THE ROLE OF CREB IN SOCIAL ISOLATION AND NATURAL REWARD BEHAVIOR

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I would like to thank the members of my Graduate Committee, my family and friends, and the animals that were used in this research.

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The role of CREB (cyclic AMP Response Element Binding protein) has been known to play a role in complex behaviors such as learning and memory, drug reward and depression. The work presented in this dissertation examines the role of CREB in an inactive stress paradigm, social isolation. Social isolation is a model which decreases CREB-mediated transcription in the nucleus accumbens and results in depressive- and anxiety- like phenotypes, all reversed by chronic but not acute administration of imipramine. In addition, aspects of social isolation can be mimicked in non-isolated animals by inhibition of CREB in the nucleus accumbens and certain deficits in isolated

animals can be reversed by wildtype overexpression of CREB. However, other behavioral deficits observed in the isolated phenotype, while reversed by imipramine administration, are unaffected by CREB manipulation in the nucleus accumbens. Potential gene targets are explored by microarray analysis comparing control double-housed animals and isolated animals, and these groups treated with wildtype CREB or chronic imipramine administration. The array analysis led to the discovery that social isolation alone increases expression of several types of potassium channels in the nucleus accumbens. Investigating the electrophysiological properties of these neurons, social isolation results in a greater hyperpolarization of the resting membrane potential, and decreased potassium channel-mediated membrane resistance. Lastly, it is shown that inhibiting CREB leads to increases of potassium channel expression, and the anxiety-like effects observed in isolated animals can be mimicked in non-isolated animals by overexpression of the inward rectifying potassium channel kir2.1. These studies further the field of depression and anxiety research with a model sensitive to chronic but not acute antidepressant treatment and reveal potential novel mechanisms for the reversal of anxiety-like behaviors.

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5-HIAA – 5-Hydroxyindole Acetic Acid

5-HT – 5-Hydroxytryptamine

α-MSH – alpha-melanocyte stimulating hormone

AAV – adeno-associated virus

ACTH – adrenocorticotropic hormone

ATF – activating transcription factor

BDNF – brain derived neurotrophic factor

BLA – basolateral amygdala

cAMP – cyclic adenosine monophosphate

CaMK – calcium/calmodulin kinase calcium modulating kinase

c-Fos – cellular oncogene Fos

CBP – CREB binding protein

CPP – conditioned place preference

CRE – cAMP response element

CREB – cAMP response element binding protein

CREM – cAMP response element modulating protein

CUS – chronic unpredictable stress

DARPP-32 – dopamine- and cAMP-regulated phosphoprotein -32KD

DG – dentate gyrus

EEG – electroencephalogram

FSL – Flinders sensitive line

FST – forced swim test

GABA – Gamma-aminobutyric acid

GPCR – G-protein coupled receptor

HPA – hypothalamic-pituitary-adrenal

HSV – herpes simplex virus

ICER – inducible cAMP early repressor

kcnd – potassium voltage-gated channel, Shal-related

kcnj – potassium inwardly-rectifying channel, subfamily J

kcns – potassium channel (kv9.2)

KID – kinase inducible domain

KIX – KID interaction domain

kir –inward-rectifying potassium channel

LC – locus ceruleus

LPS – lipopolysaccharide

MAPK – mitogen activating protein kinase

mCREB – dominant negative mutant CREB

MPOA – medial preoptic area

MSK1 – mitogen and stress kinase

NAc – nucleus accumbens

NAcSh – nucleus accumbens shell

NE – norepinephrine

NGF – nerve growth factor

nNOS – neuronal nitric oxide synthase

PAG – periaqueductal gray

p-CREB – phosphorylated CREB

PDE – phosphodiesterase

PEI – post-ejaculation interval

PKA – protein kinase A

PKC – protein kinase C

PP1 – protein phosphatase 1

PP2A – protein phosphatase 2A

RSK2 – ribosomal S6 kinase 2

TH – tyrosine hydroxylase

TORC2 – transducer of regulated CREB activity 2

TPH – tryptophan hydroxylase

TrkA – tyrosine receptor kinase A

TST – tail suspension test

VTA – ventral tegmental area

CHAPTER ONE
Introduction

THE TRANSCRIPTION FACTOR CREB

The Basics of CREB Signaling

CREB (cyclic AMP Response Element Binding protein) is a 43KD basic leucine zipper (bZIP) transcription factor that was first discovered in the initiation of gene transcription of the somatostatin gene (Montminy and Bilezikjian, 1987). A CRE sequence consists of 5'TGACGTCA-3' and is found in the promoter region of various types of genes (Shaywitz and Greenberg, 1999) including immediate early genes, such as cellular oncogene Fos (c-fos) (Sheng et al., 1990), as well as genes for the growth factors nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) (Shieh et al., 1998; Tao et al., 1998), neuronal nitric oxide synthase (nNOS) (Sasaki et al., 2000), ion channels (Mori et al., 1993), and many others. In response to a variety of stimuli, CREB is phosphorylated by protein kinases, binds CRE sequences as dimers and activates gene transcription (Lonze and Ginty, 2002). Alternatively, if CREB is already bound to the CRE sequence as a dimer, phosphorylation by these kinases can activate gene transcription (Shaywitz and Greenberg, 1999).

