of depression in mice. IMI-treated mice also mimicked mice that remained resilient to the defeat stress (as determined by a behavioral read-out), in terms of their pCREB binding pattern. What is remarkable in this study is that, while pCREB binding was increased overall after chronic treatment with IMI, there were a number of genes whose promoters had *decreased* pCREB binding after such treatment. These genes were often those who had shown *increased* binding following CSD, suggesting that IMI treatment reversed the changes brought about by stress. For example, pCREB binding to the promoter of interleukin-2 was increased by CSD, but decreased to baseline levels after chronic treatment with IMI. The number of such genes with decreased CREB binding following IMI treatment in this study was much higher than the number showing decreased binding following ECS, suggesting that CREB activity plays an important role in returning the system to baseline after stress, which may be missed in a study in which

AD treatment is administered to "healthy" mice. Further, these bimodal changes in CREB activity, depending on the target, suggest that measuring only global expression and activity levels obscures some of the complexity of CREB regulation and function. Additionally, this type of study provides a valuable list of novel targets to which CREB binds, adding to the "usual suspects," such as BDNF, mentioned above.

Effects of altering CREB expression and activity in behavioral tests of antidepressant efficacy and models of depression

Modeling behavior in rodents

To allow for analysis of molecular correlates of both depression and AD response, several rodent models of depression-like behaviors and AD response have been developed. As depression is a complex psychological disorder, including some symptoms, such as feelings of worthlessness or suicidal ideation, which rodents are unlikely to be capable of, models of depression have been difficult to develop and remain imperfect. These models can be assessed for various levels of validity, including construct, face, and predictive validity (Willner, 1984). Some rodent models, such as learned helplessness (LH), have face validity and therefore can be considered actual models of depression. Most rodent models, however, have only predictive validity, in this case the ability to identify compounds which are likely to have therapeutic effects in humans, and should therefore be thought of more as tests of AD efficacy rather than models of depression itself. Several models with predictive validity, such as the forced-swim test (FST), tail-suspension test (TST) and LH have been used to identify new

compounds which were later found to be efficacious in human depressives. However, as the predictive validity of these paradigms is determined by the ability of currently-available AD drugs to cause a change in behavior, it is possible that newer compounds, which are different in structure or mechanism of action, may give false negatives as the paradigm may be sensitive only to drugs of a particular class (ie those currently available).

As the entire syndrome of depression is unlikely to be wholly modeled in rodents, another approach has been to model aspects of the disease, or endophenotypes, such as anxiety or anhedonia (lack of interest in pleasurable activity) (Cryan et al., 2002). These models, which can be made more ethologically relevant to rodents, include measuring levels of exploratory behavior in conflict tests such as the elevated plus or zero maze or the open field test, as well as measuring motivation for highly palatable foods or sexual behavior. This approach also allows for the identification of therapeutic compounds, which in this case would be used to treat specific symptoms that may be shared across multiple psychiatric disorders.

The forced swim and tail suspension tests

The most widely-used test of AD efficacy is the FST, which exists in various forms (Porsolt et al., 1977, Lucki, 1997). In this paradigm, rats or mice are placed in a cylindrical tank of water and their behavior is scored as either immobility or swimming and climbing. Immobility, passive behavior just necessary to stay afloat, has been interpreted as "behavioral despair," in that the animal is not actively trying to escape

from the water. Clinically effective AD reduce the latency to immobility and duration of immobility in this test after acute treatment. The TST is a related test in which mice are suspended by their tail (Steru et al., 1985). Immobility in this case is the absence of active escape-oriented behavior, and is reduced by acute treatment with clinically efficacious AD. It is important to note that despite the term "behavioral despair," behavior in these paradigms is likely not analogous to depression; these tests have only predictive validity. Additionally, the response to acute treatment with AD in these paradigms, while predictive of clinical efficacy, is distinct from the much longer treatment necessary to achieve therapeutic benefit in human patients.

The learned helplessness model

The LH paradigm is a model of a stress-induced state which may mimic some aspects of depression, such as feelings of helplessness and lack of motivation. In this paradigm, animals are continuously exposed to an inescapable and uncontrollable shock, and later demonstrate deficits when escape from the shock becomes possible (Overmier and Seligman, 1967, Seligman and Beagley, 1975). In rodents, these deficits are reduced by AD drugs, often only after a longer course of treatment than is effective in the FST or TST (though effective dosing paradigms differ amongst studies) and are usually still shorter than those necessary to cause therapeutic benefit in human patients (Leshner et al., 1979, Petty and Sherman, 1979, Sherman et al., 1982).

The novelty-induced hypophagia (NIH) paradigm

The long delay in therapeutic benefit is a defining feature of current AD drugs, as well as one of the mysteries of their mechanism of action. To investigate what long-term changes are occurring during this delay, as well as to address the disparity between the onset of efficacy of AD in humans and animal models, the NIH paradigm was developed as a test of chronic, but not acute AD response (Merali et al., 2003, Dulawa et al., 2004, Dulawa and Hen, 2005). In this test, animals show significant increases in latency to approach and consume a highly-palatable food in a novel, anxiety-provoking environment, compared to latencies in their home cage. Chronic, but not acute, AD (both selective serotonin reuptake inhibitors (SSRIs) and tricyclic compounds) reduce latencies in the novel environment without affecting behavior in the home cage. Of note, this paradigm is also sensitive to acute treatment with benzodiazepines, and has been described as a measure of anxiety behavior (Merali et al., 2003). As anxiety is often co-morbid with depression, and chronic treatment with SSRIs is effective in treating many anxiety disorders in humans, it may be difficult to interpret behavior in the NIH as anxiety or depression-related. However, it remains clinically relevant in its sensitivity only to chronic treatment with AD.

The effects of AD in this paradigm may require the increased hippocampal neurogenesis they produce, which also occurs after chronic, but not acute, treatment. In one study in which irradiation was used to eliminate dividing cells in the hippocampus, the behavioral effects of chronic AD treatment in the novelty-suppressed feeding paradigm (a test similar to the NIH), were blocked (Santarelli et al., 2003). However, in

a later study by the same group, increased hippocampal neurogenesis was found to be unnecessary for the behavioral effects of chronic FLX in the NIH in a different strain (Balb/cJ) of mice (Holick et al., 2008). Interestingly, Balb/cJ mice do not show increased neurogenesis following chronic AD treatment, as is seen in other strains of mice and rats, and the authors of the study suggest that other changes in neuronal plasticity in the hippocampus may comprise the response to chronic AD treatment in this strain.

Tests of anxiety-related behavior

Several paradigms exist to measure anxiety-like behavior in rodents, most of which have some ethological relevance. These paradigms are often designed around a conflict between the rodents' desire to explore vs. its fear of predation. Animals are placed in an apparatus, such as the elevated plus/zero maze (Pellow et al., 1985, Lister, 1987), open field (Hall, 1934), or light/dark box (Crawley and Goodwin, 1980), in which a portion of the area is "safe" (dark, enclosed) and a portion is "unsafe" (brightly-lit, open). A tendency of the animal to spend most of its time in the "safe" portions is seen as increased anxiety-like behavior in comparison to animals that spend more time exploring "unsafe" areas. These paradigms have been validated by a number of anxiolytic drugs, such as benzodiazepines, which increase time spent in "unsafe" areas.

Measures of anhedonia

In addition to anxiety, anhedonia is an endophenotype of depression often examined in rodent models. Preference or motivation for pleasurable activities can be

measured by providing the animal with an opportunity to consume a highly palatable food, such as a sucrose solution (Papp et al., 1991). Intracranial self-stimulation (ICSS) has also been used as measure of anhedonia (see (Zacharko and Anisman, 1991) for a review). In this paradigm, animals are implanted with electrodes in the medial forebrain bundle, and allowed to activate stimulation (which is pleasurable) by spinning a wheel or pressing a lever. Increased threshold for ICSS is seen as a sign of anhedonia: it takes more stimulation to interest the animal.

Effects of gain of function strategies on behavior

AD drugs alter CREB expression and activity in the hippocampus and cortex, and potentially several other brain regions (see Antidepressant drugs alter CREB expression and activity). To address the functional relevance of these changes, gain of function studies have been conducted to investigate what effect increasing CREB expression/activity might have on behavior (Table 1). One can experimentally mimic the effects of AD by increasing CREB function and activity, and assess whether this change in CREB is sufficient to bring about AD-like changes in behavior. In some cases, exaggerating the function of a protein by overexpression can provide information about the endogenous function. However, it should be noted that, when overexpressed, proteins may take on novel functions not usually carried about by the endogenous protein. In the case of CREB, this is especially important to keep in mind as CREB not only heterodimerizes with other CREB-family proteins (CREM and ATF-1), but competes with many transcription factors (CREM, ATFs 1-4, cJun) for DNA binding sites. Thus,

Table 1. Animal models for behavioral analysis of CREB function

Model	Strategy[a]	Species	Brain Region	Phenotype[b]	References
Gain of function	Viral expression HSV-CREBα	Rat	Hippocampus	• Antidepressant in FST, LH	Chen, et. al. 2001
Gain of function	Viral expression HSV-CREBα	Rat	Nucleus accumbens	• Pro-depressant in FST • Anhedonic in sucrose drinking	Pliakas, et. al. 2001
Gain of function	Viral expression HSV-CREBα	Rat	Basolateral amygdala	• Pro-depressant in FST • Antidepressant or pro-depressant in LH[c] • Anxiogenic in OF, EPM	Wallace, et. al 2004
Gain of function	Transgenic NSE-tTA/TetOp CREBα	Mouse	Forebrain[d]	•Pro-depressant in LH	Newton, et. al. 2002
Loss of function	Viral expression HSV-mCREB	Rat	Nucleus accumbens	• Antidepressant in FST, LH • Anxiogenic in OF, EPM • Hedonic in ICSS	Barrot, et. al. 2002, Dinieri, et. al. 2009; Green, et. al. 2006, Newton; et. al. 2002; Pliakas, et. al. 2001;
Loss of function	Transgenic NSE-tTA/TetOp mCREB	Mouse	Forebrain[d]	•Antidepressant in LH •Decreased dendritic length in immature neurons in DG	Newton, et. al. 2002; Fujioka, et. al. 2004
Loss of function	Transgenic CamKII-tTA/TetOp mCREB	Mouse	Forebrain[e]	• Decreased proliferation in DG	Nakagawa, et. al. 2002
Loss of function	Viral expression MoMLV-aCREB	Mouse	Hippocampus	• Decreased maturation and survival in DG	Jagasia, et. al. 2009
Loss of function	Gene ablation of CREBα and Δ	Mouse	Global	• Antidepressant in FST, TST • Accelerated response to antidepressants in NIH • Anxiogenic in EZM • Increased proliferation and survival in DG	Conti, et. al. 2002; Graves, et. al. 2002; Gur, et. al. 2007
Loss of function	Inducible knockout (transgenic) Nescre Creb1$^{loxP/loxP}$	Mouse	Whole brain	• Anxiogenic in EPM (no affect in EZM)	Valverde, et. al. 2004
Loss of function	Inducible knockout (viral expression) AAV-Cre Creb$^{loxP/loxP}$	Mouse	Hippocampus	• Accelerated response to antidepressants in NIH • Increased proliferation and survival in DG	Chapter 2

[a] Abbreviations: HSV, herpes simplex virus; NSE, neuronal-specific enolase; tTA, tetracycline transactivator; TetOp, tetracycline operon; mCREB, a dominant negative mutant form of CREB; CamKII, Calcium-calmodulin-dependent kinase IIa; MoMLV, Moloney murine leukemia virus; aCREB, a dominant negative mutant for m of CREB; Nescre, Cre recombinase driven by nestin promoter; loxP, locus of cross-over from the P1 bacteriophage; AAV, adeno-associated virus.

[b] Abbreviations: FST, forced swim test; LH, learned helplessness; OF, open field test; EPM, elevated plus maze; ICSS, intracranial self-stimulation; DG, dentate gyrus; TST, tail-suspension test; NIH, novelty-induced hypophagia; EZM, elevated zero maze.

[c] Timing of CREB expression affects behavioral phenotype; expression of CREB before training led to pro-depressant phenotype, whereas expression of CREB after training led to antidepressant phenotype.

[d] Leads to expression in dorsal striatum, nucleus accumbens, some areas of cerebral cortex, and some subfields of hippocampus.

[e] Leads to expression in olfactory bulb, cerebral cortex, caudate putamen, nucleus accumbens, amygdala, and hippocampus.

overexpression of CREB could alter function, as well as expression, of other transcription factors; the effect of overexpressing CREB on the expression of other CREB family proteins is not known.

The effects of AD treatment on CREB expression differ depending on the brain region examined (see Antidepressant drugs alter CREB expression and activity), therefore viral overexpression of CREB protein in specific areas of the brain was used to examine the behavioral significance of these region-specific changes in CREB. In the rat hippocampus, increasing CREB expression through this method led to decreased immobility in the FST and fewer escape failures in LH, both antidepressant-like effects (Chen et al., 2001, Wallace et al., 2004). Specifically, increased CREB expression in the dentate gyrus was sufficient to cause this antidepressant-like effect in LH, whereas increased CREB in the CA1 region of the hippocampus or fctx did not affect the number of escape failures in this paradigm (Chen et al., 2001). The effects of CREB overexpression in the hippocampus on the endophenotypes of anxiety and anhedonia have not been examined.

In contrast to antidepressant-like effects observed in the hippocampus, viral overexpression of CREB in the nucleus accumbens (NAc) of rats caused decreased latency to immobility in the FST, a pro-depressant effect (Pliakas et al., 2001). While there was no effect of increased CREB expression in the NAc on anxiety-related behaviors, it did cause a decrease in sucrose intake, an anhedonic phenotype (Barrot et al., 2002, Wallace et al., 2009).

In transgenic mice in which overexpression of CREB is under the control of a neuronal-specific enolase (NSE) promoter-driven system, strong CREB expression is observed in the dorsal striatum and NAc, as well as the dentate gyrus and CA1 regions of the hippocampus and the parietal cortex (Newton et al., 2002). These transgenic mice show increased escape latencies and an increased number of escape failures in LH, a pro-depressant effect. It is important to keep in mind the differences between virally- and transgenically-mediated overexpression strategies. Viral overexpression leads to more specific expression patterns, both spatially and temporally. As CREB plays an important role in development, any manipulation of CREB that is constitutively present may cause effects on neuronal development and organization, beyond the stimulus-dependent role CREB plays in adulthood. The transgenic line discussed here contains overexpressed CREB in the hippocampus and NAc, regions in which it appears to play opposite roles (based on the viral studies discussed above). In this case, the phenotype appears to be driven by increased CREB expression in the NAc.

While the hippocampus and NAc appear to be important brain regions in establishing antidepressant-related behaviors, other brain regions are likely involved in such complex behaviors. In the basolateral amygdala of rats, overexpression of CREB caused a pro-depressant effect in the FST, as well as increased anxiety-related behavior (Wallace et al., 2004). In learned helplessness, differential effects were observed, depending on when viral injections were given. If given before training, CREB overexpression caused a pro-depressant effect, whereas an antidepressant effect was seen

when viral injection occurred after training (Wallace et al., 2004). Anhedonia was not examined in this study.

CREB upregulation after AD administration occurs only after chronic, and not acute, treatment. However, the effects of virally-increased CREB expression have not been tested in a paradigm that responds to chronic, but not acute AD treatment such as the NIH. Additionally, whether CREB overexpression alters the level of hippocampal neurogenesis, which is also sensitive only to chronic AD treatment, is unknown.

Effects of dominant negative strategies on behavior

Overexpression of dominant negative mutant forms of CREB can be used to reduce CREB function in specific brain regions (Table 1). One such dominant negative, mCREB, contains a point mutation of Ser133, which is phosphorylated in the wild-type protein and necessary for transcriptional activation, at least in most cases (Gonzalez and Montminy, 1989). Thus, mCREB can dimerize with endogenous CREB protein, rendering it insensitive to phosphorylation and activation, and reducing its function. While the effects of hippocampal mCREB overexpression on behavior have not been examined, many studies have looked at the effects of expressing this dominant negative in the NAc, both through viral overexpression and transgenic strategies. Viral overexpression of mCREB in the NAc of rats increased latency to immobility in the FST, as well as decreasing escape latencies and failures in LH, both AD effects (Pliakas et al., 2001, Newton et al., 2002, Green et al., 2006). In rats, viral overexpression of mCREB in the NAc also caused increased anxiety in the open field and elevated plus maze, as well

as increased hedonic behavior, as shown by increased preference for sucrose and lower thresholds for ICSS (Barrot et al., 2002, Dinieri et al., 2009).

The same transgenic approach discussed above was used to express mCREB under control of a NSE-promoter (see CREB overexpression studies), leading to expression of mCREB not only in the NAc and dorsal striatum, but also in the parietal cortex, dentate gyrus, and CA1 subfield of the hippocampus (Newton et al., 2002). Transgenic expression of mCREB in these areas mimicked the viral expression of mCREB in the NAc, causing reduced escape latencies and failures in LH, an antidepressant effect (Newton et al., 2002). However, unlike viral expression of mCREB in the NAc, there was no change in ICSS thresholds in mice with transgenically-expressed mCREB (Dinieri et al., 2009); either the additional brain regions in which mCREB was expressed or the species difference (mice vs. rats) could contribute to this discrepancy. Behavioral models sensitive to chronic, but not acute, AD treatment have not been examined with regard to the effects of dominant negative CREB expression. Overall, the AD and hedonic phenotypes resulting from mCREB expression in NAc are parallel to, but opposite, results from CREB overexpression studies, which show the pro-depressant and anhedonic phenotypes.

Two dominant negative strategies have been applied to study the effects of reduced CREB function on hippocampal neurogenesis. Overexpression of mCREB under control of a NSE-promoter-driven system (described above) led to decreased dendritic length in immature neurons in the subgranular zone of the dentate gyrus (Fujioka et al., 2004). Using a calcium/calmodulin-kinase II (CamKII) promoter-driven

system, which caused expression of mCREB throughout the forebrain of mice, including the hippocampus, decreased proliferation of hippocampal neural progenitor cells (NPCs) was observed (Nakagawa et al., 2002). A different dominant negative CREB, aCREB (Ahn et al., 1998), in which the DNA-binding domain is mutated, thus blocking CREB's ability to activate transcription, decreased maturation and survival of adult-generated hippocampal neurons in female mice when virally overexpressed in the hippocampus (Jagasia et al., 2009). While each of these studies showed an effect of a dominant negative CREB on a slightly different aspect of hippocampal neurogenesis, it appears that CREB function is important for the process in general, as might be expected from previous studies that showed CREB is necessary for expression of pro-survival genes such as *Bcl-2* in cultured neurons (Riccio et al., 1999).

The dominant negative approaches discussed here are subject to similar caveats to overexpression of CREB itself. mCREB, with its mutated phosphorylation site, is capable of binding to and functionally inactivating not just CREB, but also CREM and ATF-1 (Hummler et al., 1994). Additionally, as mCREB maintains DNA-binding ability, inactive dimers that include mCREB may occupy CRE sites, thereby preventing the binding of other proteins, including those outside of the CREB family (Gonzalez and Montminy, 1989). mCREB would also retain any functions of CREB that do not require phosphorylation at Ser133, including the ability to be phosphorylated at serines 142 and 143 (Sun et al., 1994, Kornhauser et al., 2002). aCREB, in contrast to mCREB, maintains the ability to be phosphorylated, but contains a mutated DNA-binding domain. When overexpressed, it acts as a dominant negative by binding endogenous CREB and

CREB binding partners (such as CREM and ATF-1) and preventing their binding to DNA. While slightly more specific in this way than mCREB, aCREB could still function in ways other than simply blocking the activity of endogenous CREB.

Effects of constitutive knockout strategies on behavior

Not surprisingly, given the important role of CREB in development, constitutive deletion of CREB is lethal perinatally (Rudolph et al., 1998). However, a CREB-deficient line, in which the α and Δ (but not the β) isoforms of CREB are deleted ($Creb^{\alpha\Delta}$ mice), survive to adulthood (Hummler et al., 1994) and have been used to study the necessity of CREB for various behaviors (Table 1). While overexpression studies like those mentioned above have looked only at baseline behavior after altering levels of CREB, studies in the $Creb^{\alpha\Delta}$ mice have examined response to AD, which are important to determine if this response requires CREB. $Creb^{\alpha\Delta}$ mice show an antidepressant-like phenotype in the FST and TST, and additionally respond to acute AD treatment with a further reduction in immobility (Conti et al., 2002). In a model in which chronic, but not acute, AD treatment causes changes in the behavior of wildtype mice, the NIH, $Creb^{\alpha\Delta}$ mice respond to both chronic *and* acute AD treatment (Gur et al., 2007). Additionally, these mice show increased latency to consume in the novel environment of the NIH at baseline when compared to wildtype mice, suggesting an increased level of anxiety-like behavior, which is also observed in the elevated zero maze, another measure of anxiety-like behavior (Graves et al., 2002, Valverde et al., 2004, Gur et al., 2007). It is unknown

whether Creb$^{\alpha\Delta}$ mice have alterations in hedonic behaviors, such as sucrose preference or ICSS, which might accompany their AD and pro-anxiety phenotype.

In addition to their behavioral phenotypes, Creb$^{\alpha\Delta}$ mice show increased levels of hippocampal neurogenesis (Gur et al., 2007). It has been hypothesized that this increased neurogenesis may be driving the accelerated response to AD in the NIH, in which behavioral response to chronic AD treatment may require the concomitant increase in neurogenesis that occurs (Santarelli et al., 2003, Gur et al., 2007). This increase in neurogenesis is opposite to the effects on neurogenesis seen with mCREB. Behavioral results showing AD baseline effects in the FST in Creb$^{\alpha\Delta}$ mice may be consistent with the effects of expression of mCREB in the NAc (see Effects of dominant negative strategies on behavior), assuming that the loss of CREB in the NAc is driving the phenotype in Creb$^{\alpha\Delta}$ mice.