CREB family members

CREB can dimerize not only with itself, but also with CREB-like family members including Activating Transcription Factor-1 (ATF-1) and the cyclic AMP Response Element Modulator protein (CREM) (Lonze and Ginty, 2002). ATF-1 and CREM are

also bZIP transcription factors and can bind CREs (Shaywitz and Greenberg, 1999). The CREM gene encodes the activator isoforms, CREMτ and α, and the repressor forms, S-CREM and inducible cAMP early repressor (ICER) (Lonze and Ginty, 2002). The CREB gene is composed of 11 exons, with the splice variants α and Δ as the most abundant isoforms, and the β isoform, a relatively minor product (Blendy and Maldonado, 1998). The CREB protein consists of a Q1, alpha, KID, Q2/CAD and bZIP domains (Lonze and Ginty, 2002), which will be discussed later in greater detail.

CREB activation

CREB responds to a wide variety of extracellular stimuli such as neuronal activity, neurotransmitters, peptide hormones, and growth factors that activate various different pathways (Shaywitz and Greenberg, 1999). The three most commonly studied pathways for CREB activation include: 1) cAMP/protein kinase A (PKA) (Figure 1.1A), 2) growth factor activation of any of several mitogen-activating protein kinase (MAPK) pathways (Figure 1.1B), and 3) calcium/calmodulin kinase (CaMK), (Figure 1.1C) which all lead to the phosphorylation of CREB and activation of CREB-mediated gene transcription (Lonze and Ginty, 2002). It was first discovered by Gonzalez and Montminy (Gonzalez and Montminy, 1989) that Ser-133 phosphorylation on CREB is essential to its activation.

Upstream of CREB, in response to peptides and neurotransmitters, the first activation pathway involves G protein-coupled receptors (GPCRs) which couple to adenylate cyclases (ACs), thereby increasing levels of cAMP (Gilman, 1987) (Figure 1.1A). PKA

is the main target of cAMP and, upon binding, the catalytic subunits of PKA dissociate from the regulatory subunits enabling phosphorylation of target proteins (Shaywitz and Greenberg, 1999). Activated PKA is then able to phosphorylate Ser-133 on CREB (Gonzalez and Montminy, 1989). Additionally, levels of cAMP can be decreased by phophodiesterases, (PDE) which hydrolyze the molecule (Hagiwara et al., 1992; Wadzinski et al., 1993).

Signaling pathways involved in CREB activation

CREB not only responds to increases in cAMP due to GPCRs, but also to kinases activated by growth factors (Shaywitz and Greenberg, 1999) (Figure 1.1B). Using the c-fos gene (which is normally very responsive to growth factors), with a mutation in the CREB-binding sites, it was found that c-fos induction was greatly reduced in response to growth factors, indicating a role for CREB in this second pathway (Ginty et al., 1994). CREB signaling is known to occur via two pathways in response to growth factors. The first pathway involves growth factors which activate CREB signaling via the ribosomal S6 kinase 2 (RSK2) kinase (Ginty et al., 1994; Xing et al., 1996). RSK2 was later identified as the kinase induced by nerve growth factor (NGF), which is the culmination of an activation pathway involving TrkA signaling. NGF signals via its receptor tyrosine kinase A (TrkA) to guanine-nucleotide exchange factors (GEFs), leading to the activation of Ras (a small G protein), which then activates Raf (a Ser/Thr kinase), which then activates the MAPK kinase (MEK) and its target, ERK, which then activates RSK (Shaywitz and Greenberg, 1999) (see Figure 1.1).

The second pathway involved with growth factor signaling in CREB activation is via p38 MAPK, which is activated by MAP kinase kinase 6 (MKK6) (Han et al., 1996). p38 MAPK activates MAPK-activating protein kinase 2 (MAPKAP-K2) (Rouse et al., 1994) and MAPKAP-K3 (McLaughlin et al., 1996), which have been shown to lead to CREB phosphorylation (Figure 1.1B). In addition, p38 MAPK can also activate mitogen and stress kinase 1 (MSK1), which leads to phosphorylation of CREB (Deak et al., 1998). Therefore, growth factor signaling culminating in CREB phosphorylation can occur via two distinct pathways, the Ras/Raf/MEK/ERK and the MKK6/p38MAPK, with three types of kinases acting on CREB, MAPKAP-K2/3, MSK1 and RSK1-3 (reviewed in (Shaywitz and Greenberg, 1999) (see Figure 1.1B).

The third mechanism involved in CREB activation is via calcium signaling. (Figure 1.1C) L-type calcium channels and N-Methyl-D-Aspartate (NMDA) receptors are activated by various stimuli which then recruit a Ca^{2+} binding protein, calmodulin (CaM), and the CaM kinases (CaMK) I, II and IV, leading to the phosphorylation of CREB (Lonze and Ginty, 2002). CaMKIV has emerged as the CaMK which plays the most important role in phosphorylating Ser-133CREB, leading to activation, (Dash et al., 1991; Tokumitsu et al., 1994), while CaMKII appears to play a repressive role in CREB activation by phosphorylating CREB at Ser142 (Shaywitz and Greenberg, 1999). In addition, the Ras/MAPK/RSK-mediated pathway also responds to increases in calcium, but the dynamics are more slowly developing and longer lasting (West et al., 2001; Wu et al., 2001; Deisseroth and Tsien, 2002).