While there are caveats to dominant negative approaches, constitutive knockouts, too, have their disadvantages. In the Creb$^{\alpha\Delta}$ mice, CREB is deleted throughout the body, as well as throughout development. The lack of CREB during development could set up altered brain circuitry, leading to, among other things, the changes in adult neurogenesis observed. Additionally, CREM, a CREB-family protein, is known to be upregulated significantly after the deletion of CREB, in an apparent compensatory change (Hummler et al., 1994). Increased CREM expression could be responsible for the phenotype of Creb$^{\alpha\Delta}$ mice, and might explain the opposing findings in these mice versus mCREB studies; in the Creb$^{\alpha\Delta}$ mice there is increased CREM, and in the mCREB model, the activity of both CREB and CREM are inhibited.

Effects of inducible knockout strategies on behavior

Inducible knockouts increase spatial and temporal specificity of gene deletion. The Cre/lox system is one method of generating such inducible knockouts. In this system, part of the gene to be deleted is flanked by loxP (locus of X-over from the P1 bacteriophage) sites, eight base-pair consensus sequences recognized for excision by Cre recombinase. Once this recombination and excision by Cre recombinase takes place, the effect is permanent; excision does not occur until the onset of Cre expression, however once excision has occurred, the gene does not regain its function, even if Cre is no longer expressed. Knockout can also be limited spatially to wherever Cre recombinase is expressed.

Using a transgenically-expressed Cre under the control of both nestin (*Nescre)* and CamKII (*CamKIIcre)* promoters, loss of CREB can be limited to the brain, and to postnatal development in the latter case (Mantamadiotis et al., 2002). Only the *Nescre* *Creb1*$^{loxP/loxP}$ mice were examined behaviorally, and showed increased anxiety in the elevated plus maze (though they did not show increased anxiety in the elevated zero maze, a similar test) (Valverde et al., 2004). Other behaviors associated with depression and AD response were not examined in these mice.

As with other models of reduction of CREB expression, an increase in CREM expression was observed in this model (Mantamadiotis et al., 2002). To assess what contribution this upregulation of CREM might make to the phenotype (or lack thereof), C*reb1*$^{loxP/loxP}$ mice were crossed with mice harboring a constitutive knockout of CREM (*CamKIIcre Creb1*$^{loxP/loxP}$*Crem-/- and Nescre Creb1*$^{loxP/loxP}$*Crem-/-)*. With Cre expressed

under control of the nestin promoter, the combination was perinatally lethal (Mantamadiotis et al., 2002). With Cre expression under control of a CamKII promoter, a "neurological" phenotype emerged, accompanied by significant neurodegeneration, neither of which was observed when a single copy of CREM was present (Mantamadiotis et al., 2002). These findings are consistent with the idea that CREB function is necessary for cell survival (Riccio et al., 1999), although levels of the known CREB target and anti-apoptotic gene *Bcl-2* and other related anti- and proapoptotic genes (Bcl2l, Bcl2l2, Bak1, Bax and Bad) were not changed in this study (Mantamadiotis et al., 2002). It was reported that there was no change in neurogenesis in the subventricular zone at day E18.5 in the *Nescre Creb1*$^{loxP/loxP}$*Crem-/-* mice (Mantamadiotis et al., 2002). Adult neurogenesis, specifically in the hippocampus, was not examined. Additionally, it also noted that the transgenic promoter may not have caused expression of Cre recombinase in neural progenitor cells in the hippocampus. These studies suggest that there is dynamic regulation within the CREB family, and that such compensatory changes in CREM play a significant role in mitigating the effects of CREB deletion.

Using the Cre/lox system, the extent of an inducible knockout can be further limited to adulthood, as well as to a specific brain region, if Cre recombinase is expressed virally, rather than as a transgene. For example, a similar line of *Creb*$^{loxP/loxP}$ mice injected with an adeno-associated virus expressing Cre recombinase (AAV-Cre) directly in the hippocampus showed a robust deletion of CREB specifically in this region (see Chapter 2). Hippocampal-specific CREB deletion did not alter anxiety-related behavior in the elevated zero maze, anhedonia in a sucrose preference test, or baseline behavior in

the FST. Additionally, response to acute treatment with an AD in the FST was intact in these mice, as was response to chronic AD treatment in the NIH. Interestingly, loss of CREB from the hippocampus was sufficient to phenocopy the $Creb^{\alpha\Delta}$ mice as far as their response to acute treatment with AD in the NIH as well as their increased level of hippocampal neurogenesis at baseline. C$reb^{loxP/loxP}$ mice injected with AAV-Cre in their hippocampi exhibited an increase in baseline latency to consume in the novel environment, as well as a decrease in latency after acute AD treatment. Like the $Creb^{\alpha\Delta}$ mice, mice with CREB deleted from their hippocampi also show increased levels of hippocampal neurogenesis, providing further evidence that this increased neurogenesis may mediate the response to acute AD in the NIH paradigm. The fact that the $Creb^{loxP/loxP}$ mice phenocopy the $Creb^{\alpha\Delta}$ mice suggests that the phenotype of the $Creb^{\alpha\Delta}$ mice is not due to changes that occurred during development as a result of the loss of CREB; loss of CREB only in the hippocampus and only in adulthood is sufficient to produce an identical phenotype.

As with other models of CREB deletion, $Creb^{loxP/loxP}$ mice injected with AAV-Cre show increased expression of CREM after only 8 weeks, suggesting that such compensatory changes are more dynamic than previously believed. It is hypothesized that this increased CREM contributes to the phenotypes observed in these mice by altering CRE-mediated gene expression. In particular, upregulation of the pro-survival and CREB target gene *Bcl-2* was observed, which may contribute to the increased number of new neurons in the dentate gyrus in these mice. However, further studies are necessary to explore this hypothesis.

Conclusion

CREB is located at the convergence of several signaling pathways activated by AD treatment. Indeed, as a transcription factor, CREB seems uniquely poised to transduce the immediate effects of AD drugs on synaptic monoamine levels into long-term changes brought about by altered gene expression and synaptic plasticity. Studies in which CREB expression and activity are altered following chronic, but not acute AD treatment suggest that CREB may indeed be activated by these drugs, however this evidence does not prove that CREB plays a pivotal role in mediating the downstream effects of these drugs.

Studies of rodent models of depression have suggested that CREB does play a causal role in the behavioral effects of these drugs. However, the picture is far from clear. Increasing CREB in some brain regions has an AD effect, while in other brain regions, it causes an *increase* in depression-like behavior. These studies do not provide conclusive evidence that increasing or decreasing CREB activity would ameliorate the symptoms of depression. Additionally, altering CREB function likely causes changes in the expression and activity of other CREB family proteins, clouding the interpretation of results of many of these studies: when you decrease CREB expression, are you really decreasing the activation of its targets? None of the models presently used to evaluate the importance of CREB is without flaws, although technology is allowing for greater specificity in the temporal and spatial extent of such manipulations. The best assessment of the endogenous function of CREB is likely to be gained from evaluating converging evidence obtained from multiple models.

As CREB itself is unlikely to provide a useful target for pharmacological agents (due to its ubiquitous expression and diverse functions), the important endpoint of this research is to identify the genes whose expression is affected by CREB. There may be as many as 10,000 CREs in the mammalian genome (Impey et al., 2004, Zhang et al., 2005). As there are other proteins that bind to CREs, additional verification, beyond the presence of a CRE in its promoter, is necessary before concluding that a gene is indeed a CREB target. Activation of different sets of target genes likely underlies the differential effects of CREB in different brain regions, although the mechanism by which this specificity is achieved is not understood. Identifying which targets are behind the cellular and behavioral changes brought about by CREB, *in a specific brain region and after a specific stimulus*, will identify targets that are not only more likely to allow for effective therapy, but reduce side effects as well. It is possible that some of these targets are well-studied proteins for which agonists or antagonists already exist, and may provide therapeutic benefit. The identification of novel targets would also be useful to broaden the lens through which depression research is currently conducted.

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Chapter 2: Increased hippocampal neurogenesis and accelerated response to antidepressants in mice with specific deletion of CREB in the hippocampus

Abstract

The transcription factor CREB has been implicated in the pathophysiology of depression as well as in the efficacy of antidepressant (AD) treatment. Expression and activity of CREB are increased by treatment with antidepressant drugs, and CREB target genes include those involved in the antidepressant response. However, altering CREB levels appears to have differing effects on depression-related behaviors, depending on which brain region is examined. To further investigate the region-specific importance of CREB in depression-related behavior and AD response, we used *Creb*$^{loxP/loxP}$ mice injected with an adeno-associated virus (AAV) expressing Cre recombinase to limit the deletion of CREB to the hippocampus. Eight weeks after Cre virus injections, we observed a robust and significant reduction in CREB protein throughout the hippocampus (including CA1, CA3, and dentate gyrus). At this time point, hippocampal CREB deletion did not alter behavior in contextual fear conditioning, locomotor activity, anxiety-related behavior, or response to acute AD in the forced swim test. In the novelty-induced hypophagia (NIH) paradigm, an assay sensitive to chronic, but not acute AD response, hippocampal CREB loss did not block response to chronic treatment with AD. However, *Creb*$^{loxP/loxP}$ mice injected with AAV-Cre in their hippocampi responded to acute treatment with AD, unlike control mice. This accelerated response was

accompanied by an increase in hippocampal neurogenesis in these mice. Upregulation of the CREB-family protein CREM was observed after CREB deletion, and appears to functionally compensate for CREB loss, leading to maintained or increased expression of CREB-target genes. Some of these genes, such as Bcl-2 and BDNF, are known to regulate cell survival and differentiation, and may contribute to the increased hippocampal neurogenesis observed after CREB deletion. These findings indicate that CREB family proteins are important in regulating hippocampal neurogenesis and associated behaviors.

Introduction

The mechanism by which the initial pharmacological action of antidepressants, namely increasing synaptic levels of monoamine neurotransmitters (Frazer, 1997), translates into their much slower onset of therapeutic efficacy (Wong and Licinio, 2001, Nestler et al., 2002), is not fully understood. The transcription factor CREB (cyclic-AMP response-element binding protein) is thought to play a role in the long-term effects of antidepressants, as it regulates expression of many genes, including those involved in neuroplasticity and cell survival, which have themselves been implicated in depression and antidepressant response (Nair and Vaidya, 2006, Tardito et al., 2006). The expression and activity of CREB are increased by chronic, but not acute treatment with antidepressants in both rodent (Nibuya et al., 1996, Thome et al., 2000, Blom et al., 2002, Tiraboschi et al., 2004) and post-mortem human brain (Dowlatshahi et al., 1998).

However, some researchers have reported decreases in CREB protein following antidepressant treatment, and it appears that the regulation of CREB by antidepressants differs depending on the brain region examined (Frechilla et al., 1998, Manier et al., 2002, Laifenfeld et al., 2005).

Numerous studies have examined the effects of changing the level of CREB expression in the brain on behavioral models of depression in rodents. CREBαΔ mice, in which the alpha and delta forms of CREB are knocked out throughout development, show baseline increases in anxiety-like behavior, as well as antidepressant-like behavioral responses in the forced -swim (FST) and tail suspension tests (TST) (Conti et al., 2002, Graves et al., 2002). In the novelty-induced hypophagia paradigm, in which chronic, but not acute antidepressants are effective in wild-type animals, CREBαΔ mice show an accelerated response to antidepressant treatment (Gur et al., 2007). CREB appears to differentially affect behavior, depending on where it is expressed (Carlezon et al., 2005). For example, viral overexpression of CREB has an antidepressant effect if it is localized to the hippocampus (Chen et al., 2001), or a pro-depressant and pro-anxiety effect when localized to the nucleus accumbens (Pliakas et al., 2001, Barrot et al., 2002) or amygdala (Wallace et al., 2004). Viral expression of a dominant negative form of CREB, mCREB, had the opposite effect of CREB overexpression (Pliakas et al., 2001, Barrot et al., 2002, Newton et al., 2002, Barrot et al., 2005). However it should be noted that such overexpression strategies may affect activity of other CREB-family proteins, which can heterodimerize with CREB and mCREB, as well as bind to the same consensus sequences (CRE sites) in DNA.

Hippocampal neurogenesis, which is increased after chronic, but not acute, treatment with antidepressants (Malberg et al., 2000, Sairanen et al., 2005), may also be regulated by CREB. CREB is expressed by immature neurons in the subgranular zone (SGZ) of the dentate gyrus (DG) (Nakagawa et al., 2002a, Fujioka et al., 2004, Jagasia et al., 2009). Overexpression of a dominant negative form of CREB leads to reductions in proliferation, survival, and differentiation of neurons in the DG (Nakagawa et al., 2002b, Jagasia et al., 2009). However, CREBαΔ mice show increased levels of hippocampal neurogenesis (Gur et al., 2007), suggesting that further study is necessary to determine the role of CREB in regulating hippocampal neurogenesis.

To allow for a specific examination of the role of CREB in the adult hippocampus, in regulating behavioral and cellular correlates of anxiety, depression, and antidepressant response, we deleted CREB specifically in the hippocampi of $Creb^{loxP/loxP}$ mice using an adeno-associated virus (AAV) expressing Cre recombinase. A similar line of $Creb^{loxP/loxP}$ mice, in which Cre was expressed under the control of either a CamKII or nestin promoter, showed increased anxiety (Mantamadiotis et al., 2002, Valverde et al., 2004). Additionally, when combined with a constitutive knockout of the related protein CREM, a moderate neurodegenerative phenotype was observed (Mantamadiotis et al., 2002). In our studies, expression of Cre began in adulthood, and was limited to the hippocampus.

Methods

Animals. Mice containing the *creb1* gene with exon 10/11 flanked by loxP sites were generated by John Lelay in the laboratory of Klaus Kaestner (University of Pennsylvania), and maintained on a C57BL/6 background. For all behavioral studies, $Creb^{loxP/loxP}$ mice were injected with AAV-Cre virus in the hippocampus to cause specific deletion of CREB in this region. Control mice were $Creb^{loxP/loxP}$ or $Creb^{loxP/+}$ animals injected with AAV-GFP. Male and female mice were group-housed with food and water available *ad libitum* (except as noted) and maintained on a 12-hour light/dark cycle (lights on at 07:00) according to the University of Pennsylvania Animal Care and Use Committee. Mice weighed 20-40g and were three to five months old at the time of behavioral testing.

Drugs. Desipramine (DMI) was dissolved in 0.9% saline immediately before use and a volume of 10 mL/kg was injected intraperitoneally.

Adeno-associated virus production. The University of Pennsylvania Vector Core generated AAV constructs expressing Cre recombinase (AAV-Cre) (AAV2/9.CMV.PI.Cre, titer $2.84*10^{13}$ gc/ml) or enhanced green fluorescent protein (AAV-GFP) (AAV2/9.CMV.eGFP, titer $3.74*10^{13}$ gc/ml). Each expression cassette contained AAV2 terminal repeats flanking the cytomegalovirus (CMV) promoter-PI-Cre recombinase or CMV promoter-eGFP sequences, packaged into AAV9. Vector purification was performed using a CsCl sedimentation method, and quantification of

vector genome copies (gc) was performed by an RT-PCR method. AAVs are diluted in sterile PBS for microinjections.

Stereotaxic surgery and intrahippocampal microinjection. Surgery was performed on adult mice (6-8 weeks). After anesthesia with isofluorane, mice were secured in a stereotaxic frame (Kopf, Tujuna, CA). Holes were drilled bilaterally in the skull at the injection sites (four total). Stereotaxic coordinates used for intrahippocampal injections were (from Bregma) anterior-posterior -2.1, lateral +/- 1.4, dorso-ventral -2.0, and anterior-posterior -2.9, lateral +/- 3.0, dorso-ventral -3.8. A 33 gauge needle attached to a 5 μL Hamilton syringe (Hamilton, Reno, NV), mounted to the stereotaxic frame and under control of a KDS310 Nano Pump (KD Scientific, Holliston, MA), was used to inject 0.5 μl of $1*10^9$ gc/μl AAV at each site. Injections occurred at a rate of 0.15 μl/min, after which the needle was left in place for an additional four minutes. After injections were completed, the skin was sutured and the animals were allowed to recover for 1hr on a heating pad before returning to the home cage. Mice remained in the home cage for an additional eight weeks prior to the start of behavioral testing, unless otherwise noted.

Behavioral studies. Behavioral experiments occurred between the hours of 08:00 and 15:00. Treatment conditions were assigned randomly and animals were tested in counterbalanced order. All drug injections were given at least 1hr prior to the start of behavioral testing, and animals were allowed to acclimate to testing rooms during this period.

Locomotor activity. Mice were placed into a clean home cage (one mouse/cage) resting within the photobeam frame (Med Associates, St. Albans, VT, USA). Locomotor activity was measured by beam-breaks and recorded by Med Associates software in 5-minute bins for 1hr. As changes in activity levels can affect other behaviors, measuring locomotor activity served as an important control.

Contextual fear conditioning. The fear conditioning chamber was a rectangular box with a metal grid floor capable of delivering footshocks Med Associates, St. Albans, VT). In the training session, mice were placed in the box for three minutes, receiving a two-second, 1.5mA scrambled shock between 2:28-2:30. After training, the mice were returned to their home cages until testing. Testing of long-term contextual fear memory was conducted 24 hours after training, at which time the mice were placed back in the context in which they had received the footshock. Freezing behavior was assessed from videotapes by a blinded observer using a five-second sampling technique.

Forced-swim test (FST). Mice were placed in plastic cylinders (23cm tall × 14cm diameter) containing 15cm of water (22-24°C). The 6-minute test was video-recorded and time spent in passive floating behavior ("immobility") vs. active escape behaviors such as swimming and climbing was scored by a blinded observer. Mice received three injections of either saline or DMI (15, 15, and 20 mg/kg) 24hr, 5hr, and 30 min before the test. This dosing paradigm was shown to be effective both in previous studies (Conti et al., 2002) as well as in pilot studies in this strain.

Elevated zero maze (EZM). Mice were given a 5-minute exposure to the zero maze (Stoelting, Wood Dale, IL), which consisted of two open areas (wall height, 0.5") and

two closed areas (wall height, 12”), and was elevated 24” from the ground. Lighting in the maze was 15 lux, and mice began the 5-minute exposure in one of the closed areas. The test was video-recorded and the Viewpoint Tracking System (Viewpoint, Champagne au Mont d’Or, France) was used to quantify the amount of time spent in the open areas, the number of entries into the open areas, and the distance traveled in each area.

Marble burying. The arena for marble-burying consisted of a small plastic mouse cage (26 × 20 × 14 cm) covered in bedding to a depth of 5cm. 20 marbles were equally distributed around the edge of the cage. Mice were placed in the cage (covered with a plastic lid), and left undisturbed for 15 min. At the end of the testing period, mice were carefully removed from the cage and the number of marbles buried (covered three quarters or more in bedding) was counted by an experimenter blind to group.

Novelty-induced hypophagia (NIH). Mice were pair-housed for at least one week before the start of and throughout the study. Training began six weeks after stereotaxic surgery, such that drug treatment and testing occurred at least eight weights post-surgery. During training and home cage testing, plastic dividers were used to separate the two mice in each cage, and mice were allowed to acclimate to the divider and the testing room for one hour prior to the beginning of the experiment. Training and home cage testing consisted of a 15-minute exposure to a highly palatable food (peanut butter chips) (Nestle, Glendale, CA) in a small petri dish (15cm in diameter) in the home cage. Latency to approach and consume the food was measured. Mice were trained for 12-14 days, or until their latencies had fallen to a consistent plateau.

In experiments with acute antidepressant treatment, testing began on the day following the last day of training, and mice received drug treatment in the morning and afternoon (09:00 and 17:00) of each of the three testing days (home, novel, home), such that by the time of the novel test, mice had received three administrations of drug. In experiments with chronic antidepressant treatment, mice were given 21 days of twice-daily injections beginning after the last day of training. Following the three-week treatment period, mice were tested in home, novel, and home environments, during which time they continued receiving twice-daily drug treatment at similar time points as in the acute study.

The novel environment test involved exposing mice to the food in an anxiogenic environment, which consisted of an empty standard cage, lacking bedding, which was placed in a white box with bright illumination (2150 lux) and with an added novel scent (Pine Sol or mint extract) applied to the cage. As in the home test, latency to consume food was measured by an experimenter blind to condition, with a maximum latency of 15 min. Novel testing in both acute and chronic experiments occurred 1 hour after separators were placed in the home cage and 1 hour after mice received their last injection, parallel to the timing of home cage testing.

Sucrose preference test. To assess preference for a sucrose solution, mice were single-housed and given 24-hour access to two bottles from which to drink, both equipped with sipper tubes with ball-bearings (Sta-Pure Systems, Pittsburgh, PA) to prevent leaking. Bottles were weighed daily to assess the amount of fluid consumed, and their position was switched daily to prevent development of a place preference. After a week of

acclimation to single-housing, mice were given another week during which both bottles were filled with water, in order for them to acclimate to drinking from each bottle, and to establish that there was no preexisting preference for one side or the other. To assess preference for sucrose solution, mice were exposed to a 0.5% and then a 1% sucrose solution (w/v) for four days each (concentrations were chosen based on pilot studies in this strain). Sucrose preference was defined as volume of sucrose solution consumed (over four days) divided by the total volume of liquid consumed over 4 days.

Bromo-deoxyuridine (BrdU) injection. Eight weeks following stereotaxic surgery, mice were administered a BrdU solution (Roche, Mannheim, Germany), by i.p. injection, to label dividing cells. To evaluate cell proliferation, mice received a single bolus of 200 mg/kg BrdU and were killed 24 hours later. To evaluate the survival of newly-generated cells, mice received 4 injections of 100 mg/kg BrdU (one per day for four days) and were killed four weeks after the last injection.

Tissue collection for immunohistochemistry. Mice were anesthetized with sodium pentobarbital (10 mg/kg) and transcardially perfused with 40 mL phosphate-buffered saline (PBS), followed by 30 mL of 4% paraformaldehyde in PBS. Brains were removed and post-fixed in the same fixative overnight at 4°C, after which they were transferred to a 30% sucrose solution (in PBS), which contained 0.1% sodium azide (NaN_3) for at least 48 hr at 4°C. Brains were frozen on dry ice and 40 μm sections were generated using a cryostat. Sections were placed in PBS with 0.1% NaN_3 and stored at 4°C until further processing. A series of every ninth section was used for immunohistochemistry.

BrdU immunohistochemistry. Immunohistochemistry was performed on slide-mounted sections encompassing the entire anterior-posterior axis of the hippocampus (10-12 sections per brain). Sections were boiled in heated citric acid (0.1M, pH 6.0) for 20 min for antigen retrieval. After washing in PBS, sections were treated with 0.1% trypsin solution with 0.1% $CaCl_2$ for 10 min to permeabilize cells. Following additional rinses, sections were treated for 30 min in 2N HCl. Sections were again rinsed, after which they were incubated with mouse anti-BrdU (1:200; Becton Dickinson, Franklin Lakes, NJ) with 0.5% Tween 20 overnight at room temperature. After rinsing, sections were incubated for 60 min in secondary antiserum (1:200 biotinylated horse anti-mouse IgG; Vector Laboratories, Burlingame, CA). Sections were rinsed, and incubated in avidin–biotin complex (Vector Laboratories) for 60 min. After additional rinses, visualization was achieved with 3,3'-diaminobenzidine-4HCl (DAB) tablets (Sigma). Sections were counterstained with 0.1% cresyl violet, dehydrated, and coverslipped.

Doublecortin (DCX) Immunohistochemistry. Free-floating sections were rinsed in PBS and incubated in a blocking solution of 3% Normal Horse Serum (Vector Laboratories, Burlingame, CA) with 0.5% Tween + 0.2% Triton in PBS for 1hr. Sections were then incubated for 72hr at 4°C in goat-anti-DCX (#8066, Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:500 in blocking solution. After several rinses in PBS, sections were incubated in secondary antiserum (Horse-anti-goat, Vector Laboratories, Burlingame, CA), diluted 1:200 in blocking solution. Following additional rinses, sections were exposed to 0.75% H_2O_2 in PBS for 20 min to block endogenous peroxidases. After several rinses, sections underwent a 60min incubation in avidin-biotin complex (ABC

elite kit, Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS. Staining was visualized with .04% DAB (Sigma, St. Louis, MO) containing 0.01% H_2O_2 and 0.06% nickel sulfate in Tris Buffer for ten minutes, yielding a black reaction product. Sections were mounted on glass slides, dehydrated, and coverslipped.

CREB Immunohistochemistry. Free-floating sections were incubated in 0.75% H_2O_2 in PBS for 20 min. After several rinses in PBS containing 0.3% Triton X-100 and 0.04% bovine serum albumin (PBS-Tx-BSA), sections were incubated overnight at 4°C in rabbit anti-CREB (#9197, Cell Signaling, Beverley, MA), diluted 1:800 in PBS-Tx-BSA containing 0.1% NaN_3. After several rinses in PBS-Tx-BSA, sections were incubated in secondary antiserum (biotinylated donkey anti-rabbit; Jackson ImmunoResearch, West Grove, PA), diluted 1:200 in PBS-Tx-BSA for 90 min. Sections were rinsed in PBS-Tx-BSA before incubation in avidin-biotin complex (ABC elite kit, Vector Laboratories, Burlingame, CA) for 90 min. After additional rinses in PBS, sections were treated with .04% DAB (Sigma, St. Louis, MO) containing 0.01% H_2O_2 and 0.06% nickel sulfate in Tris Buffer for five minutes, yielding a black reaction product. The DAB reaction was terminated by additional rinses in PBS. For double-labeling of DCX in addition to CREB, the procedure for DCX staining was carried out as above, immediately following CREB visualization with DAB. DCX was visualized in this case with 0.04% DAB containing 0.01% H_2O_2 in PB for a brown reaction product. After processing, sections were mounted on glass slides, dehydrated, and coverslipped. Immunoreactivity was visualized using a Nikon Eclipse E600 microscope (Melville, NY) and images were

captured with a QImaging Retiga 1300 (Surrey, British Columbia, Canada) using Image-Pro Plus software (MediaCybernetics, Bethesda, MD).

eGFP visualization. Untreated sections were wet-mounted and visualized with a fluorescent microscope (Leica) and images collected via an AxioCam HRc camera.

BrdU and DCX cell counting. BrdU cell counting was carried out using a 100x oil immersion lens on a Nikon Eclipse E600 microscope (Melville, NY). All BrdU-labeled cells in the dentate gyrus granule cell layer (including cells within two cells' distance of the granule cell layer) and hilus (all other cells within the dentate gyrus) were counted in each section by an experimenter blinded to condition. The total number of BrdU-labeled cells was normalized to a harmonic mean of the number of hippocampal sections counted, as well as multiplied by 9 to result in the total number of BrdU-labeled cells per dentate gyrus. DCX-labeled cells were counted in a similar manner to BrdU-labeled cells, with the exception that all cells present in the granule cell layer were counted, and hilar cells were not quantified.

CREB immunohistochemical quantification. To assess the amount of CREB protein expressed throughout the hippocampus, three sections from each hippocampus were quantified, with the following A/P coordinates (from Bregma): -1.5 (anterior to the anterior injection site), -2.5 (between the two injection sites), and -3.5 (posterior to the posterior injection site). For each of these sections, one picture each of the dentate gyrus, CA1 and CA3 subfields, taken at 10x, were used. The regions of interest were outlined and the integrated density of staining, normalized both to the area selected and the level of background staining, was measured. The average of the three sections was then

calculated to produce one number for each of the three subfields. Levels of staining in the AAV-Cre-injected animals were normalized to AAV-GFP-injected animals to give a percent reduction in CREB protein.

RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction. Mice were killed by cervical dislocation either directly from their home cages in the mouse colony, at least one week following any behavioral testing, or 10 minutes following a forced-swim for the evaluation of immediate early gene expression. Brains were rapidly removed, whole hippocampi hand-dissected, and frozen in liquid nitrogen. RNA was extracted from hippocampal tissue using TRIzol/chloroform (Invitrogen) and the RNeasy Mini kit (Quiagen). CDNA was synthesized from RNA using an Oligo dT primer (Operon) and Superscript II reverse transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction (QPCR) was carried out using SYBR-green master mix (Applied Biosystems) and 300 nM primers (final concentration), and run on the Stratagene MX3000 using MXPro QPCR software. Cycling parameters were 95°C for 10min followed by 40 cycles of 95°C (30s) and 60°C (1min), ending with a melting curve analysis to control for the amplification of a single gene product. All reactions were performed in triplicate, with the median cycle time used for analysis. TATA-box binding protein (TBP) was used as a housekeeping gene against whose levels all experimental genes were normalized. Primer sequences can be found in Table 1.

Statistical analysis. For the elevated zero maze, marble burying, DCX immunohistochemistry, and gene expression studies (excluding *cfos*), Student's t-test was used to assess statistical significance. For the forced-swim test, *cfos* expression, and

Table 1. Primers used for RT-QPCR analysis (see Methods) are listed.

Gene	Forward Primer	Reverse Primer
Bcl-2	5'-CCATGCATCTCAGCATTGTTT-3'	5'-AAAGCTGTTCCCACCTTTTCA-3'
BDNF exon I	5'-CAGTGACAGGCGTTGAGAAAG-3'	5'- AACGCCCTCATTCTGAGAGAC -3'
BDNF exon IV	5'-GCCTCTGCCTAGATCAAATGG-3'	5'-AGTCTTTGGTGGCCGATATGT-3'
c-FOS	5'- TCCTTCTATGCAGCAGACTGG-3'	5'-AGTACAGGTGACCACGGGAGT-3'
CREM	5'-CAGAGGAAGAAGGGACACCA-3'	5'-TTGTATTGCCCCGTGCTAGT-3'
ICER	5'- ATGGCTGTAACTGGAGATGAAACT-3'	5'- GTAGGAGCTCGGATCTGGTAAGT-3'

CREB immunohistochemisty, ANOVA and Bonferroni *post hoc* tests were used to assess significant differences between treatment groups. For locomotor activity, fear conditioning, and BrdU immunohistochemistry, repeated measures ANOVA was performed with either time bin (locomotor activity), testing period (pre-shock, post-shock, and recall for fear conditioning), or brain region (granule cell layer or hilus for BrdU immunohistochemistry) as the repeated measure. For the NIH experiments, three-way, repeated measures ANOVA was performed with day (home, novel, home) as the repeated measure. Bonferroni *post hoc* tests were used to determine statistical differences in specific pair-wise comparisons. Statistical significance was set at $p<0.05$.

Results

Hippocampal injection of adeno-associated virus expressing Cre recombinase in *Creb*$^{loxP/loxP}$ mice leads to robust and specific decrease in CREB expression

CREB plays varying roles, depending on when it is expressed, and which brain region is examined. Therefore, to assess the role of CREB specifically in the adult hippocampus, we utilized a mouse in which deletion of CREB was inducible (*Creb*$^{loxP/loxP}$ mice). To limit the temporal and spatial extent of CREB deletion, we injected an adeno-associated virus expressing Cre recombinase (AAV-Cre) directly into this region in adult mice. An identical AAV, which expresses enhanced green fluorescent protein (AAV-GFP) in place of Cre recombinase was used to determine the spatial extent of viral transduction and served as a control virus for all behavioral studies. Two weeks following

surgery, GFP expression could be seen in the hippocampi of mice injected with AAV-GFP, and by four weeks, there was robust expression throughout the anterior-posterior axis of the hippocampus, including both dorsal and ventral aspects (Figure 1). Importantly, GFP was not observed outside of the hippocampus, suggesting that the virus had not spread to infect cells outside of this region. Additionally, we observed little damage at the injection site, and no obvious neurodegeneration as a result of viral infection, based on gross visual inspection, as expected from previous reports (Kaspar et al., 2002, Ahmed et al., 2004).

Viral expression of GFP in the hippocampus does not guarantee that Cre recombinase is expressed at adequate levels to effectively excise the floxed region of CREB. Therefore, to measure the amount of CREB protein in the hippocampus, we performed immunohistochemical labeling using an anti-CREB antibody at several time points after the viral injection (Figure 2). In this and in subsequent experiments, $Creb^{loxP/loxP}$ and $Creb^{loxP/+}$ animals injected with AAV-GFP were used as controls. At four weeks post-injection, levels of CREB protein were significantly reduced in each of the three subfields of the hippocampus examined (dentate gyrus (DG), CA1, and CA3), but still approximately 50% of the levels found in controls (DG: main effect of viral injection, $F_{(1,64)}=164.8$, $p<0.0001$; No significant effect of time or interaction. CA1: main effect of viral injection, $F_{(1,60)}=149.3$, $p<0.0001$; main effect of time, $F_{(3,60)}=3.751$, $p=0.0155$; significant virus x time interaction, $F_{(3,60)}=3.751$, $p=0.0155$; GFP vs. Cre, $p<0.01$ for four weeks, $p<0.001$ for eight weeks, $p<0.001$ for nine weeks, $p<0.001$ for twelve weeks, Bonferroni *post hoc* test. CA3: main effect of viral injection, $F_{(1,61)}=97.68$,

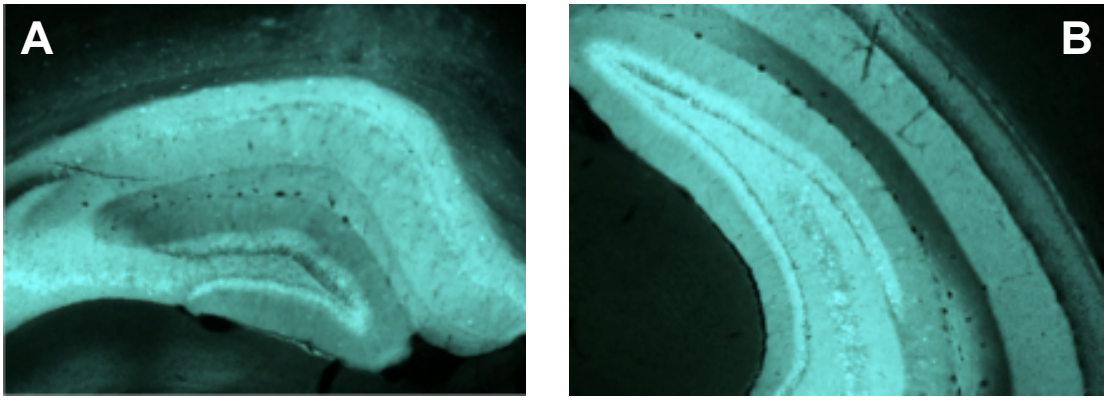

Figure 1. AAV-GFP spreads throughout, but not beyond hippocampus. Sections from dorsal (**A**) and ventral (**B**) hippocampus showing strong expression of eGFP throughout the hippocampus, 4 weeks after injection with AAV-GFP.

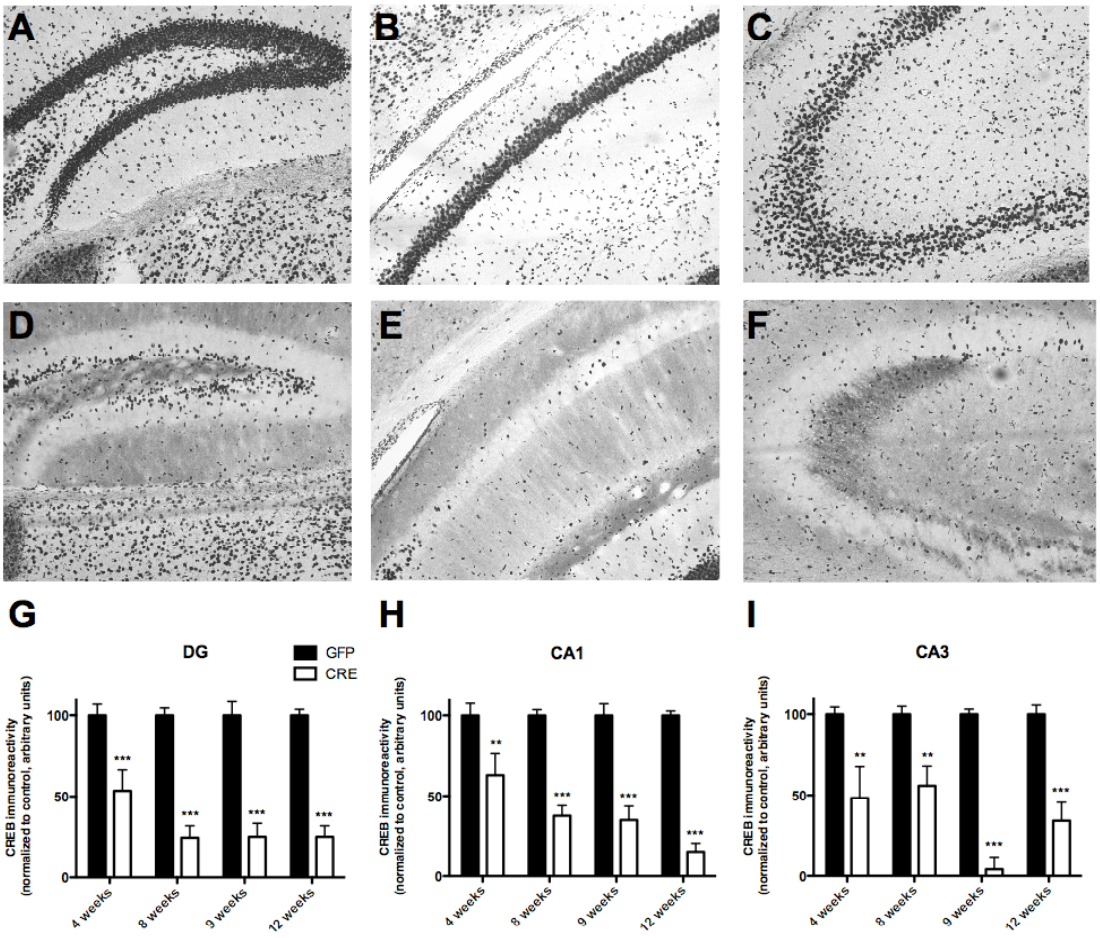

Figure 2. Intrahippocampal injection with AAV-Cre leads to robust and significant deletion of CREB in the hippocampus of *Creb*$^{loxP/loxP}$ mice 8 weeks after injection. Hippocampal sections from the brains of *Creb*$^{loxP/loxP}$ mice 4, 8, 9, and 12 weeks after intrahippocampal administration of AAV vectors were stained with an anti-CREB antibody. **A-F**, Representative photomicrographs of the dentate gyrus (**A** and **D**), CA1 (**B** and **E**) and CA3 (**C** and **F)** subfields of the hippocampus. Extensive CREB staining is seen is seen in the AAV-GFP-injected animals (**A-C**), but is lacking in the AAV-Cre-injected animals (**D-F**). **G-I**, Quantification of average staining in three hippocampal sections (approx. A/P coordinates -1.5, -2.5, and -3.5 from Bregma) showing significant reduction in CREB expression in DG (**G**), CA1 (**H**), and CA3 (**I**) subfields at all time points examined, with greatest extent of deletion occurring by 8-9 weeks after surgery (4 weeks: n=7-8; 8 weeks: n=7-8; 9 weeks: n=8-10; 12 weeks: n=9-12). **p<0.01, ***p<0.001 vs. equivalent AAV-GFP group; +p<0.0001 main effect of viral injection. Error bars indicate SEM.

$p<0.0001$; main effect of time, $F_{(3,61)}=3.017$, $p=0.0366$; significant virus x time interaction, $F_{(3,61)}=3.017$, $p=0.0366$; GFP vs. Cre, $p<0.01$ for four weeks, $p<0.01$ for eight weeks, $p<0.001$ for nine weeks, $p<0.001$ for twelve weeks, Bonferroni *post hoc* test.) (Figure 2G-I). By eight weeks post-injection, levels of CREB protein were reduced further in the DG and CA1 regions, and there was no consistent additional decrease in levels after nine weeks post-injection (Figure 2G-I). Based on these data, we began our behavioral studies in mice eight weeks after they received surgery.

Hippocampal-specific CREB deletion does not affect long-term memory of contextual fear conditioning

Both CREB and the hippocampus are known to be important for some types of learning and memory. Therefore, we assessed long-term contextual memory in a fear-conditioning paradigm, which requires hippocampal function. Eight weeks after intrahippocampal injection with AAV-GFP or AAV-Cre, $Creb^{loxP/loxP}$ mice were trained to associate a context with an aversive event (a single foot-shock), and tested 24 hours later for their recall of this association. As shown by the amount of freezing behavior (a typical fear response in rodents), mice in which CREB was deleted in the hippocampus did not show altered contextual memory in this paradigm (Main effect of time period, $F_{(2,38)}=52.72$, $p<0.0001$; No main effect of viral injection or significant virus x time interaction) (Figure 3). Importantly, they also responded similarly to control mice in their initial response to the context (pre-shock period), as well as their response to the aversive stimulus (post-shock period).

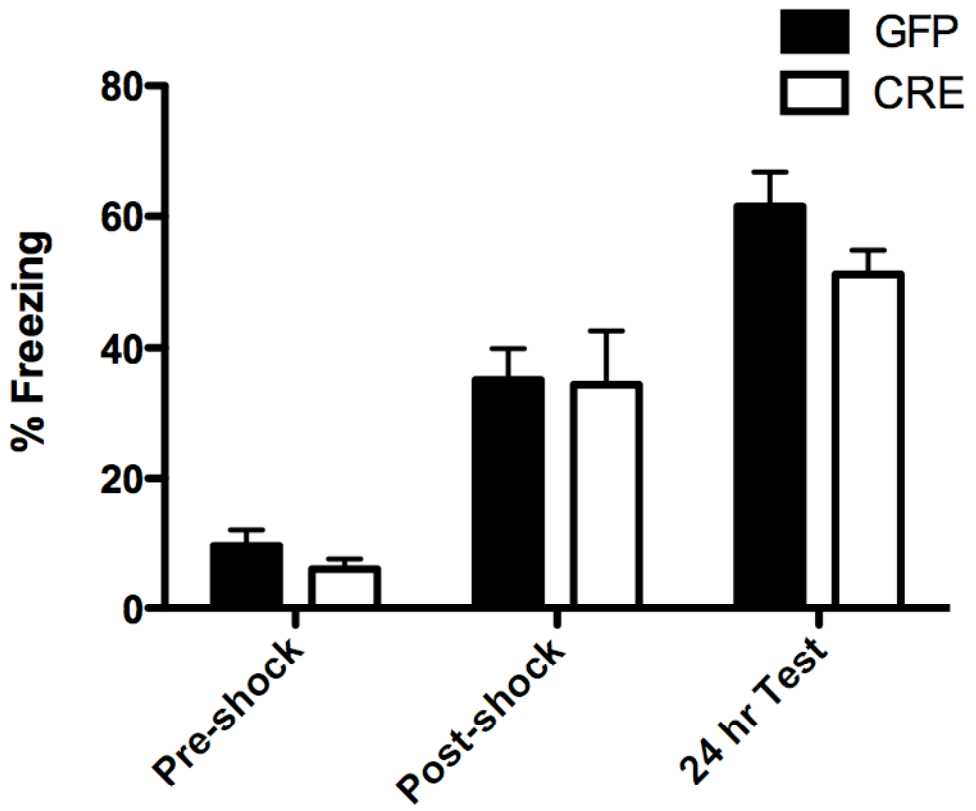

Figure 3. Hippocampal CREB deletion does not lead to long-term memory deficit in contextual fear conditioning. Mice were trained in a one-shock contextual fear conditioning paradigm and tested 24 hours later. Percent time spent freezing is shown. Mice injected with AAV-Cre did not show a significant deficit in recall 24 hours after training (n=10-11). Error bars indicate SEM.