Cofactors involved in CREB signaling

In addition to promoting dimerization and CRE binding, phosphorylation of CREB at serine 133 is required for gene activation (Shaywitz and Greenberg, 1999). Most importantly, the serine 133 is located within the kinase inducible domain (KID) of CREB, and when phosphorylated allows binding to the KID interaction domain (KIX) of the CREB binding protein (CBP) (Lonze and Ginty, 2002). The CBP is a 265 kDA nuclear protein which functions to recruit and stabilize the RNA polymerase II (Pol II) transcription complex at the TATA box (Shaywitz and Greenberg, 1999). RNA helicase A (RHA) binds to the C/H3 domain of the CBP and mediates the interaction of CBP with Pol II (Nakajima et al., 1997) (Figure 1.2). CBP also has intrinsic histone acetyl transferase (HAT) activity and associates with another HAT containing factor (pCAF). This association alters the chromatin structure and makes DNA more accessible to the transcription machinery (Shaywitz and Greenberg, 1999). Another important region of CREB is the Q2 or CAD domain (Brindle et al., 1993; Quinn, 1993). This domain is necessary for basal CREB activity, but is not involved in signal induced activation (Xing and Quinn, 1994) and also interacts with Pol II which is necessary for transcription (Ferreri et al., 1994; Xing et al., 1995).

Inactivation of CREB

Inactivation of CREB takes place by dephosphorylation of Ser-133 by either protein phosphatase I (PPI) or protein phosphatase 2A (PP2A) (Hagiwara et al., 1992; Wadzinski et al., 1993; Alberts et al., 1994). In addition, activation of NR2B-containing NMDA

glutamate receptors also can act as a CREB shutoff mechanism by actively dephosphorylating CREB (Hardingham et al., 1999). After inactivation, there can be a refractory period in which CREB is no longer able to activate gene transcription, due to downregulation of the catalytic PKA subunit and the induction of ICER (Armstrong et al., 1995; Lamas et al., 1997; Lamas and Sassone-Corsi, 1997). Conversely, dopamine- and cAMP-regulated phosphoprotein -32KD (DARPP-32), which is also phosphorylated by PKA, can inhibit PP-1 thereby prolonging CREB phosphorylation (p-CREB) which prolongs CREB activation (Hemmings et al., 1984; Hemmings et al., 1989).

Gene Targets of CREB

Broadly speaking, a CREB target gene is any gene with one or more functional CRE binding sites in the promoter region. However, many genes which contain CRE-like sites may not be regulated by CREB, which has made it difficult to identify which genes are truly CREB targets in vivo. Over 100 putative CREB target genes have been studied directly, and these play diverse roles in cell function, including basic cell structure and metabolism, as well as complex signal transduction and neurotransmission (Kornhauser et al., 2002; Lonze and Ginty, 2002) (see Table 1).

Specificity of CREB

While these genes play various roles, it is believed that CREB probably responds in a cell-specific and stimulus-specific manner, with coactivators regulating which target genes are induced (Shaywitz and Greenberg, 1999). This is especially noted in the example of c-fos activation, which is an immediate early gene activated by CREB

(Berkowitz et al., 1989). Even when p-CREB activation is prolonged, c-fos expression degrades quickly, peaking within a few minutes of the stimulus and returning to baseline with an hour (Greenberg and Ziff, 1984), suggesting that p-CREB alone is not responsible for the kinetics of gene expression. The action of cofactors, and the general state of activation of chromatin around a particular gene, may explain how cell type affects CREB-mediated gene transcription. For instance, BDNF expression has been shown to be CREB mediated in various cell types (Shieh et al., 1998; Tao et al., 1998), but can be solely activated in certain hippocampal cell types (Patterson et al., 1992) but not others despite CREB activation in most neuron types upon stimulation. This suggests that CREB must be acting with cell type specific transcription factors and coactivators to bring about BDNF expression in only certain cell types (reviewed in (Lonze and Ginty, 2002).

One of the cofactors known to play a role in CREB target gene expression is transducer of regulated CREB activity (TORC2), which associates with CBP/p300. p300, like CBP, is a HAT and increases the recruitment of CBP to a subset of CREB target genes in response to stress (Ravnskjaer et al., 2007). TORC2 itself is not activated in response to stress, however, without it p-CREB is unable to drive CRE-dependent transcription, due to the absence of CBP recruitment to the CRE domain (Ravnskjaer et al., 2007). Another mechanism of CREB specificity may involve the interaction of the KID domain of CREB with the KIX domain of CBP, via the interaction of TORC. TORC appears to play a role in genes that are capable of recruiting CBP independent of KIX (Xu et al., 2007). The

discovery of other cofactors and how they function in regard to different CREB-target genes may elucidate the mechanisms by which CREB activates specific gene targets.

The Role of CREB in Complex Behaviors

CREB in Development

CREB is phosphorylated by many growth factors and neurotrophins which are important to development. Therefore, the role of CREB in development of the nervous system has been extensively studied. Using CREB null mice, it was discovered that CREB and CREB family members are necessary for survival of various neuronal subtypes (Rudolph et al., 1998), an in vivo finding which supported various in vitro experiments (Bonni et al., 1999; Riccio et al., 1999). It is believed that CREB mediates pro-survival effects through B cell lymphoma 2 (Bcl-2) (Riccio et al., 1999) and possibly by inhibiting apoptosis through a Bax-depenedent mechanism (Lonze et al., 2002). However, the development of neurons in the CNS is not dependent on CREB alone, but also other CREB family members. Knockout of both CREB and CREM is perinatal lethal, while knockout of CREB alone affects survival on some genetic backgrounds but not others (Mantamadiotis et al., 2002). In addition, if CREB is deleted postnatally in the CREM null background, various brain regions undergo massive neuronal degeneration (Mantamadiotis et al., 2002). While other CREB members such as CREM may compensate for late embryonic CNS developmental deficits without the presence of CREB, it does not appear that in early development either is required, since the embryo is able to form to the perinatal stage.