Hippocampal deletion of CREB does not affect behavior in two tests of anxiety

CREB has been implicated in anxiety behavior (Barrot et al., 2002, Graves et al., 2002, Wallace et al., 2004). To assess the role of CREB specifically in the hippocampus, we subjected *Creb*$^{loxP/loxP}$ mice to two tests of anxiety, eight weeks after injection with AAV-GFP or AAV-Cre in the hippocampus. In the elevated-zero maze (EZM), AAV-Cre injected mice did not show differences in level of anxiety, as indicated by a similar number of entries into open quadrants (Figure 4A) as well as a similar amount of time spent in open quadrants (Entries: $t_{(16)}$=0.2414, p=0.81; Time: $t_{(18)}$=0.0515, p=0.95) (Figure 4B). In a marble-burying paradigm, another measure of anxiety behavior, AAV-GFP and AAV-Cre mice again exhibited similar levels of anxiety, with no significant difference between the groups in the number of marbles buried ($t_{(18)}$=0.7732, p=0.44) (Figure 4C).

Sucrose preference in a two-bottle choice paradigm is not affected by hippocampal CREB deletion

CREB in the mesolimbic reward pathway appears to play a role in hedonic behavior, such as preference for high-sugar foods (Barrot et al., 2002). To determine if hippocampal CREB is involved in such behavior, we measured preference for a sucrose solution using a two-bottle choice paradigm. *Creb*$^{loxP/loxP}$ mice did not show differences in preference for either of two concentrations of sucrose eight weeks after hippocampal injection with AAV-Cre, compared to corresponding mice injected with AAV-GFP

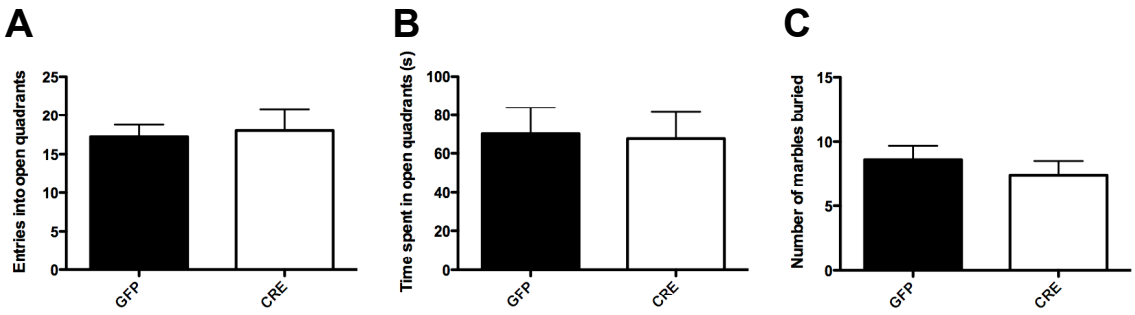

Figure 4. Hippocampal CREB deletion does not alter anxiety behavior in the elevated zero maze (EZM) or marble-burying. **A** and **B**, Mice were placed in the EZM for 5 min. Mice injected with the Cre-expressing virus did not differ from those injected with GFP-injected virus in number of entries into open quadrants (**A**) or time spent in open quadrants (**B**) (n=9). **C**, Anxiety behavior was also assessed in a marble-burying paradigm. There was no significant difference in number of marbles buried between AAV-GFP-injected and AAV-Cre-injected mice (n=10). Error bars indicate SEM.

(Main effect of sucrose concentration, $F_{(1,14)}$=10.47, p=0.006; no significant effect of viral injection or virus x concentration interaction) (Figure 5).

Response to acute antidepressant treatment in the forced-swim test is not altered by deletion of CREB in the hippocampus

Antidepressant drugs cause decreases in immobility in the forced-swim test after acute administration (Lucki, 2001, Porsolt et al., 2001, Cryan et al., 2002). To determine if hippocampal CREB is necessary for this response to antidepressants, we tested *Creb*$^{loxP/loxP}$ mice injected with AAV-GFP and AAV-Cre in the hippocampus in this paradigm. Mice injected with AAV-Cre did not show any difference in baseline levels of immobility in this paradigm, and both groups of mice spent less time immobile if they had been acutely treated with the antidepressant desipramine (DMI), regardless of viral injection (Main effect of drug treatment, $F_{(1,26)}$=53.2, p<0.0001; no significant effect of viral injection or virus x drug interaction) (Figure 6A). Changes in levels of general locomotor activity can confound interpretation of results in the FST. There was no difference between animals injected with AAV-GFP and AAV-Cre in their levels of activity in a home-cage environment over a one-hour period (Main effect of time, $F_{(11,242)}$=11.50, p<0.0001; no significant effect of viral injection or virus x time interaction) (Figure 6B).

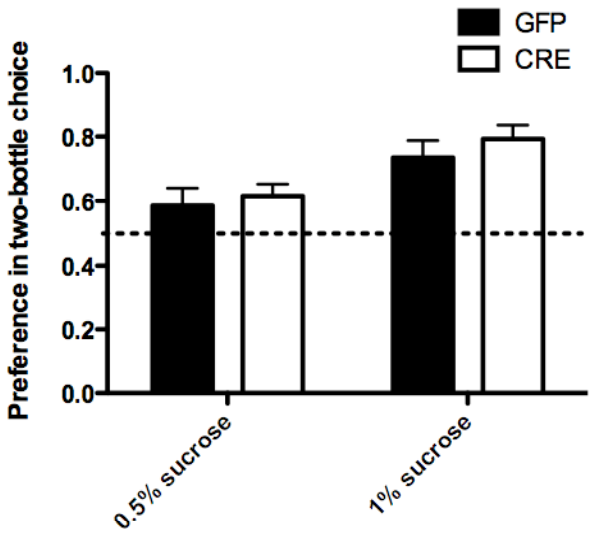

Figure 5. Hippocampal-specific CREB deletion does not affect sucrose preference. Mice were exposed to two concentrations of sucrose (0.5 and 1%) in a two-bottle-choice paradigm. Preference for sucrose (over water) based on average consumption over four days is shown. There was no significant difference between AAV-GFP- and AAV-Cre-injected mice at either concentration (n=7-9). Dotted line indicates 0.5; values greater than 0.5 indicate preference. Error bars indicate SEM.

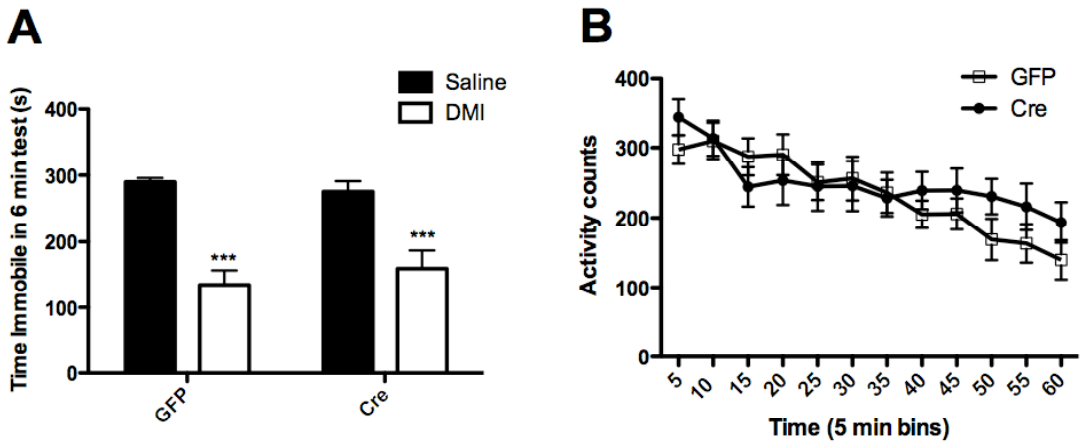

Figure 6. Forced swim test (FST) behavior and locomotor activity are not altered after hippocampal CREB deletion. **A**, Immobility during a 6-minute forced swim test is shown. Mice received a series of three injections of either saline or DMI over the 24 hours prior to testing. DMI-treated groups of both AAV-GFP- and AAV-Cre-injected mice showed a significant reduction in immobility (n=7-8). There was no significant difference in immobility between AAV-GFP- and AAV-Cre-injected animals. *$p<0.05$ vs. saline-treated animals. **B**, Activity counts in a home-cage environment are shown. Activity counts were measured in 5-minute bins over 60 min. Although activity decreased significantly in both groups over time, there were no significant differences between AAV-GFP- and AAV-Cre- injected animals (n=12). Error bars indicate SEM.

Response to chronic antidepressant treatment in the NIH paradigm is unaffected by loss of CREB in the hippocampus

Because CREB is thought to play a role in long-term changes in gene expression and plasticity that might result from long-term antidepressant treatment, we assessed the role of hippocampal CREB in a paradigm sensitive to treatment with chronic, but not acute, antidepressants, the NIH paradigm (Merali et al., 2003, Dulawa and Hen, 2005). In wild-type animals, latency to consume a highly palatable food is increased in a novel environment. Chronic, but not acute, antidepressants reduce latency to consume in the novel environment. In this study, $Creb^{loxP/loxP}$ mice behaved similarly, regardless of the viral injection they received. Viral injection did not affect training behavior (Main effect of day, $F_{(4,36)}=3.341$, $p=0.011$; No significant effect of viral injection or virus x day interaction) (Figure 7A). In the testing phase, all groups showed increased latency to consume in the novel environment, as compared to the home cage, with the AAV-Cre-injected mice showing a non-significant trend towards higher latencies after only saline treatment, as compared to their saline-treated AAV-GFP counterparts (Main effect of day, $F_{(2,58)}=35.44$, $p<0.0001$; trend toward main effect of viral injection, $F_{(1,58)}=2.76$, $p=0.10$; non-significant virus x day interaction) (Figure 7B). Both AAV-GFP- and AAV-Cre-injected mice exhibited decreased latencies in the novel environment after 3 weeks' treatment with DMI (Main effect of drug, $F_{(1,58)}=27.18$, $p<0.0001$; significant drug x day interaction, $F_{(2,58)}=11.37$, $p<0.0001$, no significant virus x drug x day interaction) (Figure 7B). Thus, even after deletion of CREB in the hippocampus, response to chronic treatment with DMI in this paradigm remains intact.

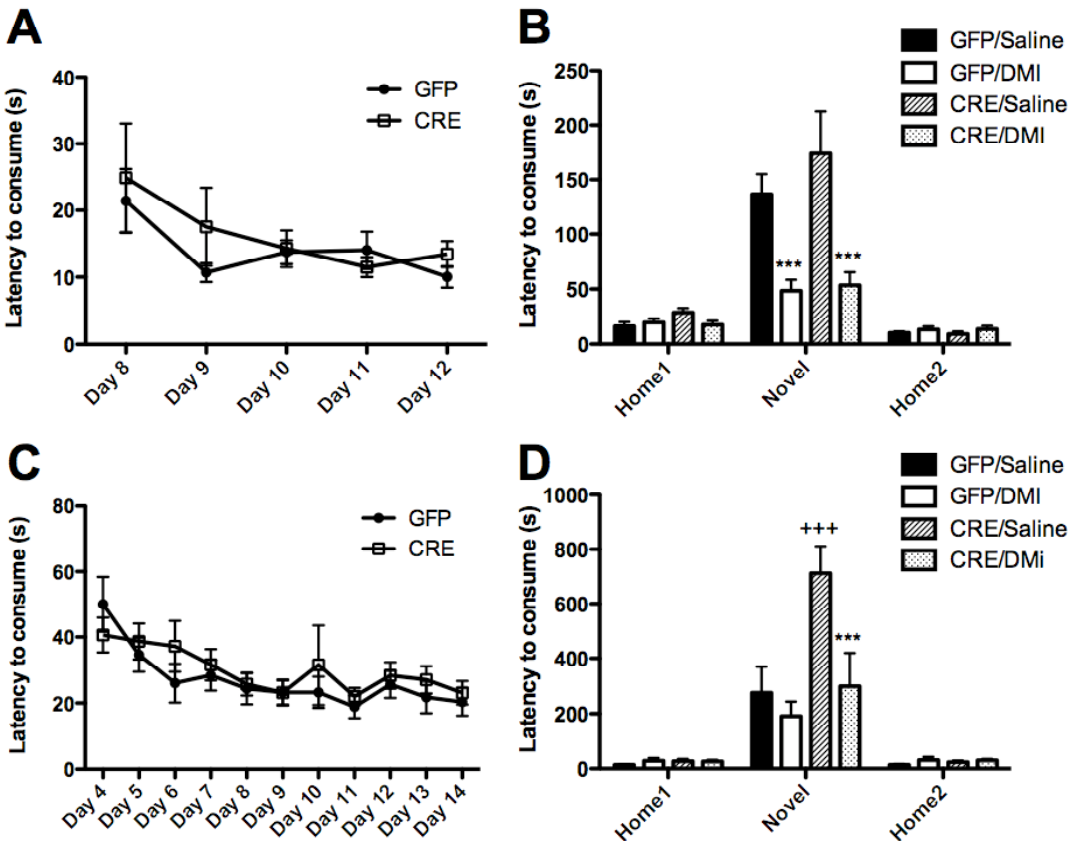

Figure 7. Hippocampal CREB deletion does not affect response to chronic antidepressant treatment in the novelty-induced hypophagia (NIH) paradigm, but allows for response to acute antidepressant treatment in this paradigm. Mice were trained and tested in the NIH paradigm, with one cohort receiving 3 weeks of treatment with DMI (chronic, **A** and **B**), and one cohort receiving only 3 administrations before testing (acute, **C** and **D**). Latency to consume is shown. AAV-GFP- and AAV-Cre-injected mice did not show significant differences in training in either experiment (**A** and **C**, n=17-21). After chronic treatment, AAV-Cre-injected mice treated with saline showed a non-significant increase in latency to consume in the novel environment, but both AAV-GFP- and AAV-Cre-injected mice showed a significant reduction in latency to consume in the novel environment after 3 weeks of treatment with DMI (**B**). There were no significant differences amongst the groups in home cage behavior (n=6-10). After acute treatment, AAV-Cre-injected mice treated with saline showed a significant increase in latency to consume in the novel environment as compared to their AAV-GFP-injected counterparts (**D**). Additionally, AAV-Cre-injected mice showed a significant reduction in latency after 3 administrations of DMI, as compared to their saline-treated counterparts, whereas AAV-GFP-injected animals did not show any change in response to DMI treatment. There were no

significant differences amongst the groups in home cage behavior (n=8-10). ***$p<0.001$ vs. analogous saline-treated group; +++$p<0.001$ vs. AAV-GFP-injected group. Error bars indicate SEM.

Hippocampal CREB deletion allows for response to acute antidepressant treatment in the NIH paradigm

CREB-deficient $CREB^{\alpha\Delta}$ mice, unlike wild-type mice, show a reduction in latency to consume in the novel environment of the NIH paradigm after only three antidepressant administrations (Gur et al., 2007). Therefore, we examined whether the more specific loss of CREB in our model would recapitulate this phenotype. $Creb^{loxP/loxP}$ mice were tested in the NIH paradigm, eight weeks after intrahippocampal injection with AAV-GFP or AAV-Cre. As with the chronic experiment, there were no differences in training between mice injected with either virus (Main effect of day, $F_{(10,340)}=5.268$, $p<0.0001$; no significant effect of viral injection or virus x day interaction) (Figure 7C). All groups exhibited increased latency to consume in the novel environment, when compared to the home cage, with saline-treated AAV-Cre-injected animals having significantly higher latencies in the novel environment compared to their saline-treated AAV-GFP counterparts (Main effect of day, $F(2,64)=56.6$, $p<0.0001$; main effect of viral injection, $F(1,64)=8.3$, $p=0.006$; main effect of drug, $F(1,64)=5.4$, $p=0.02$; significant day x virus x drug interaction, $F(2,64)=2.968$, $p=0.05$; AAV-GFP-sal vs. AAV-Cre-sal, $p=0.0061$, Bonferroni *post hoc* test) (Figure 7D). Just three doses of DMI significantly reduced latencies in the novel environment of AAV-Cre-injected mice, compared to their saline-treated counterparts (AAV-Cre-sal vs. AAV-Cre-DMI, $p=0.02$, Bonferroni *post hoc* test). AAV-GFP-injected animals had similar latencies regardless of drug treatment. Thus, loss of CREB in the hippocampus allows for a response to short-term antidepressant treatment in the NIH.

Hippocampal neurogenesis is increased after deletion of CREB

In addition to a response to acute treatment with antidepressants in the NIH, CREB-deficient $CREB^{\alpha\Delta}$ mice show increased rates of hippocampal neurogenesis (Gur et al., 2007). Indeed, the antidepressant-induced reduction in latency to consume in the novel environment of the NIH may require concomitant increases in hippocampal neurogenesis (Santarelli et al., 2003). To assess levels of hippocampal neurogenesis in mice after hippocampal CREB deletion, we administered bromo-deoxyuridine (BrdU), a marker of cell division, to $Creb^{loxP/loxP}$ mice eight weeks after they were given hippocampal injections of either AAV-GFP or AAV-Cre. The number of BrdU-labeled cells in the dentate gyrus (DG) was determined in mice killed either 24 hours or 4 weeks after BrdU administration. As shown in Figure 8A, the DG of AAV-Cre-injected mice contained greater numbers of BrdU-labeled cells than those from mice injected with AAV-GFP in mice killed 24 hours after treatment with BrdU, indicating higher levels of cell proliferation in this group (Main effect of viral injection, $F_{(1,54)}=52.86$, $p<0.0001$; main effect of area, $F_{(2,54)}=221.2$, $p<0.0001$; significant virus x area interaction, $F_{(2,54)}=8.614$, $p=0.0006$). Four weeks after BrdU administration, AAV-Cre-injected mice retained a significant increase in BrdU-labeled cells compared to corresponding AAV-GFP-injected mice, suggesting that larger numbers of newly-produced cells had survived in this group (Main effect of viral injection, $F_{(1,30)}=11.29$, $p=0.0043$; main effect of area, $F_{(2,30)}=171.1$, $p<0.0001$, significant virus x area interaction, $F_{(2,30)}=15.54$, $p<0.0001$) (Figure 8B). The significant increase in the total number of BrdU-labeled cells in AAV-Cre-injected mice was driven by a significant increase in labeled cells in the granule cell

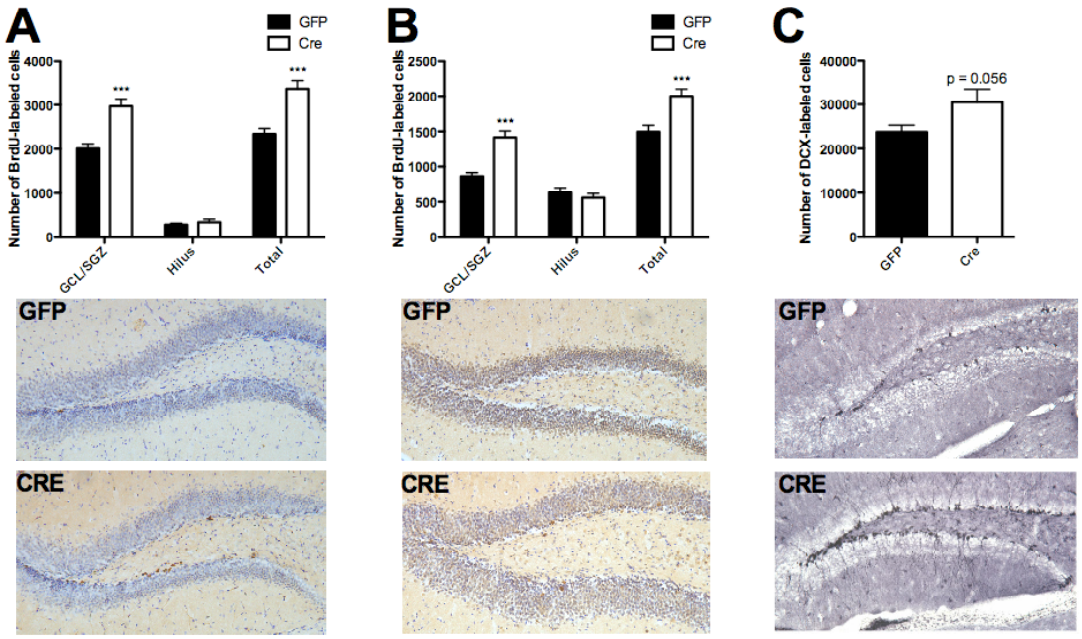

Figure 8. Deletion of CREB in the hippocampus causes significant increase in hippocampal cell neurogenesis. **A** and **B** Quantification of the number of bromodeoxy-uridine (BrdU)-labeled cells in the dentate gyrus granule cell layer/subgranular zone (GCL/SGZ) and hilus are shown. Example images of the DG from AAV-GFP-injected (middle) and AAV-Cre-injected animals (bottom) after staining with anti-BrdU antibody (brown) and counterstaining with cresyl violet (purple) are shown. **A** Mice were killed 24hr following a single injection of bromodeoxyuridine (BrdU). There was a significant increase in BrdU-labeled cells in the GCL/SGZ, but not the hilus, of AAV-Cre-injected mice, as compared to AAV-GFP-injected mice, indicating an increase in cell proliferation (n=14-15). **B** Mice were killed four weeks following a series of four BrdU injections. AAV-Cre-injected mice showed significantly higher levels of BrdU-injected cells in the GCL/SGZ, but not the hilus, as compared with AAV-GFP-injected controls, suggesting a sustained increase in the number of newly-generated (surviving) cells (n=7-10). ***$p<0.001$ vs. AAV-GFP-injected controls. **C** The number of doublecortin (DCX)-labeled cells in the dentate gyrus granule cell layer of mice killed 12 weeks after hippocampal microinjection is shown (top). Deletion of CREB in the hippocampus caused a sizable and nearly-significant increase in the number of DCX-labeled cells, as compared to AAV-GFP-injected controls (n=8-9). Example images of the DG from AAV-GFP-injected (middle) and AAV-Cre-injected (bottom) animals stained with antibody against doublecortin are shown. Error bars indicate SEM.

layer/subgranular zone (GCL/SGZ) of the dentate gyrus, whereas numbers of labeled cells in the hilus were similar between the groups (24hr: AAV-GFP vs. AAV-Cre, $p<0.001$ for GCL/SGZ, $p>0.05$ for hilus, $p<0.001$ for total, Bonferroni *post hoc* test; 4wk: AAV-GFP vs. AAV-Cre, $p<0.001$ for GCL/SGZ, $p>0.05$ for hilus, $p<0.001$ for total, Bonferroni *post hoc* test).