Although the survival of neurons may not depend solely on CREB, proper development of axons and dendrites is affected by the absence or inhibition of CREB with decreased growth and projections (Rudolph et al., 1998; Mantamadiotis et al., 2002; Redmond et al., 2002). In addition, several diseases appear to involve CREB-related deficits. Coffin-Lowry syndrome, characterized by mild retardation and physical abnormalities is caused by a mutation in the RSK-2 gene, which encodes one of the kinases involved in CREB signaling (Trivier et al., 1996). Rubinstein-Taybi syndrome, a similar disease also characterized by mental retardation and physical abnormalities such as broad thumbs and keloid development, is caused by a loss-of-function mutation in CBP (Petrij et al., 1995). CBP dysfunction is also implicated in some forms of Huntington's disease (HD) with the mutant huntingtin protein aggregating with CBP, thereby affecting CREB-CBP dependent transcription (Dawson and Ginty, 2002). Therefore, not only is CREB signaling important in the survival and development of the CNS, but its dysfunction can have various mental and physical affects.

CREB in Learning and Memory

Early studies showed that cAMP plays a role in facilitating short- and long-term memories in *Aplysia* (reviewed in (Kandel, 2001), and later it was found that CREB plays a role in formation of long-term memory (Dash et al., 1990). This study showed the cAMP-CREB pathway is activated in a learned behavior and that CREB is necessary for the induction of long-term facilitation (LTF) (Kandel, 2001). In addition, genetic screens for *Drosophila* mutants with deficits in learning and memory yielded two genes related to

CREB-mediated transcription, those encoding an adenylate cyclase and phosphodiesterase (Waddell and Quinn, 2001). Also in *Drosophila*, CREB repressors and enhancers were found to alter long-term memory processes in olfaction tasks (Yin et al., 1994; Yin et al., 1995).

In addition, CREB has been implicated in rodent models of learning and memory with several studies showing that CREB-related transcription can alter learning and memory processes and/or long term potentiation (LTP). Mice with mutations in adenylate cyclase (AC1) and those with a transgene leading to PKA inhibition both caused deficits in spatial learning and late LTP (L-LTP) (Wu et al., 1995; Abel et al., 1997). Also in hippocampus slice culture, cAMP antagonists blocked L-LTP (Frey et al., 1993) while cAMP agonists induced L-LTP in the absence of a tetanus stimulus (Huang et al., 1994). Infusions of CREB antisense oligonucleotides into the hippocampus caused spatial learning deficits in rats (Guzowski and McGaugh, 1997). Mutant mice which lack the α and Δ isoforms of CREB also show deficits in spatial memory and LTP in the hippocampus (Bourtchuladze et al., 1994).

CREB in Addiction

Long-term changes in neuronal signaling after drugs of abuse have led to the study of the role CREB plays in addiction. CREB was first discovered to play a role in opiate dependence by modulation of the locus ceruleus (LC), a major noradrenergic nucleus located in the brainstem (Aghajanian, 1978). Acute opiate exposure decreases CREB signaling and neuronal firing (Duman et al., 1988; Beitner et al., 1989; Guitart and

Nestler, 1989; Guitart et al., 1992). However, chronic exposure increases CREB related signaling proteins and returns firing to normal (Nestler et al., 1994; Widnell et al., 1994). Under withdrawal conditions, firing is upregulated and the withdrawal state can be affected behaviorally by CREB manipulation in the LC (Lane-Ladd et al., 1997; Han et al., 2006). The CREBαΔ mutant mice, which make neither the α nor Δ splice variants of CREB, but retain the β CREB isoform, also show decreased opiate tolerance and withdrawal responses (Maldonado et al., 1996; Blendy and Maldonado, 1998) and appear to be insensitive to the reinforcing properties of morphine (Walters and Blendy, 2001). Conversely, these mutants are hypersensitive to positive reinforcing effects of cocaine (Walters and Blendy, 2001) .

The LC is not the only structure in which opiates affect CREB signaling. The nucleus accumbens (NAc) and ventral tegmental area (VTA) are also important structures influenced by drug administration. Chronic administration of opiates or amphetamine increases CREB activity in the NAc (Turgeon et al., 1997; Shaw-Lutchman et al., 2002; Shaw-Lutchman et al., 2003) and morphine withdrawal produces even more robust CREB changes (Shaw-Lutchman et al., 2002).

PKA modulation in the NAc has also been shown to affect self-administration of cocaine in rats. Decreasing PKA signaling, thereby decreasing CREB phosphorylation, leads to decreased lever pressing and an increase in the interval between self-injections, which is indicative of enhanced reward, while increased PKA signaling had the opposite effects (Self et al., 1998). Conversely, decreasing levels of Gi/Go proteins that inhibit cAMP

levels (thereby increasing cAMP activity) in the NAc produces tolerance-like increases in cocaine and heroin self-administration (Self et al., 1994). In the conditioned place preference paradigm (CPP), direct CREB manipulation in the NAc, via use of viral-mediated gene transfer, alters the way in which animals respond to the rewarding effects of cocaine and morphine; an increase in CREB decreases the rewarding properties of the drugs and a decrease in CREB increases the rewarding properties (Carlezon et al., 1998; Barrot et al., 2002).

CREB levels are increased in the VTA in response to chronic morphine and cocaine administration (Terwilliger et al., 1991). However, in response to morphine withdrawal, CREB activity is decreased mainly in the lateral component of the VTA (Shaw-Lutchman et al., 2002). The effects of CREB overexpression in the VTA depend on the anatomical location of the virus, with rostral overexpression of CREB leading to increased drug reward of cocaine and morphine and caudal overexpression of CREB having the opposite effect (Olson et al., 2005). These studies underscore the diverse anatomical subdivisions of the VTA and the role these subdivisions play in addiction-related behaviors.

In addition to the role of CREB in the LC, NAc and VTA, there are several other brain areas in which CREB has been implicated in drug addiction. These include the amygdala (Jentsch et al., 2002; Alleweireldt et al., 2006), hippocampus (Duman et al., 2001; Carlezon et al., 2005) and prefrontal cortex (Everitt and Robbins, 2000; Ferrer-Alcon et al., 2004).

CREB in Antidepressant Action and Depression-like Behavior

The regulation of CREB by antidepressants is dependent not only on the type of antidepressant but also on the brain region and the methods used (Blendy, 2006). The hippocampus shows an increase in CREB both at the mRNA and protein level, as well as an increase in the activity of CREB, in response to chronic antidepressant administration (Nibuya et al., 1996; Thome et al., 2000). The frontal cortex also shows increased phosphorylation of CREB after antidepressant treatment (Laifenfeld et al., 2005), although this study did not find an increase in p-CREB in the hippocampus. However, the methods differed with respect to time after the last administration compared to other studies. CREB binding was also found to be increased with administration of fluoxetine (a standard serotonin-selective reuptake inhibitor antidepressant) in the frontal cortex (Frechilla et al., 1998) as well as the hippocampus (Nibuya et al., 1996; Frechilla et al., 1998). Using a CRE-LacZ reporter mouse line, CRE activity was found to be increased with chronic fluoxetine in the cortex, amygdala, hypothalamus hippocampus (Thome et al., 2000). Chronic exposure to tranylcypromine, a monoamine oxidase inhibitor antidepressant, produced similar effects.

Not only do antidepressants increase CREB expression in the hippocampus, but overexpression of CREB in the dentate gyrus (DG) of the hippocampus can alone produce antidepressant-like effects. Using the forced swim test (FST or Porsolt swim test) (Porsolt et al., 1978) immobility time (the time in which the animal is not struggling to escape, a measure of despair) was reduced in animals with CREB overexpression in the DG, but not CA1 or prefrontal cortex regions (Chen et al., 2001). Similarly, CREB

overexpression in the DG decreased the number of escape failures (failure to escape an electric shock), in the learned helplessness model (Chen et al., 2001). It must be noted however that CREB in the ventral striatum (the NAc and the basolateral amygdala (BLA) has the opposite effect. CREB overexpression in the BLA produced pro-depressive like effects in the learned helplessness model (Wallace et al., 2004), while overexpression in the NAc showed pro-depressive like effects in the FST with increases in immobility time and decreased time to become immobile (Pliakas et al., 2001). Conversely, overexpression of mCREB (a mutant form of CREB which lacks serine 133 and functions as a dominant negative antagonist) in the NAc of rats has antidepressive-like effects in the learned helplessness model (Newton et al., 2002) and FST (Pliakas et al., 2001). Experiments using mCREB overexpressing mice or CREB deficient mice also demonstrate antidepressant like effects in learned helplessness, FST and the tail suspension test (TST) (Conti et al., 2002). However, antidepressant drugs still have effects on these CREB-deficient mice (Conti et al., 2002), suggesting CREBαΔ is not required for antidepressant action.

In addition to the aforementioned depression models, sucrose preference and sucrose intake are measures of natural reward-related behavior, which are sensitive to stress paradigms and antidepressant treatment (Willner et al., 1987; Sampson et al., 1992). CREB overexpression in the NAc decreases sucrose preference, while mCREB overexpression increases it (Barrot et al., 2002). It is interesting to note, however that not only positive rewarding stimuli but also negative aversive stimuli are affected by CREB overexpression. Overexpression of CREB in the NAc decreases the rewarding response

to morphine as measured by CPP, but also decreases the aversive responses to foot shock (Barrot et al., 2002). Conversely, mCREB overexpression increases responses to both rewarding stimuli, and to aversive stimuli such as foot shock (Barrot et al., 2002). These studies indicate a role for CREB in the NAc in mediating emotional responsiveness not only to positive but also to negative external stimuli.

DEPRESSION

The prevalence of depression is 10-30% in women and 7-15% in men, depending on the diagnostic criteria used, and is considered to be one of the most burdensome diseases in terms of loss in productivity and strain on society worldwide (Nestler et al., 2002a; Blendy, 2006). Depression is characterized by a loss of interest in pleasurable activities, defined as anhedonia, and also presents itself with disturbances in sleep, appetite and sexual libido. In addition, feelings of guilt, hopelessness and despair can be symptoms (American Psychiatric Association 2000). Both anxiety disorders and drug abuse have high comorbidity with depression (Stein, 2001; Schuckit, 2006).