In wild-type animals, the vast majority of newly-generated cells in the DG differentiate into neurons. To determine if this was true of the increased number of newly-generated cells in mice with CREB deletion in the hippocampus, we conducted immunohistochemistry with an antibody recognizing doublecortin (DCX), a marker of immature neurons. 12 weeks post-surgery, AAV-Cre-injected mice showed an increase in DCX-positive cells similar in magnitude to the increase in BrdU-positive cells, though the difference between AAV-GFP- and AAV-Cre-injected groups did not quite reach statistical significance ($t_{(15)}=2.067$, $p=0.0565$) (Figure 8C).

Because AAV2/9 is known to infect neurons more efficiently than glial cells (Cearley and Wolfe, 2006, Klein et al., 2008), and because neural progenitor cells (NPCs) are glia-like (Seri et al., 2001), we investigated whether CREB expression remained in newly-created neurons. To examine whether immature neurons also expressed CREB, we labeled hippocampal sections taken from mice killed 12 weeks after microinjection with AAV-Cre in the hippocampus with antibodies against both DCX and CREB (Figure 9). Sections from AAV-GFP-injected animals, in which CREB is not deleted, show CREB staining in the dentate gyrus too dense to resolve individual cells. In mice injected with AAV-Cre, we found large numbers of cells labeled for DCX (mean: 914, standard

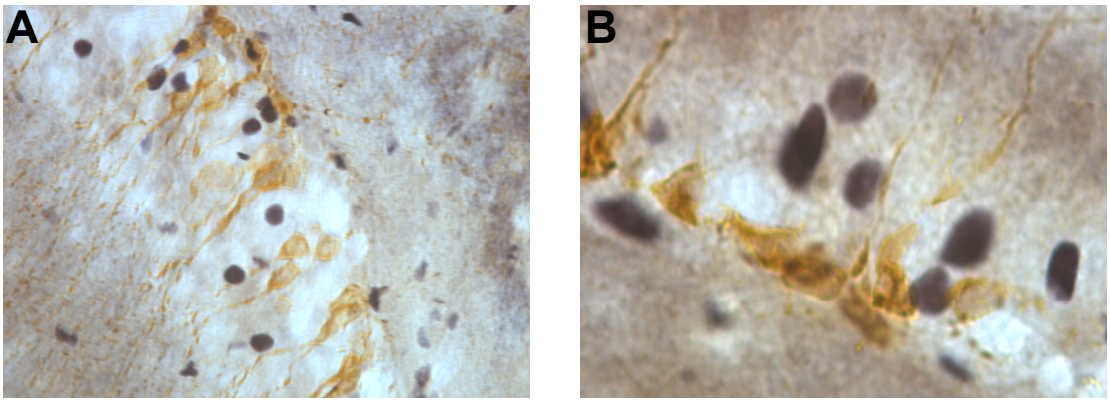

Figure 9. Most immature neurons in AAV-Cre-injected dentate gyrus do not express CREB. Example picture of the dentate gyrus from an AAV-Cre-injected animal after staining with antibodies against doublecortin (brown) and CREB (black) at 40x (A) and 100x (B) magnification.

deviation: 346) and CREB (mean:1592, standard deviation: 841), but few cells labeled with both antibodies (mean: 28, standard deviation: 16). Thus, a very small percentage of DCX-expressing cells also expressed CREB (2.9%), suggesting that NPCs are being infected efficiently.

Deletion of CREB in the hippocampus leads to selective upregulation of CREM, a related CRE-binding factor

In other models with reduced CREB expression, compensatory upregulation of the CREB-family protein cyclic-AMP-response-element modulator (CREM) has been observed, and is thought to affect the severity of phenotypes resulting from reduction of CREB (Hummler et al., 1994, Mantamadiotis et al., 2002). Using quantitative RT-PCR, we measured levels of CREM mRNA in the hippocampi of $Creb^{loxP/loxP}$ mice eight weeks after hippocampal injection with either AAV-GFP or AAV-Cre. As in other models of CREB reduction, we saw a robust and significant increase in CREM mRNA in the hippocampi of AAV-Cre-injected mice, compared to AAV-GFP-injected controls ($t_{(13)}$=3.858, p=0.002) (Figure 10A). There are many isoforms of CREM, including those acting as transcriptional activators and repressors. The above experiment used primers designed to amplify most isoforms, including the main activator form, CREMτ, which is normally expressed only at low levels in brain (Foulkes et al., 1992). One isoform *not* amplified by these primers is the main repressor form of CREM, inducible cyclic-AMP early repressor (ICER). Using primers specifically designed against ICER, we saw no

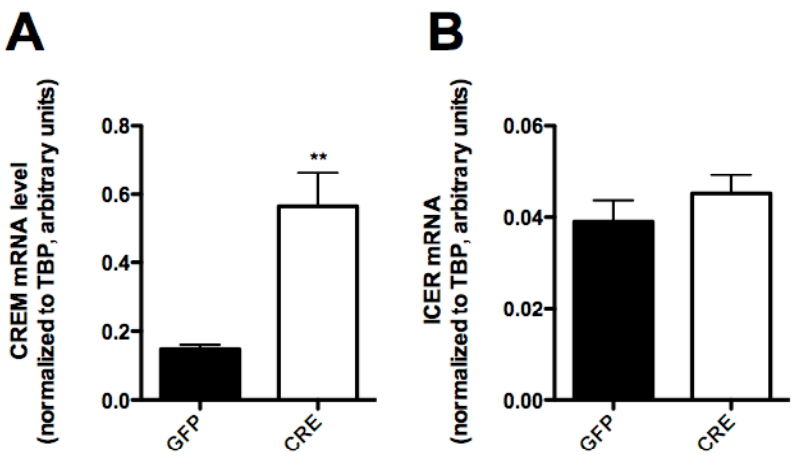

Figure 10. Deletion of CREB in the hippocampus leads to a robust increase in expression of cyclic-AMP-response-element modulator (CREM) (**A**), but not of inducible cyclic-AMP early repressor (ICER) (**B**) in the hippocampus (n=7-8). Levels of CREM and ICER mRNA in the hippocampus, as determined by RT-PCR, were normalized to the housekeeping gene TBP. Error bars indicate SEM.

change in mRNA levels in *Creb*$^{loxP/loxP}$ mice, regardless of the viral injection they received ($t_{(13)}$=0.9947, p=0.33) (Figure 10B).

CRE-mediated gene transcription is intact or increased after loss of hippocampal CREB

Because increased levels of CREM activators like CREMτ might be compensating for the lack of CREB at the level of transcriptional activation, we examined the expression of several genes known be targets of CREB regulation after deletion of CREB in the hippocampus. The immediate early gene *cfos* (FBJ murine osteosarcoma viral oncogene homolog) is a marker of neuronal activity, increasing in expression after a variety of stimuli, including stress (Greenberg et al., 1986, Morgan et al., 1987, Cole et al., 1989, Senba et al., 1993). CREB is known to be important for the upregulation of *cfos* (Ginty et al., 1994, Ahn et al., 1998, McClung and Nestler, 2003, Lemberger et al., 2008). 10 min following a forced-swim stress, there was an increase in *cfos* expression in the hippocampus compared with non-stressed controls (Main effect of stress, $F_{(1,28)}$=81.45, p<0.0001) (Figure 11A). This stress-induced increase did not depend on the presence of CREB; indeed, AAV-Cre-injected mice showed overall higher levels of *cfos* expression than AAV-GFP-injected controls (Main effect of viral injection, F(1,28)=4.345, p=0.046; no significant interaction).

CREB regulates many genes necessary for cell proliferation, survival, and differentiation, such as the cell-survival gene B-cell lymphoma 2 (Bcl-2) (Riccio et al., 1999). Surprisingly, after hippocampal CREB deletion, levels of Bcl-2 mRNA were

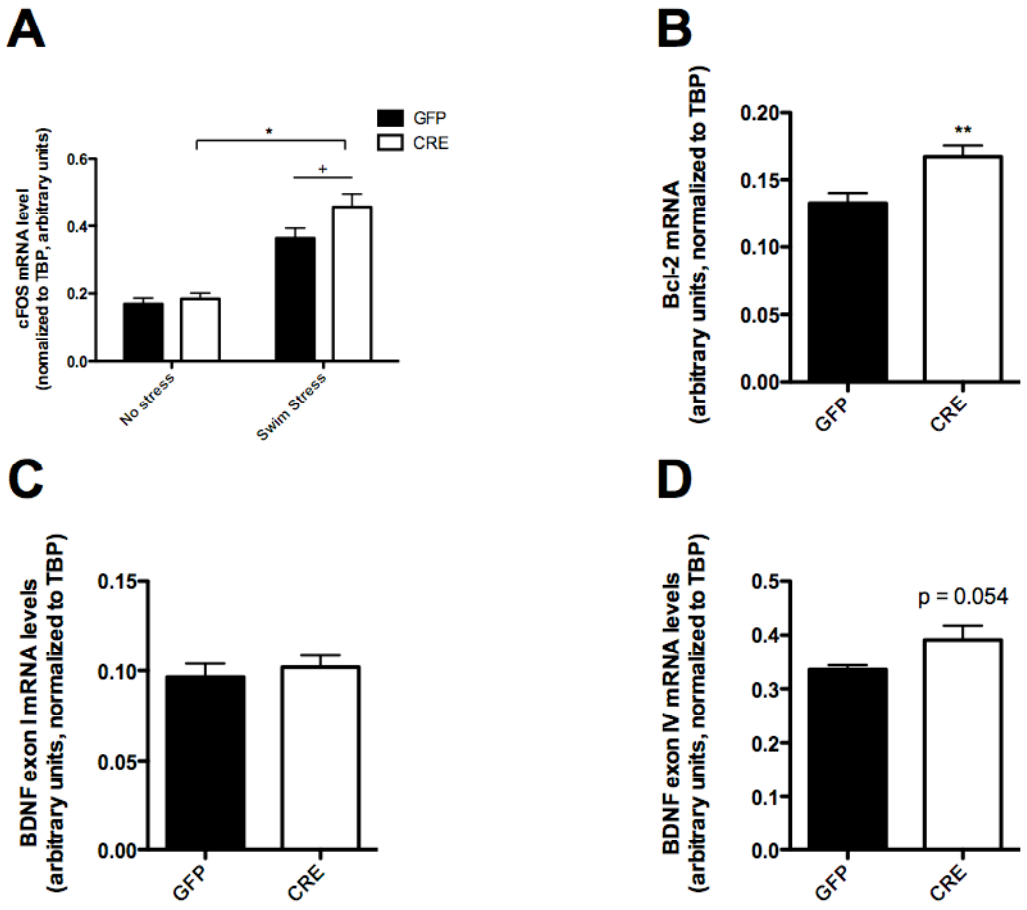

Figure 11. CRE-mediated gene transcription in the hippocampus is not decreased after deletion of CREB. mRNA levels of several genes in the hippocampus were measured by RT-PCR and normalized to the housekeeping gene TBP. **A** Levels of *cfos* mRNA in the hippocampus were measured 10 min following a forced-swim stress. Stress caused a significant increase in *cfos* expression. Mice injected with AAV-Cre also showed overall higher levels of *cfos* mRNA, though there was no stress x virus interaction (n=6-9). +$p<0.05$ main effect of stress; *$p<0.05$ main effect of viral injection. Error bars indicate SEM. **B** Bcl-2 mRNA was present at significantly higher levels in AAV-Cre-injected hippocampi than their AAV-GFP-injected counterparts (n=8-9). **C** Levels of transcripts for brain-derived neurotrophic factor (*Bdnf*) containing exon I were not significantly different in the hippocampi of AAV-GFP- and AAV-Cre-injected mice (n=8-9). **D** Levels of *Bdnf* transcripts containing exon IV were nearly significantly increased in the hippocampi of mice injected with AAV-Cre as compared to those injected with AAV-GFP (n=8-9). Error bars indicate SEM.

significantly increased in the hippocampus ($t_{(15)}$=2.993, p=0.0091) (Figure 11B). We also assessed expression of another CREB-target known to be involved in cell survival and differentiation, and implicated in the response to antidepressants, brain-derived neurotrophic factor (BDNF). The *Bdnf* gene is a complex locus, with eight upstream exons (I-VIII), each with their own promoter, which can be alternately spliced to the single downstream exon (IX) containing the coding region (Aid et al., 2007). Two *Bdnf* exons, I and IV, have identified CREs in their promoters (Tao et al., 1998, Shieh and Ghosh, 1999, Tabuchi et al., 2002), and thus we chose these two to examine. Levels of *Bdnf* exon I-containing mRNA were unchanged in the hippocampi of $Creb^{loxP/loxP}$ mice after injection with AAV-Cre ($t_{(15)}$=0.5433, p=0.59), whereas transcripts containing *Bdnf* exon IV were nearly significantly increased compared to AAV-GFP-injected animals (t(15)=2.084, p=0.0547) (Figure 11C and D). The expression of CREB target genes was either unaffected or increased after loss of hippocampal CREB.

Discussion

To determine the role of hippocampal CREB in behaviors associated with depression, anxiety, and antidepressant response, we generated a mouse in which CREB was deleted specifically in this region, beginning in adulthood. Surprisingly, despite demonstrating robust and significant loss of CREB in the hippocampus, we found no effect of this restricted loss of CREB in a number of behavioral paradigms, including contextual fear conditioning, tests of anxiety-like behavior, a sucrose preference test,

general locomotor activity, and response to acute antidepressant administration in the forced-swim test (FST). Additionally, deletion of CREB in the hippocampus did not affect response to chronic antidepressant treatment in the novelty-induced hypophagia (NIH) paradigm. However, loss of hippocampal CREB allowed for a response to short-term antidepressant treatment in this test, which is not observed in wild-type animals. This accelerated response to antidepressant treatment was associated with increased levels of hippocampal neurogenesis. The deletion of CREB in the hippocampus also led to upregulation of a CREB-family protein, CREM, though the CREM repressor isoform ICER was unchanged. In line with potential compensation by CREM, we observed no decreases in the expression of genes known to be regulated by CREB, showing instead that expression of some of these genes was increased after loss of CREB.

CREB has been implicated in anxiety- and depression-related behavior, with the strongest evidence coming from studies of the CREB-deficient $CREB^{\alpha\Delta}$ mice. These mice, which have a constitutive deletion of the alpha and delta forms of CREB, show increased anxiety, as well as an "antidepressant" phenotype in models of acute antidepressant response, such as the FST and tail-suspension test (TST) (Conti et al., 2002, Graves et al., 2002). Despite lower baseline levels of immobility in $CREB^{\alpha\Delta}$ mice in the FST and TST, these mice respond to acute antidepressant treatment with a further reduction in immobility in these paradigms. In our model of hippocampal-specific CREB deletion, we did not observe changes in anxiety paradigms or in baseline behavior in the FST. These differences in phenotype between our model and the $CREB^{\alpha\Delta}$ mice could be due to several factors. In our model, the onset of CREB deletion is in adulthood, as

compared to embryonic in the constitutive deletion in CREB$^{\alpha\Delta}$ mice. Additionally, in our model we do not see a complete lack of CREB expression, and although this is also the case in the CREB$^{\alpha\Delta}$ mice, there may be slightly more CREB expression in the *Creb*$^{loxP/loxP}$ model, which could be sufficient to mediate wild-type behaviors in these paradigms. Finally, CREB in the hippocampus may not be involved in mediating these behaviors, and other brain regions, such as the nucleus accumbens, may be responsible for the phenotype seen in the CREB$^{\alpha\Delta}$ mice. The ability of both CREB$^{\alpha\Delta}$ mice and our mice with CREB deleted in the hippocampus to respond to acute treatment with antidepressants in the FST might suggest that CREB does not play a role in antidepressant response. However, it is important to keep in mind that CREB's role as a transcription factor would likely be to bring about long-term changes in gene expression and synaptic plasticity involved in the response to *chronic* antidepressant treatment, and therefore may not be reflected in this test of *acute* antidepressant response.

In a test of chronic antidepressant efficacy, the NIH paradigm, CREB$^{\alpha\Delta}$ mice show a similar reduction in latency to consume in the novel environment after chronic treatment with desipramine (DMI), a tricyclic antidepressant (Gur et al., 2007). In addition, these mice respond to short-term antidepressant administration in the NIH paradigm, unlike wild-type animals. This response to a shorter course of antidepressants was attributed to increased levels of hippocampal neurogenesis in CREB$^{\alpha\Delta}$ mice. In our model, mice with a deletion of CREB restricted to the hippocampus almost exactly phenocopied the CREB$^{\alpha\Delta}$ mice. We observed a response to both chronic and acute

antidepressant treatment in the NIH in our mice, accompanied by an increase in hippocampal neurogenesis of equivalent magnitude to that seen in the CREB$^{\alpha\Delta}$ mice. These results suggest that the hippocampus is an important locus for determining behavior in the NIH, particularly CREB's influence on this behavior. Additionally, our results provide further evidence for a connection between the level of hippocampal neurogenesis and behavior in the NIH. Previously, the antidepressant-induced increase in hippocampal neurogenesis was seen to be necessary for the behavioral response to chronic antidepressant treatment in the novelty-suppressed feeding paradigm, which is similar to the NIH (Santarelli et al., 2003). If hippocampal neurogenesis is necessary for the behavioral response to antidepressants, it may be one cause for the lengthy time course of response, as only chronic, not acute, treatment with antidepressants causes increased hippocampal neurogenesis (Malberg et al., 2000). In this case, as with the CREB$^{\alpha\Delta}$ mice, the increase in baseline levels of hippocampal neurogenesis may be "priming" the brain to respond to antidepressant treatment, allowing for an accelerated behavioral response.

Importantly, both CREB$^{\alpha\Delta}$ mice and mice with CREB deleted only in the hippocampus show increased latency to consume in the NIH after saline treatment as compared to saline-treated controls. Therefore, the reduction in latency seen after short-term treatment with antidepressants could be interpreted as bringing latencies back down to the level of saline-treated control mice. The increased baseline latency in the novel environment may be seen as a reflection of increased anxiety-like behavior, and as acute treatment with anxiolytic compounds is effective to reduce novel environment latency in

this paradigm (Merali et al., 2003, Dulawa and Hen, 2005), the effect of acute DMI in this case could be construed as an anxiolytic effect. However, acute treatment with DMI (of equivalent dose and time course) failed to produce an anxiolytic effect in the elevated zero maze (EZM), another test of anxiety-like behavior, even in the $CREB^{\alpha\Delta}$ mice (Gur et al., 2007), suggesting that the effect of acute DMI in the NIH is more related to its antidepressant properties. In addition, hippocampal CREB deletion did not lead to increased anxiety in other paradigms, suggesting that the phenotype in the NIH may be specific, and influenced by other aspects of the paradigm, such as motivation to consume a highly-palatable food. In future studies, it would be interesting to determine if acute DMI is efficacious to decrease latencies in the novel environment of the NIH when they are increased at baseline due to other manipulations (e.g. other mouse lines with increased levels of anxiety).

Although this report is the first to use a virally-expressed Cre recombinase to cause deletion of CREB in a specific brain region, other studies have suggested that CREB plays a different role in depression- and anxiety-related behaviors depending on where it is expressed (see (Carlezon et al., 2005) for a review). In particular, viral overexpression of CREB led to reduced anxiety and antidepressant-like behavior in the FST and learned helplessness, another model used to screen pharmacological compounds for antidepressant efficacy (Chen et al., 2001, Wallace et al., 2004). There are procedural differences between that study and ours, including the species used (rats were used for the other two studies), and time course of overexpression. Additionally, overexpressed CREB protein may act as a dominant negative by monopolizing CRE sites and excluding

other Cre-binding proteins, leading to effects on behavior not strictly due to the endogenous functions of CREB. Finally, aspects of our model may be preventing a phenotype in the FST from emerging, including the presence of some residual CREB protein, or the compensatory upregulation of CREM, something that was not examined in the CREB overexpression studies.