Animal Models of Depression

Animal models of depression have been beneficial in shedding light on the mechanisms of depression, however, they are limited and do not always represent all facets of the human condition (McKinney, 1984; Nestler et al., 2002b; Berton and Nestler, 2006; Duman and Monteggia, 2006). Tests for measuring antidepressant efficacy in animals often show response to acute antidepressant drugs (McKinney, 1984; Dalvi and Lucki,

1999), whereas in humans 3-4 weeks of chronic drug administration is required. Such rodent tests for antidepressant efficacy include the FST (Porsolt et al., 1978), learned helplessness test (Petty and Sherman, 1979; Sherman et al., 1979) and TST (Chermat et al., 1986; Thierry et al., 1986) mentioned earlier. Despite their limitations, these tests are very useful in predicting antidepressant efficacy. However, they do not always work well for testing an animal for depressive-like phenotypes. While the FST may show the efficacy of fluoxetine in animals, in behavioral paradigms which produce depressive-like phenotypes (such as chronic unpredictable stress, discussed in greater detail below) the "depressed" animals may not always perform with the predicted "depressed" like characteristics in the FST or TST (increased time immobile and decreased time to become immobile) (Mineur et al., 2006). This limits these tests for examining depression-like phenotypes. Other tests for antidepressant efficacy that do require chronic administration to exhibit its effects are novelty suppressed feeding (Bodnoff et al., 1988) and aggressor interaction tests after social defeat (Berton et al., 2006).

Active Stress Models of Depression

Of the behavioral assays that reliably produce depressive-like phenotypes in rodents, the most common effect on the animals is active stress. For example, one common paradigm is chronic unpredictable stress (CUS), in which an animal is subjected to different types of stress over a 5-7 day time period including social stress (being put in a cage with unfamiliar animals), foot shock, restraint stress, cold forced swim, etc. (Dalvi and Lucki, 1999; Barrot et al., 2002; Duman and Monteggia, 2006). These animals show "depressed-like" phenotypes in various behavioral tests (most commonly in sucrose

drinking), as well as endocrine changes noted in depression, such as activation of the hypothalamic-pituitary-adrenal (HPA) axis (Dalvi and Lucki, 1999; Barrot et al., 2002; Duman and Monteggia, 2006). A challenge with CUS is that the behavioral phenotypes have been difficult to replicate among several laboratories. Another active stress model, learned helplessness, induces depression-like symptoms by retaining an animal on one side of a cage with a closed door and an electric grid (Sherman et al., 1979). When the electric shock is given, the animal finds it cannot escape and after several exposures the animal will stop attempting escape even when the door is opened. A control animal, by contrast, has not learned that it is helpless, and when a shock is presented with an open door, it immediately escapes. Antidepressant administration, acute or subchronic, acts to reverse this learned helplessness phenotype (Petty and Sherman, 1979).

Physical Manipulations in Depression Models

Other models of depression involve physical rather than behavioral manipulations to bring about depressive-like phenotypes. These include olfactory bulbectomy, in which an animal's olfactory bulbs are severed thereby depriving it of olfaction cues (Wang et al., 2007). Systemic injections of lipopolysaccharides (LPS) induces an immune response in an animal and also leads to depression-like phenotypes (Yirmiya, 1996). Both of these paradigms are sensitive to antidepressant administration in reversing depressive-like phenotypes (Yirmiya, 1996; Wang et al., 2007). However, concerns about these models focus on their lack of relevance to human depression, which do not involve abnormalities in olfactory mechanisms or acute inflammatory responses.

SOCIAL ISOLATION IN ANIMAL MODELS

Although all of the previously mentioned paradigms induce depressive-like phenotypes, they are based on exposure to active stressors and external stimuli or physical perturbations. While traumatic stress is associated with a significant percentage of human depression cases, another significant feature of depression in humans is oftentimes loneliness, isolation and neglect (Blazer and Hybels, 2005; Heinrich and Gullone, 2006; Mahon et al., 2006). A relevant model to this type of depression is the absence of physical and emotional stimuli, which can be termed social isolation. It also should be noted that social isolation can exacerbate the symptoms of active stress paradigms such as social defeat, and social rehousing can even mitigate the effects of social defeat (de Jong et al., 2005). In addition, social isolation can also further impact the differences observed between genetically "depressed" rodent models such as the Flinders sensitive line (FSL). In comparison to group housed FSL and their control "non-depressed" counterparts, the isolated FSL animals have even greater decreases in levels of D2 receptors, but not D1 in brain reward regions (Bjornebekk et al., 2007).

Social isolation has been studied in many species, among them *Drosophila* (Ehrman, 1990), angelfish (Gomez-Laplaza and Morgan, 1991), songbirds (Leitner and Catchpole, 2007), zebrafinch (Lauay et al., 2005), domestic fowl chicks (Feltenstein et al., 2003), prairie voles (Grippo et al., 2007; Ruscio et al., 2007), hamsters (Crawley, 1985), gerbils (Starkey et al., 2007), tree shrews (Fischer et al., 1985), degus (Gruss et al., 2006), dairy cows (Herskin et al., 2007), chimpanzees (Reimers et al., 2007) but most extensively in

rodents (Hall, 1998). A great deal of attention has been given to the effect of early life isolation, such maternal separation, on psychopathology in later adulthood. However, in humans, psychopathology such as depression and anxiety disorders, is also seen after adulthood social isolation, especially among the elderly (Owen, 2007), prison inmates (Tanay, 1973; Dorman et al., 1993), widows/widowers (Costello and Kendrick, 2000; Chou et al., 2006), and aspects of these disorders can be modeled by animal models of adulthood social isolation.