The role of CREB in regulating hippocampal neurogenesis has also been examined in models other than the $CREB^{\alpha\Delta}$ mouse. In one study, the dominant negative mCREB (which lacks the ability to be activated by phosphorylation) was expressed under control of a Ca^{2+}-calmodulin-dependent protein kinase II (CamKII) promoter (Nakagawa et al., 2002b). mCREB expression was observed throughout the forebrain, including hippocampus, and caused a modest reduction in the number of BrdU-labeled cells in the dentate gyrus (Nakagawa et al., 2002b). In a similar study, a different dominant negative, aCREB, which cannot bind DNA, caused a decrease in survival and differentiation of newly-generated cells in the dentate gyrus when virally-expressed in the hippocampus (Jagasia et al., 2009). These studies would suggest that CREB function is necessary to maintain normal levels of hippocampal neurogenesis. However, we found *increased* neurogenesis after deleting CREB in the hippocampus, as did previous studies in $CREB^{\alpha\Delta}$ mice. To explain this seeming discrepancy, it is important to note the differential effect of CREB deletion and a dominant negative with regard to other CRE-binding proteins. The dominant negative mCREB, by binding to CRE sites and remaining unactivated, would inhibit the activity of not only CREB, but any CRE-binding protein, including CREM, the ATF family of proteins, and others. aCREB would

still block the activity of CREM and ATF-1, with which it could heterodimerize, but does not bind DNA, thus leaving unoccupied CRE sites available for other CRE-binding proteins (e.g. ATF-2, -3 and -4). In contrast to the decreased CREB-family activity seen in dominant negative approaches, our model of CREB deletion actually leads to increased expression of at least one CREB-family protein, CREM. This increased CREM expression is also observed in the $CREB^{\alpha\Delta}$ mice (Hummler et al., 1994), but was not examined in studies using a dominant negative approach. Therefore, the differences in effect on hippocampal neurogenesis may be due to altered activity of CREB-family proteins, rather than actions of CREB itself.

Upregulation of CREM, which is believed to be a compensatory change, is observed in other models in which CREB expression is reduced (Hummler et al., 1994, Mantamadiotis et al., 2002). The fact that we observe increased expression of CREM only eight weeks after injection of a Cre-expressing virus in the hippocampi of $Creb^{loxP/loxP}$ mice suggests that the mechanisms responsible for such compensatory changes are much more dynamic than previously believed. The increased expression of CREM observed after deletion of CREB may be responsible for some of the phenotypes we observed, particularly the lack of a decrease in expression of a set of CREB target genes that we analyzed. While CREMτ, the main isoform of CREM with transcriptional activator activity, is not expressed at high levels in the brain under normal conditions (Foulkes et al., 1992, Mellstrom et al., 1993), it may be the main isoform driving the increase in CREM expression we observed, and may be performing transcriptional activation to compensate for the absence of CREB, as has been seen in a cancer cell line

that lacks CREB (Groussin et al., 2000). This hypothesis is supported by the fact that expression of key CREB target genes, including those involved in cell survival and differentiation, such as *Bcl-2* and *Bdnf*, is not decreased, and is in some cases increased after deletion of CREB. This increase in expression of survival genes may be responsible for the increase in hippocampal neurogenesis we observe, as well as any associated behavioral changes, e.g. in the NIH. Compensation by CREM may also be responsible for the lack of effect of hippocampal CREB deletion we observed in other behavioral paradigms that are unrelated to hippocampal neurogenesis. Additional experiments are necessary to determine if the observed upregulation in CREM is necessary and sufficient to produce the changes in hippocampal neurogenesis and behavior that we observed.

Other mechanisms could be responsible for the increase in hippocampal neurogenesis seen after CREB loss in the hippocampus. The loss of CREB in mature neurons of the dentate gyrus could lead to increased cell death in this population. Neural progenitor cells (NPCs) in the dentate gyrus might then be increasing their rate of proliferation to compensate for this increase in cell death. As the NPCs are glia-like (Seri et al., 2001), and AAV2/9 preferentially infects neurons (Cearley and Wolfe, 2006, Klein et al., 2008), they might be less likely to be infected by the injected AAV, and thus unsusceptible to the loss of CREB, allowing them to survive and proliferate. If this hypothesis is true, one would expect immature neurons to retain their ability to express CREB even after injection of AAV-Cre. We found the opposite, as DCX-labeled cells rarely co-expressed CREB. It therefore appears that the NPCs are infected by AAV-Cre,

leading to CREB deletion. This observation is consistent with another study from our laboratory in which NPCs were infected by an AAV (Onksen, 2010).

The present study defines a role for hippocampal CREB in mediating neurogenesis and accelerating response to antidepressants. Whether this occurs as a result of the upregulation of another CRE-binding protein, CREM, remains to be seen. As CREB itself is unlikely to provide a useful target for drug development, it is important to understand the downstream effects of CREB in producing antidepressant effects, such as changes in hippocampal neurogenesis and gene expression. Understanding of these later steps in the process may allow for the development of antidepressant drugs with a faster onset of efficacy.

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Chapter 3: Discussion

The mechanism by which the immediate increase in synaptic levels of monoamines produced by antidepressant drugs translates, weeks later, into therapeutic efficacy is not understood. Signaling cascades downstream of neurotransmitter receptor activation likely modulate transcription factors, whose activity leads to changes in gene expression necessary for improvement in symptoms. One such transcription factor is the cyclic-AMP response element binding protein (CREB), which is a point of convergence of multiple signaling cascades. CREB is modulated by many second messenger cascades. These include the cAMP/PKA pathway, activated by neurotransmitter receptors coupled to $G\alpha_s$, as well as kinase cascades activated by increases in intracellular calcium, caused by activation of $G\alpha_q$- or possibly $G\alpha_i$-coupled receptors, as well as ionotropic receptors that depolarize the membrane.

CREB expression, phosphorylation, and activity are increased by chronic treatment with antidepressants, suggesting that CREB may play a role in their efficacy. Studies manipulating the expression or activity of CREB have suggested that CREB does function in the antidepressant response, although its specific role is not completely understood (see Chapter 1). The work described in Chapter 2 demonstrates that CREB in the hippocampus plays a role in some, though not all, behaviors associated with anxiety, depression, and antidepressant response in mice. Using a system in which CREB is deleted in selectively in the hippocampi of adult mice, we showed no affect on long-term memory in a fear conditioning paradigm, anxiety-like behavior in the elevated zero maze

or marble burying, anhedonia in a sucrose drinking paradigm, and no change in baseline behavior or response to acute antidepressant administration in the forced swim test (FST). Loss of hippocampal CREB also did not interfere with response to chronic antidepressant administration in a paradigm developed to assess such treatment, the novelty-induced hypophagia (NIH) paradigm. However, a response to acute antidepressants in the NIH, absent in wildtype mice, emerged after CREB was deleted in the hippocampus. This accelerated response to antidepressants in the NIH was accompanied by an increase in hippocampal neurogenesis. In addition to the behavioral and cellular changes we observed, an increase in expression of the CREB-family protein, CREM, was evident in the hippocampus after CREB deletion. This compensatory upregulation of CREM may be responsible for the maintenance of expression levels of CREB target genes, including increased expression of the cell-survival gene B*cl-2* that we observed. Based on these results, we can conclude that CREB plays a role in response to antidepressant treatment, including regulation of hippocampal neurogenesis.

The regulation of CREB-family proteins by other members of the family, which includes CREM and ATF-1, has been observed previously (Hummler et al., 1994, Blendy et al., 1996, Mantamadiotis et al., 2002). Following CREB deletion, CREM is upregulated in multiple systems, including a constitutive knockout (Hummler et al., 1994) as well as in Cre/loxP systems in which Cre recombinase is expressed as a transgene early in development (Mantamadiotis et al., 2002). However, work described in this thesis is the first to demonstrate that such compensatory changes occur in such a dynamic manner; we observed increased CREM expression only eight weeks after CREB was deleted. Additionally, in our study the deletion of CREB did not begin until

adulthood, showing that such compensatory changes are not limited to the period of heightened plasticity during embryonic development. However, it does occur in a region of the brain that is known for its incredible plasticity, the hippocampus. It would be of interest to see if the same increase in CREM occurs when CREB is deleted in other areas of the brain that are not know to undergo neurogenesis, such as the cortex or nucleus accumbens, two other areas in which CREB function is known to be important. This observation reinforces the importance of considering other family members when modulating protein expression, even on a short time-scale. Additionally, it speaks to the evolutionary importance that CREB family proteins must play in order to warrant such tight regulation.

The ability of biological systems to adapt and compensate for perturbations over time is often cited as a reason why constitutive knockout mice are not the ideal strategy for investigating gene function. In the case of the CREB αΔ mice, many of their more surprising phenotypes, including their antidepressant-like behavior in the forced swim test at baseline as well as increased levels of hippocampal neurogenesis, could be ascribed to compensatory changes that occurred during development, rather than to the function of CREB in the adult brain. The results described here demonstrate that altered CREB function in the brain over the course of development is not responsible for all of the phenotypes of the CREB αΔ mice; our model deletes CREB only in adulthood, and phenocopies the CREB αΔ mice in their response to acute antidepressant treatment in the NIH and their increase in hippocampal neurogenesis at baseline. Additionally, as our model deletes CREB only in the hippocampus, our results suggest that the hippocampus,

and likely hippocampal neurogenesis, play an important role in the behavioral response to antidepressants in the NIH paradigm. A previous study suggested that the increases in hippocampal neurogenesis seen after chronic antidepressant administration are necessary for the behavioral response to antidepressants in the novelty-suppressed feeding paradigm, which is similar to the NIH (Santarelli et al., 2003). When hippocampal neurogenesis was ablated by irradiation, the behavioral response to antidepressants was blocked. However, a later study by the same group did not see any affect of irradiation on the behavioral response to chronic antidepressant treatment in the NIH in a different strain of mice (Balb/cJ) (Holick et al., 2008). Interestingly, Balb/cJ mice do not show increased neurogenesis after chronic treatment with antidepressants, suggesting that, at least in this strain, hippocampal neurogenesis is not necessary for the behavioral response to antidepressants; other changes in neuronal plasticity in the hippocampus may mediate antidepressant effects in this strain. Our work provides support for a correlation between hippocampal neurogenesis and behavior in the NIH, as increased levels of hippocampal neurogenesis were associated with a more rapid response to antidepressants, both of which were also observed in the CREB $\alpha\Delta$ mice.

Future Directions

One major similarity of all models of reduced CREB expression is the increase in CREM that is observed. Future studies will investigate the role that such compensatory changes play in producing the phenotypes observed in our model. At this point, evidence that the upregulation in CREM plays a role in producing the changes in neurogenesis,

and, by extension, behavior in the NIH, is based on measurement of CRE-mediated transcription, an indirect measure of CREB/CREM function. That CREB-target gene expression is not decreased after CREB is deleted is suggestive of a role for CREM in activating transcription of such targets, but further experiments are needed to test this theory. To test the hypothesis that the phenotypes associated with hippocampal CREB deletion occur as a result of CREM upregulation, the *Creb*$^{loxp/loxp}$ mice can be crossed with *Crem-/-* mice, to assess the effects of deleting CREB, specifically in the hippocampus, when CREM is deleted and thus cannot be upregulated in response. If the hypothesis is correct, we would expect to lose the phenotypes seen in the *Creb*$^{loxp/loxp}$ mice alone. If CREM is indeed functionally compensating for the lack of CREB, one might expect more severe phenotypes to emerge after the loss of both proteins, particularly in paradigms in which no phenotype was observed after the loss of CREB alone. Other studies have used this approach in models in which Cre recombinase is expressed transgenically, and have observed more severe phenotypes after both CREB and CREM are deleted, including neurodegeneration in some cases (Mantamadiotis et al., 2002, Bilbao et al., 2008, Diaz-Ruiz et al., 2008, Lemberger et al., 2008), but see also (Parlato et al., 2006). One caveat of these studies is that, in the C*rem-/-* mouse, expression of ICER, the major repressor isoform of CREM, would also be eliminated. In our studies, we have not seen alterations in ICER after deletion of CREB, thus any effects of its deletion might confound interpretation of results in this study.

A parallel and complementary method would test the sufficiency of CREM upregulation by virally overexpressing the main activator form of CREM, CREMτ,

which could be responsible for the functional compensation for CREB deletion that we observe in our mice. In this case, we would not alter expression of ICER, and thus would avoid any confounding effects it might produce. This method would allow us to assess whether increasing CREM without deleting CREB is sufficient to produce the phenotypes we observed. If so, it would suggest that the increased activation of genes regulated by CREB family proteins is sufficient to produce the behavioral and cellular effects we observed.

With a broader perspective, future studies should investigate the downstream mechanisms by which altering CREB function affects neurogenesis and behavior. One place to start is the overwhelming list of CREB target genes identified in a number of studies using chromatin immunoprecipitation and microarray technology, as well as newer direct-sequencing methods (Zhang et al., 2005, Tanis et al., 2008, Wilkinson et al., 2009). We have begun to look at targets of CREB, including *Bcl-2* and *Bdnf*, which are known to be important for cell survival and differentiation, but there are many more CREB targets to be investigated, as it is unlikely that one or two genes are responsible for changes in such complex systems. Additionally, the role that CREB and its targets play in other brain regions must be examined, as the hippocampus is not the only brain region to be implicated in stress response and affect, and divergent roles for CREB have been described in different areas of the brain, such as the nucleus accumbens or amygdala (Carlezon et al., 2005). Ultimately, any therapeutic intervention in human patients is likely to be systemic, therefore it is important to understand the function of a protein such as CREB and its targets throughout the brain, not only to begin to predict the efficacy of any new therapeutic intervention, but also any potential side effects.

Because CREB plays such a critical role in cell survival throughout the body, and has such diverse functions in different tissues, it is unlikely that CREB itself will be a target of future drug discovery and development. Therefore, research to pinpoint and characterize CREB genes that play key roles in depression and anxiety behavior is crucial to allow for identification of those that are more useful targets for therapeutics. Little is known about the intricacies of transcriptional activation by CREB and CREM, including how they respond to different stimuli by activating different subsets of targets. Whether CREB and CREM activate the same set of targets is also unknown; it is likely that interactions among CREB family proteins (and other transcriptional regulators) with slightly different subsets of targets may contribute to CREB's ability to cause such diverse patterns of gene expression under different circumstances.

Better understanding of the downstream effects of current antidepressant therapies is necessary to develop new, more efficacious and expeditious therapies. Many of the important changes that occur only after long-term antidepressant treatment, including the role CREB plays, but also other factors that affect gene expression over the long-term, such as chromatin modifications (see Appendix A), are only partially understood. Here, we have demonstrated that deleting CREB in the hippocampus allows for a faster response to antidepressant treatment in the NIH. Dissecting the mechanism through which these effects occur might allow us to directly target downstream effectors in order to speed-up the response to antidepressants, and possibly also reduce side effects of such treatments.

Conclusion

This work has confirmed a crucial role for CREB in the hippocampus in mediating antidepressant-like behaviors and antidepressant-induced cellular responses. Additionally, we have demonstrated the utility of directed knockout strategies in isolating the role of a protein in a specific time and place. In this case, our strategy does not eliminate compensatory changes in related proteins. The demonstration of such dynamic compensatory changes is, in this case, novel, and sheds light on the degree of regulation found within the CREB family. Additionally, this work identifies important downstream effects of altering CREB function, some of which may be important in antidepressant response. Better understanding of these mechanisms is necessary for the development of more effective and expeditious treatments for depression, which are sorely needed.

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Appendix A:

Effects of the histone deacetylase inhibitor sodium butyrate in models of depression and anxiety

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Abstract

Histone modification, which affects the rate of transcription without altering DNA sequence, occurs in response to various psychiatric drugs and in several models of psychiatric disease. As increases in histone acetylation have been seen after treatment with antidepressants, we investigated whether directly increasing histone acetylation using a histone deacetylase inhibitor would have antidepressant effects. We administered sodium butyrate (NaB, 100 mg/kg, i.p.) to mice acutely (3 injections over 24 hours) or chronically (twice daily for 21 days) and subjected them to a number of behavioral tests of antidepressant response. This dose of NaB had no effect on overall locomotor activity after either acute or chronic treatment. Acutely treated mice showed an increase in immobility in the forced-swim test (FST) and an increase in latency to consume in the novel environment of the novelty-induced hypophagia (NIH) paradigm, an anxiogenic effect. The effect of NaB on anxiety did not generalize to another test, the elevated zero maze, where it had no effect. Chronic treatment with NaB had no effect on latency to consume in the NIH or immobility in the FST. However, this dose did alter histone

acetylation in the hippocampus. While H4 acetylation increased in the hippocampus 30 min following acute NaB, chronic treatment caused a decrease in AcH4. There were no changes in AcH3 following either treatment. While changes in chromatin structure may be involved in the mechanism of action of antidepressant drugs, these data suggest that increasing histone acetylation pharmacologically is not sufficient to produce antidepressant effects.

1. Introduction

Despite its status as the most common psychiatric disorder, depression is poorly understood, both in terms of its pathophysiology and the mechanisms by which antidepressants ameliorate symptoms. The observation of decreased hippocampal volume in depressed humans (Sheline et al., 1999, Bremner et al., 2000), combined with studies showing molecular and cellular changes in the hippocampi of rodents after chronic stress, and their reversal by antidepressants, have focused depression research on this brain region. In particular, antidepressants have been shown to block stress-induced decreases in hippocampal plasticity (Popoli et al., 2002), including decreases in hippocampal neurogenesis (Dranovsky and Hen, 2006). Antidepressants have also been shown to upregulate neurotrophic factors such as BDNF, which may contribute to increased plasticity (Duman and Monteggia, 2006, Castren et al., 2007). The mechanism by which antidepressants cause these changes in gene expression and plasticity are still under investigation.

Changes in chromatin structure due to post-translational modifications of histones have been associated with a number of behavioral events, including drug addiction (Kumar et al., 2005, Levine et al., 2005, Renthal et al., 2007, Pandey et al., 2008, Schroeder et al., 2008), memory formation (Alarcon et al., 2004, Levenson et al., 2004, Lattal et al., 2007), seizures (Huang et al., 2002, Tsankova et al., 2004), and stress (Bilang-Bleuel et al., 2005, Tsankova et al., 2006, Chandramohan et al., 2007, Renthal et al., 2007). While a number of post-translational modifications to each of the four core histone proteins (H2A, H2B, H3 and H4) are possible, histone acetylation, which is associated with increased rates of transcription, has received the most attention. Because of the possible role of histone acetylation in a variety of behaviors and disease states, some studies of the effects of histone deacetylase (HDAC) inhibitors in animals have been reported. The HDAC inhibitors trichostatin-A (TSA) and sodium butyrate (NaB) have been shown to increase contextual fear conditioning and extinction (Levenson et al., 2004, Lattal et al., 2007), and both NaB and SAHA, another HDAC inhibitor, have been shown to reduce symptoms in a mouse model of Huntington's disease (Ferrante et al., 2003, Hockly et al., 2003). NaB was shown to augment the increase in histone acetylation caused by exposure to cocaine (Kumar et al., 2005), as well as to decrease the anxiety-like symptoms associated with alcohol withdrawal (Pandey et al., 2008).

Recently, chromatin modification has also been implicated as an important regulator of the expression of depression-related genes, including BDNF. Electroconvulsive shock, a model of electroconvulsive therapy, the most potent treatment for depression, was shown to increase histone H3 acetylation at the BDNF promoter,

which correlated with upregulation of BDNF mRNA (Tsankova et al., 2004). In another study, stress was shown to increase histone H3 di-methylation, a modification associated with repressed transcription, at BDNF promoters (Tsankova et al., 2006). In this same study, antidepressants opposed the effects of stress by increasing H3 acetylation at BDNF promoters, and this effect was associated with a decrease in expression of histone deacetylase 5 (HDAC5), an enzyme that acts to remove acetyl groups from histones. This downregulation in HDAC5 was shown to be necessary for the behavioral effects of chronic antidepressant treatment, but it remains to be shown whether these changes in chromatin are sufficient to produce antidepressant behavioral effects.

To test whether increasing histone acetylation in the hippocampus is sufficient to cause behavioral effects, we studied both acute and chronic treatment with NaB in behavioral models of anxiety and antidepressant response. In the present study, NaB failed to induce antidepressant-like behavioral responses in the forced-swim test (FST) or the novelty-induced hypophagia (NIH) paradigm despite observed alterations in histone acetylation in the hippocampus. Our results demonstrate that increasing histone acetylation in the hippocampus *alone* is not sufficient to drive antidepressant behavioral changes, suggesting that additional mechanisms must be involved.

2. Materials and Methods

2.1 Animals

All mice used for behavioral and biochemical experiments were F1 hybrid offspring obtained from crosses of 129SvEv and C57Bl/6 mice, a strain which has been

shown to respond to antidepressant and anxiolytic compounds in a number of behavioral paradigms in our laboratory (Conti et al., 2002, Gur et al., 2007). Mice (20-40 g, 2-6 months of age, mixed sexes) were group-housed with food and water available *ad libitum* and maintained on a 12-hour light/dark cycle (lights on at 07:00) in accordance with the University of Pennsylvania Animal Care and Use Committee. All behavioral testing sessions were performed between the hours of 08:00 and 15:00h, and animals were randomly assigned to treatment conditions and tested in counterbalanced order.

2.2 Drugs

All drugs were dissolved in 0.9% saline immediately before use and injected intraperitoneally using a volume of 10 mL/kg. For acute studies, sodium butyrate (NaB) (Sigma, St. Louis, MO) or 0.9% saline was injected on the morning and afternoon of the day preceding testing, as well as on the morning of testing, for a total of three doses before exposure to the behavioral test. For chronic studies, NaB or 0.9% saline was injected twice daily (09:00 and 17:00h) for 21 days before exposure to the behavioral test. As a positive control in the NIH, a third group received desipramine (DMI) (Sigma) (12.5 mg/kg) for chronic studies or chlordiazepoxide (CDP) (10 mg/kg) (Sigma) in the acute study. In the FST, DMI was also used as a positive control with the three injections preceding the test at doses of 10 mg/kg, 10 mg/kg, and 20 mg/kg as described previously (Conti et al., 2002).