Rodent Models of Social Isolation

In the rodent models, the time period in which the isolation takes place–as pups, post-weaning juveniles or adults–produces different phenotypes in behavior (Hall, 1998). Isolation as pups is referred to as maternal deprivation, or maternal isolation, and acute studies focus on the neurochemical, metabolic and behavioral changes of the animals while under maternal deprivation conditions. Chronic effects of maternal deprivation are less commonly studied, but investigate the changes in adulthood after deprivation as pups. This paradigm differs from post-weaning, juvenile social isolation, also referred to as isolation-rearing. These animals are raised normally with their mothers, however, upon weaning-age, are isolated until adulthood. This model differs from the maternal deprivation model in the presence of constant versus variable maternal influences during development (Hall, 1998). Additionally, the isolation-rearing model lacks the critical "play period" seen during adolescence (Einon et al., 1978).

While the chronic effects of maternal deprivation are limited, studies have shown adult rats maternally deprived have increased weight, decreased corticosterone responses and increases in glucocorticoid receptors for females and decreases in males (Ogawa et al., 1994; Matthews et al., 1996; Sutanto et al., 1996). Although behavior studies are limited, decreases in sensitivity to d-amphetamine have also been reported (Matthews et al., 1996). Isolation reared rats, by comparison, have shown increased responses to stimulants and consistent reports in hyperactivity (Gentsch et al., 1981; Jones et al., 1989), decreased weight and increased adrenal function (Gamallo et al., 1986). Additionally, increases in basal and corticosterone response to stress (Gamallo et al., 1986; Holson et al., 1991), as well as increased dopamine functioning have all been shown (Holson et al., 1991). The isolated-reared animals also have a deficit in paired-pulse inhibition, and has been proposed as a potential model for schizophrenia (Geyer et al., 1993).

Adulthood Social Isolation in Rodents

The third isolation model, adult social isolation, or isolation housing, also called the social deprivation model, focuses on animals in adulthood. These animals are raised under normal maternal conditions, and receive normal social cues as juveniles during the critical play period, but once reaching adulthood (250-300g or 8-10 weeks of age) are deprived of social contact by single housing. This model conserves developmental processes and focuses on the effects of social deprivation per se in otherwise normal animals. To date, most studies have focused on maternal deprivation and isolation-rearing, however, there have been many instrumental findings on the behavior, neurochemistry and physiology induced by adulthood social isolation paradigms.

A challenge in the field is that, although all adulthood social isolation studies involve rodents beyond juvenile age, the amount of time in which they are isolated before testing often varies. Chronic social isolation (typically greater than 4 weeks) differs from acute social isolation (greater than 24 hours but less than a week) in the behavioral and physiological changes that are induced, but some aspects are the same (Hall, 1998). For instance, acute and chronic isolation both cause an increase in anxiety-like behaviors and increase alcohol consumption (Parker and Radow, 1974; Wolffgramm and Heyne, 1991; Maisonnette et al., 1993; Ahmed et al., 1995). However, acute isolation increases social interactions and investigations of unfamiliar animals (Niesink and van Ree, 1983), while chronic isolation can cause aggressive behaviors toward unfamiliar animals (Wolffgramm and Heyne, 1991).

The effects of chronic social isolation will be the main focus presented in this dissertation, along with the effects on behavioral phenotypes and neurochemical changes. This focus is justified by the fact that: 1) adulthood social isolation has been relatively understudied, compared with early life deprivation, 2) virtually nothing is known about its biological underpinnings, and 3) this form of isolation seems particularly relevant to common subtypes of human depression which are not well represented by the large literature involving active stress or early deprivation models.

Rodent Behaviors in Adulthood Social Isolation

As previously mentioned, anxiety is a prominent behavioral phenotype of chronic social isolation, with increased anxiety observed on elevated plus maze (Jankowska et al., 1991; Vasar et al., 1993) and longer periods to enter novel environments (Ahmed et al., 1995). It was also found that rehousing can reverse this anxiety phenotype, as well as acute midazolam and chronic gepirone (Maisonnette et al., 1993). Midazolam is a benzodiazepine derivative, while gepirone, an analog of buspirone, is a 5HT1A partial agonist which has been shown to have putative anxiolytic action in humans (Taylor and Moon, 1991). Benzodiazepines such as alprazolam and diazepam, are GABA-A agonists which also reverse anxiety behavior (Whiting, 2006).

Although in isolated-reared rats, increased locomotor behavior is consistently observed, in adult animals the locomotor effect is not as clear (Hall, 1998). Although several studies found no differences in baseline locomotor behavior, increases in locomotion are found in response to stimulants and sensitization occurs with d-amphetamine compared to social controls (Ahmed et al., 1995). Like amphetamine, there is also an increased locomotor response seen in response to morphine (Deroche et al., 1994). Increased activity in isolated animals is observed in the presence of unfamiliar socially housed rats, with repeated approach and withdrawal types of behaviors (Hol and Spruijt, 1992). However, these behaviors may also be reflective of an anxiety-like phenotype, as they are eliminated with α-melanocyte-stimulating hormone (αMSH) or adrenocorticotropic hormone (ACTH) into the central amygdala (Hol and Spruijt, 1992), manipulations known to oppose anxiety responses.