2.2 Behavioral studies

For all behavioral studies, mice were given their last injection and brought into the testing room one hour preceding the start of testing.

2.21 Forced swim test

Mice were placed in 15cm of water (22–24°C) in plastic cylinders (23cm tall × 14cm diameter) for 6 min. Mice were video-recorded and time spent immobile vs. swimming and climbing was scored by the Viewpoint Tracking System (Viewpoint, Champagne au Mont d'Or, France).

2.22 Novelty-induced hypophagia

Mice were housed in groups of two for one week before the start of the training period and for the duration of the experiment. During training and home cage testing, mice had daily exposure to a highly palatable food (peanut butter chips) (Nestle, Glendale, CA) in a clear plastic dish in their home cage. Plastic dividers were placed inside the home cage to separate the mice, beginning one hour before training and testing periods. Food was placed in the cage for 15 min and latency to consume the chips was measured. By the 12th day of training, baseline latencies had been established with less than 20% variability amongst mice. For acute experiments, mice received 12 days of training, followed by testing in home cage (Home1), novel environment, and home cage (Home2) on the 3 days following training. For chronic experiments, mice received 12

days of training, followed by 21 days of injections, and then testing in home, novel, home on days 22-24.

Testing in the novel environment consisted of placing mice in an empty standard cage, lacking bedding, which was placed in a white box with bright illumination (2150 lux) and with an added scent (Pine Sol) applied to the cage. Latency to consume in the novel environment was recorded with a 15-minute maximum exposure. Novel testing in both acute and chronic experiments occurred 1 hour after separators were placed in the home cage and 1 hour after mice received their last injection, parallel to the timing of home cage testing.

2.23 Elevated zero maze

The zero maze (Stoelting, Wood Dale, IL) consisted of two open areas (wall height, 0.5") and two closed areas (wall height, 12"), and was elevated 24" from the ground. Lighting in the maze was 15 lux. At the start of testing, mice were placed into one of the closed areas and allowed to explore the maze for 300s. The Viewpoint Tracking System (Viewpoint) was used to video-record and track the amount of time spent in the open areas, the number of entries into the open areas, and the distance traveled in each area.

2.24 Locomotor activity

Locomotor activity was measured by beam-breaks in a photobeam frame (Med Associates, St. Albans, VT, USA). During the test, mice were placed individually into a clean home cage resting within the photobeam frame, and data were recorded by Med Associates Software. Ambulations, crossings, and rearings were measured in 5-minute bins

for 30 (acute study) or 60 minutes (chronic study).

2.3 Histone acetylation western blots

Mice were sacrificed by cervical dislocation 30 minutes following the last injection (both acute and chronic studies). Brains were removed; whole hippocampus was hand-dissected and flash-frozen in liquid nitrogen. Tissues were homogenized in 200 ml of ice-cold extraction buffer containing 250 mM sucrose, 50 mM Tris, pH 7.5, 25 mM KCl, 0.5 mM PMSF, 0.9 mM NaB, as well as protease inhibitors ("complete" protease inhibitor cocktail, Roche, Basel, Switzerland**)** and phosphatase inhibitors (phosphatase inhibitor cocktail 1, Sigma). The nuclear fraction (pellet) was separated by centrifugation at 7,700xg for 1 min (4°C), and re-suspended in 1 mL 0.4 N H_2SO_4 and incubated for 30 min (4°C). Samples were centrifuged at 14,000xg for 30 min (4°C). 250 μL trichloroacetic acid (with 4 mg/mL deoxycholate) was added to the supernatant, and incubated for 30 min (4°C) to precipitate protein. Samples were then spun at 14,000xg for 30 min (4°C) to pellet protein. Pellets were washed for 5 min with 1 mL acidified acetone (0.1% HCl), then for 5 min with acetone. Between washes, protein was collected by centrifuging 5 min at 14,000xg (4°C), and aspirating supernatant. After the last wash, the pellet was re-suspended in 200 μL 10 mM Tris, pH 8.0, and incubated for 15 minutes at room temperature. Protein concentrations were determined using a Bradford assay, with bovine serum albumin as the standard. Equivalent amounts of protein (10 μg) for each sample were resolved with SDS-PAGE using 4-15% gradient Tris-HCl gels. After electrophoresis, proteins were transferred to nitrocellulose membranes for 2 hours at 50V. Membranes were incubated in blocking buffer (LI-COR,

Lincoln, Nebraska, USA) 1 hr at room temperature to block non-specific binding. The blots were reacted with primary antibodies (anti-AcH3, 06-599, Millipore, USA; anti-AcH4, 06-598, Millipore; anti-H3, mAbcam 10799, AbCAM, Cambridge, MA, USA) at a concentration of 1:1000 in blocking buffer (LI-COR) overnight at 4°C. After washing (3x15 min in PBS-Tween20), the blots were incubated in secondary antibody (goat anti-mouse IRDye 680 and goat anti-rabbit IRDye 800, LI-COR) in blocking buffer for 1 hr at RT in dark boxes. Membranes were then washed (3x15 min in PBS-Tween20) and dried overnight (also in the dark). Immunolabeling was detected and quantified at two wavelengths simultaneously using the Odyssey infrared imaging system scanner and software (LI-COR). This system allows for accurate quantification of multiple bands on the same membrane at the same time, using different antibodies raised in different species (which appear at different wavelengths), thus allowing quantification of AcH3 and H3 on the same blot. The AcH3/H3 bands were detected at 17 KDa and the AcH4 band was detected at10 kDa. Ratios of AcH3 or AcH4 to total H3 fluorescence were calculated for each sample and analyzed across conditions.

2.4 Statistics

For the elevated zero maze, Student's t-test was used to assess statistical significance. For the AcH westerns and FST, ANOVA and Bonferroni *post hoc* tests were used to assess significant differences between groups. For the NIH and locomotor activity, repeated measures ANOVA was performed, with either time bin (locomotor activity) or day (home1, novel, home2 for the NIH) as the repeated-measures factor. Bonferroni and

Newman-Keuls multiple comparison *post hoc* tests were used to determine significant differences between drug-treated and saline-treated groups at specific time points.

3. Results

3.1 Acute treatment with NaB causes an increase in immobility in the FST, while chronic treatment has no effect

To determine an appropriate dose to evaluate antidepressant effects of NaB, we tested two doses of NaB, 100 mg/kg and 1.2 g/kg (the latter was previously shown to have effects on memory, depression- and reward-related behaviors, see (Levenson et al., 2004, Schroeder et al., 2006, Lattal et al., 2007, Schroeder et al., 2008), for their effects on spontaneous locomotor activity (data presented in 5-minute bins). Acute treatment (3 injections) with 1.2 g/kg of sodium butyrate caused a significant decrease in both ambulations and crossings (Ambulations: main effect of treatment, $F_{(2, 105)} = 4.690$, $p = 0.02$, RMANOVA; 1.2 g/kg NaB vs. saline, $p < 0.01$ for first 5 min, Bonferroni *post hoc* test. Crossings: main effect of treatment, $F_{(2, 105)} = 4.144$, $p = 0.03$, RMANOVA; 1.2 g/kg NaB vs. saline, $p < 0.001$ for first 5 min, Bonferroni *post hoc* test) (Figure 1a). In contrast, the lower dose of NaB (100 mg/kg) did not cause any significant changes in either measure of locomotor activity (100 mg/kg NaB vs. saline, $p > 0.05$ for all time points, Bonferroni *post hoc* test).

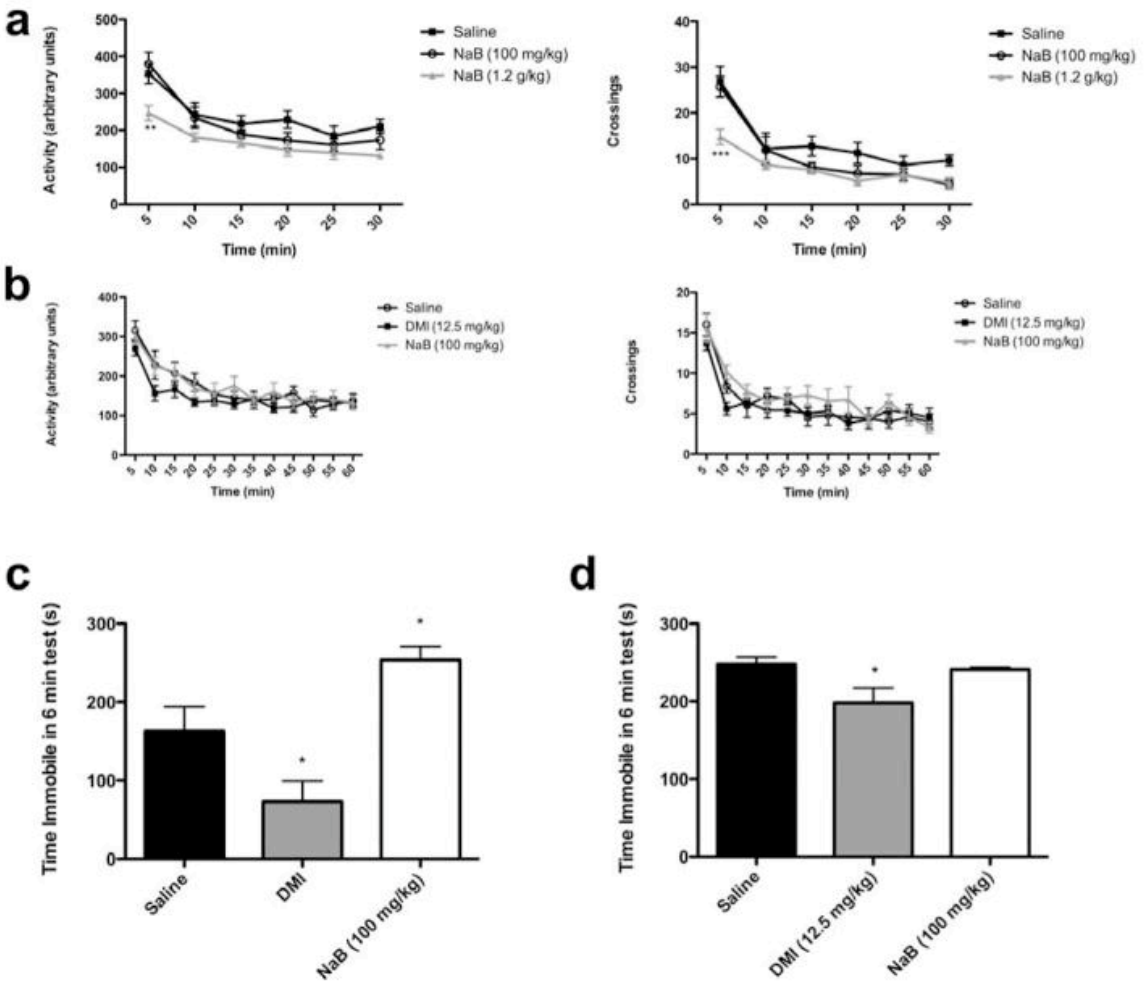

Figure 1. Acute, but not chronic, treatment with NaB increases immobility in the FST at doses that do not affect locomotor activity. Mice were given 3 injections of NaB over 24 hours (a) or treated twice daily for 21 days with NaB or DMI (b), and their locomotor activity was measured 1 hour after the last injection. Ambulations (left) and crossings (right) were measured in 5-minute bins for a total of 30 minutes in acutely treated mice (a). The higher dose of NaB (1.2 g/kg) caused significant hypolocomotion in the first 5 minutes, while the lower dose (100 mg/kg) had no effect (n=8). Chronic treatment with NaB (100 mg/kg) or DMI (12.5 mg/kg) had no significant effect on either ambulations (left) or crossings (right) over 60 minutes (b) (n = 30). A separate cohort of mice was given acute treatment with NaB or DMI, and immobility during a 6 minute FST was measured (c). There was a significant effect of treatment on immobility, with DMI significantly reducing immobility and NaB (100 mg/kg) significantly increasing immobility (n=5-6). After chronic treatment with twice-daily NaB (100 mg/kg) or DMI (12.5 mg/kg), DMI significantly reduced immobility in the FST, whereas NaB had no effect (d). Error bars indicate SEM. *p< 0.05 vs. saline; ** p< 0.01 vs. saline; ***p<0.001 vs. saline.

Changes in general activity levels can compromise interpretation of results in the FST; therefore, we examined the effect of the lower dose of NaB in this paradigm. Acute treatment (3 injections over 24 hr) with 100 mg/kg NaB caused a significant increase in immobility in the FST (main effect of drug, $F_{(2, 14)} = 12.10$, $p = 0.0009$, ANOVA; significant difference between NaB and saline, $p < 0.05$, Newman-Keuls multiple comparisons *post hoc* test) (Figure 1c). In the same study, the tricyclic antidepressant desipramine (DMI) caused the expected decrease in immobility (DMI vs. saline, $p < 0.05$, Newman-Keuls multiple comparisons *post hoc* test), an antidepressant effect. After chronic treatment (100 mg/kg, twice daily for 21 days), mice injected with NaB showed no difference in immobility from saline-injected mice one hour after the last injection (Figure 1d) (Main effect of treatment, $F_{(2, 24)} = 4.201$, $p = 0.0273$, ANOVA; no significant difference between NaB and saline, $p > 0.05$, Bonferroni's multiple comparisons *post hoc* test). The effect of DMI, however, was maintained after chronic treatment, as DMI-injected mice showed a decrease in immobility compared to saline-treated mice (significant difference between DMI and saline, $p < 0.05$, Bonferroni's multiple comparisons *post hoc* test). Locomotor activity was also measured after chronic treatment with both NaB and DMI (Figure 1b). Chronic treatment with NaB did not alter locomotor activity one hour after the last injection (a time point parallel to when other behavioral tests were carried out) (Ambulations: No main effect of treatment, $F_{(2, 209)} = 1.204$, $p = 0.3218$; significant time by treatment interaction, $F_{(22,209)} = 1.789$, $p = 0.0196$, RMANOVA; NaB vs. saline, $p > 0.05$ for all time points, Bonferroni *post hoc* test. Crossings: No main effect of treatment, $F_{(2, 209)} = 1.122$, $p = 0.3462$, trend toward

significant time by treatment interaction, $F_{(2, 209)} = 1.559$, $p = 0.0586$, RMANOVA; NaB vs. saline, $p > 0.05$ for all time points, Bonferroni *post hoc* test). Chronic treatment with DMI also did not cause any significant change in locomotor activity one hour after the last injection (Activity: DMI vs. saline, $p > 0.05$ for all time points, Bonferroni *post hoc* test. Crossings: DMI vs. saline, $p > 0.05$ for all time points, Bonferroni *post hoc* test).

3.2 NaB causes divergent effects in the NIH paradigm depending on the length of treatment

The FST is responsive to acute treatment with antidepressants; therefore, we sought to test the efficacy of NaB in the novelty-induced hypophagia (NIH) paradigm, which is both a test of anxiety and a model of chronic antidepressant response. In this paradigm, latency to approach a familiar food in a novel environment is reduced acutely by anxiolytic compounds, and by chronic treatment with antidepressants (Merali et al., 2003, Dulawa et al., 2004, Gur et al., 2007). We first sought to evaluate the acute effect of NaB in this paradigm, and did so using a dosing regimen identical to that used in the FST; mice received 3 injections of NaB in the 24 hours before testing in the novel environment, with the last injection given one hour before testing. We observed the expected increase in latency to consume in the novel environment as compared to the home cage in all groups (main effect of day, $F_{(2, 52)} = 30.81$, $p < 0.0001$, RMANOVA) (Figure 2). However, NaB caused a further increase in latency to consume, which was specific to the novel environment (significant day x drug interaction, $F_{(4, 52)} = 4.934$, $p < 0.01$; NaB vs. saline on Novel Day, $p < 0.001$, Bonferroni *post hoc* test; NaB vs. saline on

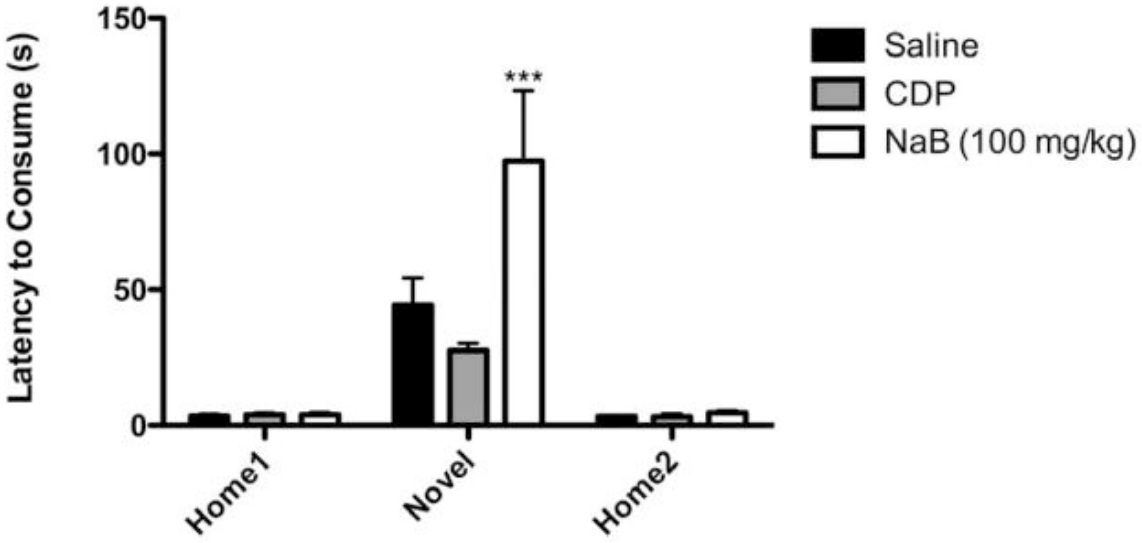

Figure 2. Acute treatment with NaB increased latency to consume peanut butter chips in the novel environment of the NIH paradigm. Mean latencies to consume in home and novel environment are shown. Mice were given 3 injections of NaB (AM and PM on Home Day 1 and AM on Novel day, 1 hr before test) or 1 injection of CDP (1 hr before test) before exposure to novel environment. There was an increase in latency to consume in the novel environment relative to the home cage ($p<0.0001$) and an increase in latency in NaB-treated animals as compared to saline-treated animals (*$p<0.001$) (n=9-10). Error bars indicate SEM.

Home1 and Home2, $p > 0.05$, Bonferroni *post hoc* tests) (Figure 2). As a control, we also examined the effects of chlordiazapoxide (CDP), a benzodiazepine that has been shown to decrease latency to consume in the novel environment (Merali et al., 2003). As reported previously in this strain (Gur et al., 2007), CDP reduced latencies; however in this study, the difference did not quite reach significance (CDP vs. saline, $p > 0.05$, Bonferroni *post hoc* test).

To further investigate this anxiogenic effect of acute treatment with NaB, we examined the effects of NaB in another test of anxiety, the elevated zero maze (EZM). In this paradigm, acute treatment with NaB did not have any significant effect on time spent in the open arms (NaB vs. saline, $p = 0.4182$, unpaired t-test), entries into the open arms (NaB vs. saline, $p = 0.570$, unpaired t-test), or distance traveled in the open arms (NaB vs. saline, $p = 0.884$, unpaired t-test) (Figure 3). In addition, NaB had no effect on total distance traveled during exposure to the EZM (NaB vs. saline, $p = 0.8339$, unpaired t-test), further confirming that this dose does not affect overall activity levels.

Changes in gene expression brought about by NaB might require longer time periods to take effect; therefore, we examined the effects of chronic treatment (2 injections/day for 21 days) with NaB in the same array of behavioral paradigms.
In the NIH paradigm, selective serotonin reuptake inhibitors and tricyclic antidepressants have been shown to reduce latency to consume in the novel environment after similar lengths of treatment, which have led to use of this paradigm as a model of chronic antidepressant response (Merali et al., 2003, Dulawa et al., 2004, Gur et al., 2007). In

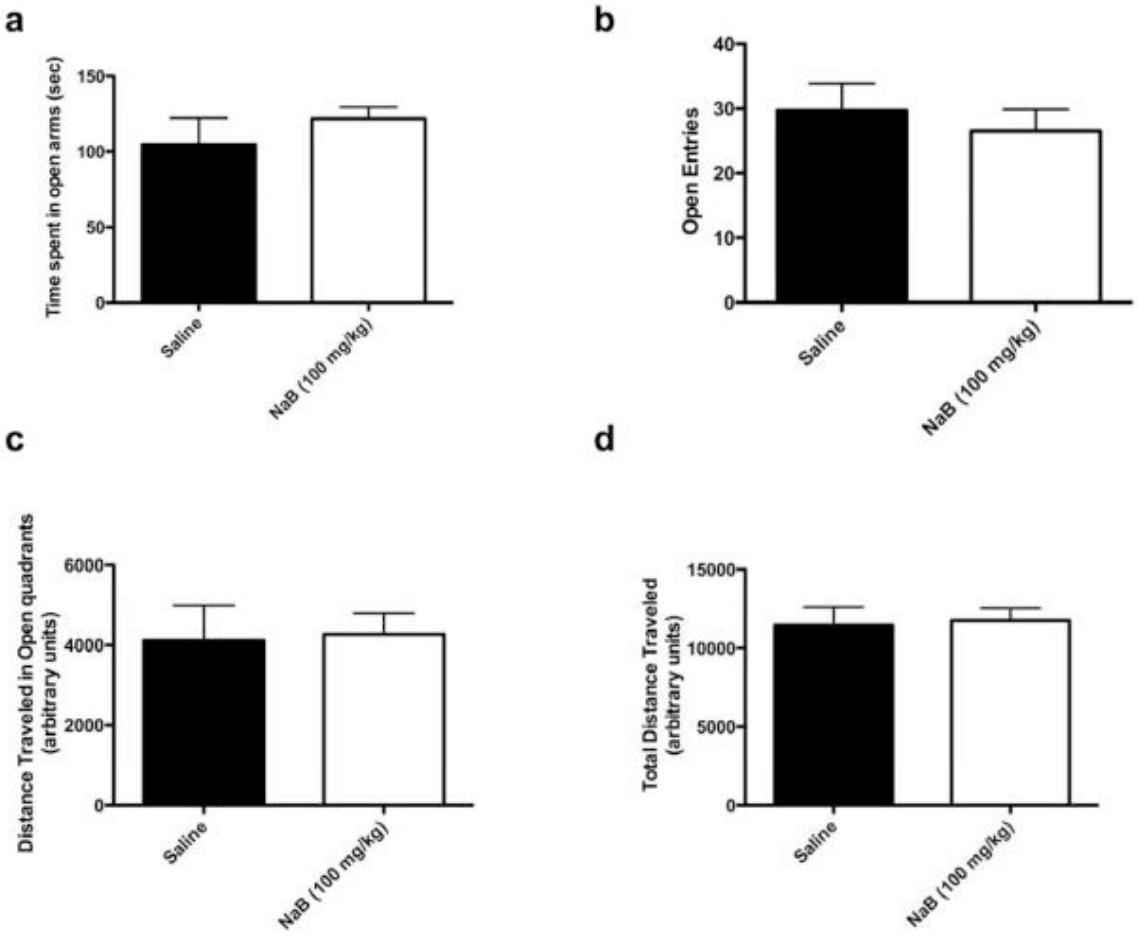

Figure 3. Acute treatment with NaB had no effect on anxiety behavior in the elevated zero maze. Mice were given 3 injections of NaB over 24 hours and tested in the EZM 1hr following the last injection. NaB had no effect on time spent in the open quadrants (a), entries into the open quadrants (b), distance traveled in the open quadrants (c), or total distance traveled in the maze (d) (n = 6-7). Error bars indicate SEM.

this study, mice were injected twice daily with a dose of 100 mg/kg NaB, which was well tolerated. We observed no changes in weight during the treatment period (data not shown), as has been previously reported (Schroeder et al., 2006). After chronic treatment with NaB, we again observed an effect of the novel environment to increase latency to consume (main effect of day, $F_{(2, 40)} = 96.30$, $p < 0.0001$, RMANOVA) (Figure 4), a main effect of treatment on latency to consume ($F_{(2, 40)} = 4.813$, $p = 0.0197$, RMANOVA), and a treatment x day interaction ($F_{(4, 40)} = 5.596$, $p = 0.0011$, RMANOVA). However, we saw no effect of chronic treatment with NaB (NaB vs. saline, $p > 0.05$, Bonferroni *post hoc* test). DMI, a tricyclic antidepressant, significantly reduced latency to consume in the novel environment (DMI vs. saline, $p < 0.001$, Bonferroni *post hoc* test), and the effect was specific to the novel environment (DMI vs. saline on Home1 and Home2, $p > 0.05$, Bonferroni *post hoc* test).

3.3 NaB causes increases in histone acetylation in the hippocampus after acute, but not chronic treatment

NaB is known to be an inhibitor of HDACs, but its effects in the brain have not been extensively characterized. Thus, we sought to examine the effects of the doses of NaB used in behavioral assays on histone acetylation in the hippocampus, a region known to be important in the activity of antidepressants. Using western blots, we examined the levels of acetylated histones H3 and H4 in the hippocampi of mice 30 min after the last of three injections of NaB (100 mg/kg or 1.2 g/kg). Acute treatment with both doses of NaB increased AcH4 levels in the hippocampus (main effect of treatment

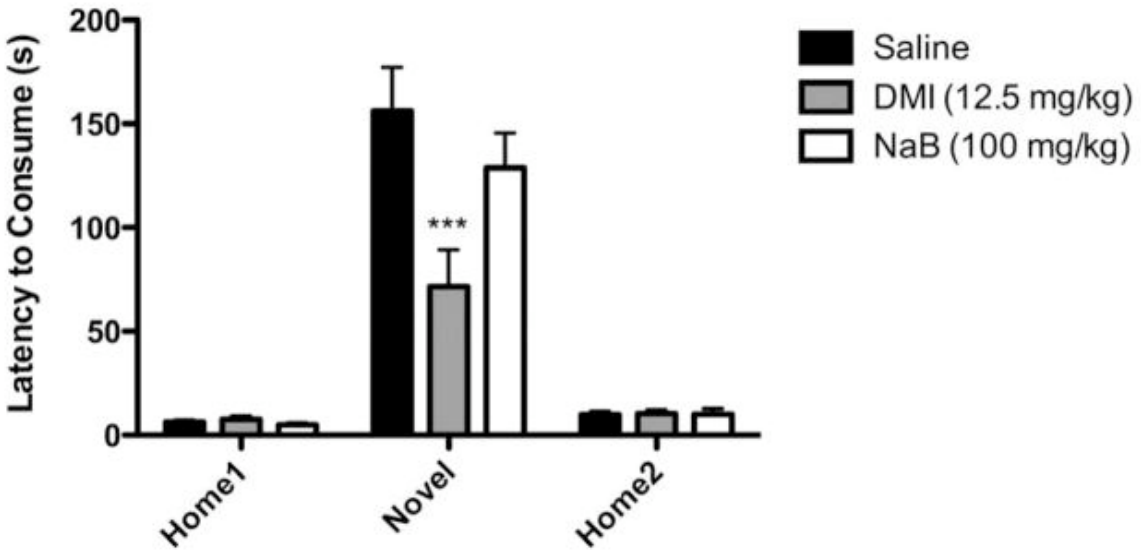

Figure 4. Chronic treatment with NaB had no effect on latency to consume peanut butter chips in the novel environment of the NIH paradigm. Mean latencies to consume in home and novel environment are shown. Mice were treated with NaB or DMI for 22 days before exposure to novel environment. There was an increase in latency to consume in the novel environment relative to the home cage ($p<0.001$). There was a significant decrease in latencies in the novel environment in DMI-treated mice as compared to saline-treated animals, but no change in NaB-treated mice (*$p<0.001$) (n=9-10). Error bars indicate SEM.

$F_{(2, 14)} = 35.78$, $p < 0.0001$, ANOVA; 100 mg/kg vs. saline, $p < 0.05$; 1.2 g/kg vs. saline, $p < 0.001$, Bonferroni *post hoc* tests) (Figure 5a). However, only the higher dose significantly increased AcH3 in the hippocampus (main effect of treatment $F_{(2, 15)} = 12.93$, $p = 0.0005$, ANOVA; 100 mg/kg vs. saline, $p > 0.05$; 1.2 g/kg vs. saline, $p < 0.01$, Bonferroni *post hoc* tests) (Figure 5b). We also examined histone acetylation after the chronic treatment (twice daily for 21 days) with 100 mg/kg NaB (30 minutes after the final injection). Here, we observed a significant decrease in levels of AcH4 in the hippocampus ($p = 0.0166$, unpaired t-test) (Figure 5c), and no change in the levels of AcH3 in the hippocampus ($p > 0.05$, unpaired t-test) (Figure 5d).

4. Discussion

The importance of chromatin remodeling in psychiatric diseases has received much attention recently. In this study we show that, despite causing changes in the level of histone acetylation in the hippocampus, the histone deacetylase (HDAC) inhibitor sodium butyrate (NaB) failed to cause any change in mouse models of chronic antidepressant response. Acute treatment with NaB did induce an increase in immobility in the FST as well as an anxiogenic effect in the novelty-induced hypophagia (NIH) paradigm, with no effects on general locomotor activity. Acute NaB had no effect on anxiety behavior in the elevated zero maze (EZM). While chromatin remodeling has been reported following chronic antidepressant treatment (Tsankova et al., 2006), these data suggest that changes in chromatin structure alone may not be sufficient to induce antidepressant behavioral effects.

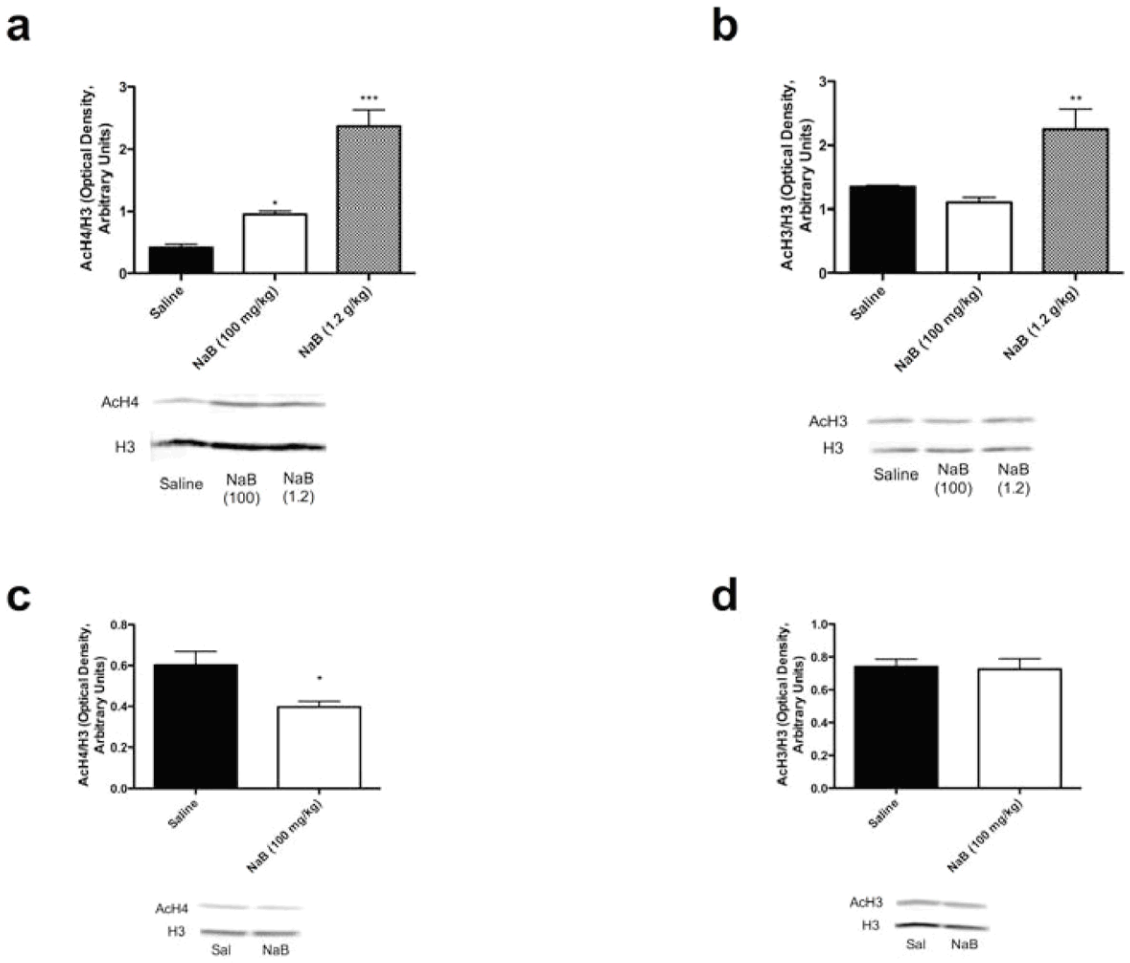

Figure 5. Optical density of AcH staining normalized to total H3 staining is shown. Mice were given 3 injections of NaB over 24 hours and sacrificed 30 min after the last injection (a and b). Acute treatment with NaB dose-dependently increased AcH4 in the hippocampus (a), while only the higher dose of NaB (1.2 g/kg) significantly increased AcH3 in the hippocampus (b) (n = 5-7). A separate cohort of mice was injected with NaB for 21 days and sacrificed 30 minutes after the last injection (c and d). Chronic treatment with NaB decreased AcH4 in the hippocampus (c), while the level of AcH3 in the hippocampus did not change after chronic treatment with NaB (n=7-8). *$p<0.05$ vs. saline; **$p<0.01$ vs. saline; ***$p<0.001$ vs. saline. Error bars indicate SEM.

We showed an increase in immobility in the FST after acute treatment with NaB. Recently, Schroeder et. al. (2006) showed a similar effect of acute treatment with NaB in the tail suspension test (TST), an analogous behavioral paradigm, although in a different mouse strain (C57BL/6, whereas we use an F1 hybrid of C57BL/6 and 129SvEv mice) and at a much higher dose (1.2 g/kg) than used here (100 mg/kg). At this higher dose (1.2 g/kg), we saw significant hypolocomotion, which could be responsible for the increased immobility seen in Schroeder et. al. (2006), but not for that seen in the present study, as the lower dose of NaB (100 mg/kg) had no such effects on locomotor activity.

We also report here that chronic treatment with 100 mg/kg NaB (twice daily for 21 days) failed to cause any changes in behavior in either the FST or NIH paradigms. Schroeder et. al. (2006) did see an antidepressant effect in the TST after chronic treatment. There are a number of methodological differences between the present study and that done by Schroeder and colleagues, including the dose of NaB (100 mg/kg twice daily here, 1.2 g/kg in the Schroeder et. al. study), the behavioral paradigm (FST vs. TST), the mouse strain (C57BL/6 vs. F1 hybrids of C57BL/6 and 129SvEv), and the length of treatment (21 vs. 28 days). Additionally, the effect observed by Schroeder et. al. (2006) was seen only when the TST was administered on the last day of a 4-day battery of testing and not when administered to behaviorally naïve mice. As the authors suggest, the previous days' tests may have been a source of stress, and the NaB may have acted to reduce the effects of this stress, rather than having antidepressant effects on its own. This activity of NaB to counteract the effects of stress fits well with results obtained by Tsankova et. al. (2006) using the chronic social defeat (CSD) paradigm. In

this study, the tricyclic antidepressant imipramine (IMI) was seen to increase H3 acetylation at BDNF promoters, but only in mice previously exposed to CSD (Tsankova et al., 2006). These stressed mice showed increases in histone methylation, a modification associated with repression of gene expression. The activity of IMI to increase AcH3 countered the decreases in BDNF expression associated with stress-induced increases in di-methylated H3 (Tsankova et al., 2006). In another study, chronic fluoxetine (21 d) was shown to increase AcH3 at the BDNF promoter, but only in mice with increased tri-methylated H3 and decreased AcH3 at the BDNF promoter due to perinatal exposure to methylmercury (Onishchenko et al., 2008). It is therefore possible that the effects of NaB require prior stress experience (or some additional manipulation that causes a repressive chromatin state), and because there was no prior stressful experience in our FST study, NaB did not exert an antidepressant effect. In addition, the FST and TST have been traditionally used to screen novel antidepressants, but these paradigms have mainly validated compounds that act to increase synaptic monoamine levels. Thus, they may not be appropriate to test the efficacy of any antidepressant acting by a novel pathway, e.g. direct changes in gene expression (Cryan et al., 2002).

Due to the potential limitations of the FST, we next examined the effects of NaB in another behavioral paradigm: the NIH. We report here that acute treatment with NaB caused an increase in latency in the novel environment, an anxiogenic effect (Soubrie et al., 1975). This response was not observed in the elevated zero maze (EZM), another test of anxiety behavior. While these results seem to contradict each other, it is important to note that the NIH may measure different aspects of anxiety than the EZM, and also

contains appetitive or hedonic components (e.g. consumption of peanut butter chips) not present in the EZM. Increases in histone acetylation (H3 and H4) have been correlated with the anxiolytic effects of alcohol, and decreases in histone acetylation (H3 and H4) in the amygdala were associated with the anxiogenic effects of alcohol withdrawal (Pandey et al., 2008). These decreases in AcH3 and AcH4 and increases in anxiety during alcohol withdrawal were reversed by the HDAC inhibitor TSA, suggesting that increases in histone acetylation are causally related to decreases in anxiety (Pandey et al., 2008). Our study shows an inverted relationship between increased histone acetylation and anxiety as compared to the Pandey et. al. (2008) study. This discrepancy may be explained by the species used (rats were used in the Pandey et. al. study) and/or the presence or absence of alcohol (our mice were alcohol naïve), which may change the valence of the effect of increasing histone acetylation on anxiety.

We also examined the effects of chronic treatment with NaB in the NIH. The NIH has been validated as a test of chronic antidepressant response by a number of laboratories, including our own, as changes in latencies in the novel environment occur after chronic, but not acute, treatment with current antidepressants (Merali et al., 2003, Dulawa et al., 2004, Gur et al., 2007). Chronic treatment with NaB (21 days) did not cause any change in latencies in the NIH. This is in contrast to the increased latencies seen after acute treatment, and also in contrast to the antidepressant effect of chronic treatment with sodium butyrate in the TST seen by Schroeder et. al (2006). In addition to the differences between our study design and the Schroeder et. al. (2006) study discussed above, an additional aspect of the NIH paradigm that could have been

influenced by NaB is the motivation of the mice to consume a highly palatable food: peanut butter chips. Based on the control of the home cage tests, the NaB dosing paradigm used in both the acute and the chronic studies did not appear to have any effect on motivation to consume the chips, as home cage latencies were unaffected by NaB. Furthermore, TSA was not seen to affect self-administration of sucrose in a fixed-ratio schedule or breaking points in a progressive-ratio schedule over a 7-day period, or consumption of sucrose in a two-bottle choice preference test after 4 days of treatment (Romieu et al., 2008). Therefore, it is unlikely that the effects of NaB on latency to consume in the NIH are based on changes in motivation for highly palatable food.

The NaB treatment regimens used in these studies were accompanied by changes in the acetylation state of H3 and H4 in the hippocampus. 30 minutes after acute treatment with NaB we observed a significant increase in AcH4 in the hippocampus but no change in AcH3. At the same time point following chronic treatment with NaB, we observed a *decrease* in AcH4 and no change in AcH3. This transition from an increase in AcH4 acutely to a decrease after chronic treatment may explain the shift in behavioral response to NaB between acute and chronic time points.

The literature contains conflicting evidence regarding the effects of NaB on histone acetylation levels in the brain, and it is important to keep in mind not only dosing regimen and area of the brain, but also whether acetylation is measured globally (e.g. western blots or immunhistochemistry) or at specific promoters (e.g. chromatin immunoprecipitation) (ChIP). In one study, ChIP analysis showed an effect of acute treatment with NaB (200 mg/kg) on AcH4 in the striatum, with AcH3 affected only by

chronic treatment with NaB (Kumar et al., 2005). At higher doses, however, increases in both AcH3 and AcH4 in the hippocampus and frontal cortex were measured by western blot 30 minutes after a single injection of NaB (Schroeder et al., 2006). Here, we show an increase in AcH4 after acute treatment with NaB, which shifts to a *decrease* after chronic treatment. This change in response may be due to a desensitization after chronic treatment with this drug or some compensatory response of neurons to prolonged increases in AcH4. Additionally, it is important to remember that changes at specific promoters may be more complex than changes in global levels of acetylation, as measured in the present study. Recently it has been suggested that regulation of gene expression by histone acetylation is more complex than previously imagined, and deacetylation in some cases may lead to activation of genes (Nusinzon and Horvath, 2005). Indeed, previous work has shown that NaB alters levels of histone acetylation at specific promoters in a time-, dose-, and brain region-specific manner (Schroeder et al., 2008), and thus there may still be increased histone acetylation at some promoters despite the global decrease in AcH4 we observed.

Though limited in number, studies have demonstrated changes in histone acetylation in the brain after chronic treatment with antidepressants. Increases in AcH3 were seen in the hippocampus after chronic treatment with two distinct antidepressants, fluoxetine (an SSRI) and imipramine (a tricyclic), but only after manipulations (stress, methylmercury exposure) that lowered AcH3 and caused increases in repressive post-translational modifications to histones (Tsankova et al., 2006, Onishchenko et al., 2008). In another study, however, chronic treatment with fluoxetine was actually seen to

decrease levels of AcH3 in the dentate gyrus of the hippocampus, as well as the frontal cortex and caudate/putamen (Cassel et al., 2006). Because of these discrepancies, it will be of increasing utility to examine histone acetylation at specific promoters, as the global acetylation state may not reflect chromatin structure at genes of interest. In addition, it is important to examine brain regions related to the behaviors in question, as there are clearly region-specific changes brought about by these drugs.

Based on recent evidence, changes in chromatin structure at specific promoters in the hippocampus are likely to play a role in the effects of classic antidepressants. We demonstrate here, however, that global changes in histone acetylation may not be sufficient to produce behavioral effects. It is possible that additional events are required for the full effects of antidepressants to be realized, such as parallel activation of transcription factors. Chromatin remodeling may act as a facilitating event, allowing specific transcription factors to more easily activate their target genes. Alternately, changes in histone acetylation may be more specific and occur only in concert with transcription factor binding, as in the case of CREB and CREB-binding protein, which has intrinsic histone-acetyl transferase activity. Thus, treatment with HDAC inhibitors may be most effective in combination with classic antidepressants, either to increase their efficacy or reduce the lag time before symptoms are ameliorated. Additionally, although BDNF has been implicated as one promoter at which changes in AcH may be involved in the action of antidepressants, it will be important to identify more genes at which these changes are taking place. It is also important to investigate how these changes in chromatin structure interact with the activity of transcription factors (such as CREB)

known to be downstream of the activity of antidepressants. Overall, while chromatin modification likely plays some role in depression, the results of this study suggest that direct modulation of histone acetylation levels alone is not sufficient to induce antidepressant behavioral effects.

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