Other behaviors affected by adulthood social isolation include decreased performance on the Morris Water maze, a measure of spatial learning, which is reversed by rehousing (Hall, 1998). In addition, isolated animals allowed unlimited access to running wheels run about the same as the double housed animals with unlimited access, however, the isolated animals have a decrease in hippocampal neurogenesis while the grouped housed runners have an increase (Stranahan et al., 2006). This study also found that it takes 48 days until the increased neurogenesis effects are seen in the isolated animals whereas in the grouped housed animals they are seen within a week (Stranahan et al., 2006).

There have been inconsistencies reported with behaviors involving drug-related reward behavior. While no differences have been found with intra-venous (i.v.) self-administration of cocaine, isolated animals actually learned to acquire self-administration of heroin at a faster rate than social controls (Bozarth et al., 1989). Morphine also produces varied results, with no differences observed with CPP (Schenk et al., 1985) but an increase in oral morphine consumption (Alexander et al., 1978). As mentioned previously, several reports consistently describe increased ethanol consumption with social isolation (Parker and Radow, 1974; Ellison, 1981; Schenk et al., 1990; Ehlers et al., 2007). It is speculated that the increase observed with alcohol intake is due to an increase in anxiety, and this increased intake may not translate to other drugs of abuse (Hall, 1998). However, with respect to ethanol intake as a response to anxiety, one report showed isolated animals had no differences in consumption of diazepam solutions (Wolffgramm and Heyne, 1991). Although to date no reports have studied sucrose consumption in adulthood isolation in rats, no differences were observed in isolation-

reared rats (Hall et al., 1997). However, social isolation in adult prairie voles does lead to a decrease in sucrose preference (Grippo et al., 2007). Some studies indicate socially isolated animals may have an increase to emotional responses, including to stress related situations (Greco et al., 1990, 1992). However, other reports indicate increased pain tolerance with increased latency to tail flick (Roske et al., 1994).

Changes in Neurochemistry with Social Isolation

Conflicting reports also exist on the neuroendocrine changes induced with social isolation, with basal corticosterone reported as increased (Lovely et al., 1972; Greco et al., 1990, 1992), decreased (Barrett and Stockham, 1963; Miachon et al., 1993) and no change (Plaut and Grota, 1971; Giralt and Armario, 1989; Ehlers et al., 1993). In response to stress, an increase is found in corticosterone levels (Plaut and Grota, 1971), although other investigators report no change (Lovely et al., 1972; Giralt and Armario, 1989). Related to the HPA axis, increases are also reported in ACTH levels induced by chronic social isolation (Miachon et al., 1993), although these changes are not seen with only two weeks of isolation (Giralt and Armario, 1989). Interestingly, in the study investigating neurogenesis in isolated animals (Stranahan et al., 2006), socially isolated runners and control group-housed runners showed an increase in glucocorticoid levels over the group-housed non-runners at the onset of their active cycle. However, 4 hours later both non-runners and runners of group-housed animals had decreased levels of corticosterone in comparison to both isolated conditions. Also during this phase, the group-housed runners did not show stress-induced increases of corticosterone, while increases occurred in all other groups. Overall, the authors hypothesize that the isolated

animals have an increased exposure to corticosterone levels over a 24-hour period, especially in response to stress. They provide evidence for this hypothesis by showing adrenalectomy in the isolated runner group increases neurogenesis in conditions which otherwise previously decreased neurogenesis.

In addition to hormones involved with the HPA axis, dopamine changes are also reported. Increased dopamine utilization in the frontal cortex was reported in response to mild footshock, as well as decreased basal DOPAC/dopamine ratios in the frontal cortex, with increased ratios in the nucleus accumbens and dorsal striatum (Blanc et al., 1980), as well as increases in dopamine metabolism in the hypothalamus (Gambardella et al., 1994). In addition, increased activity of tyrosine hydroxylase (TH),the rate-limiting enzyme in dopamine synthesis, was reported in the striatum and midbrain (Segal et al., 1973).

Overall there appears to be decreased serotonergic function in socially isolated rats, with decreases also observed in basal serotonergic metabolism (Hall, 1998). Changes in tryptophan hydroxylase (TPH) activity, which is rate-limiting for serotonin synthesis, are observed in the septum (Segal et al., 1973) and decreased affinity for the 5HT-1A receptor was found in frontal cortex, striatum, hippocampus and hypothalamus (Petkov et al., 1987). However, no change was found in the density of 5HT-1D receptors in the dorsal raphe (Ahmed et al., 1995). In comparison, increased binding was found with socially isolated male mice (6 weeks) in CA1, septum, amygdala, periaquadal gray (PAG) and various cortical regions of 5HT-1AR (Schiller et al., 2006), although this

same group previously reported decreased 5HT-1A and 5HT-2A binding in male mice isolated for 4 weeks in similar regions (Schiller et al., 2003).

Decreases in norepinephrine (NE) have been reported in hippocampus tissue, with increases in NE synthesis in hippocampus, cortex and cerebellum (Miachon et al., 1993), as well as an increase in basal NE turnover (Stolk et al., 1974). There have also been reports in regards to GABAergic function, with increased benzodiazepine binding observed in the hippocampus and frontal cortex (Miachon et al., 1990), however, others did not observe this change (Vasar et al., 1993). Other changes of possible interest include decreases in preproenkephalin mRNA levels in the NAc and dorsal striatum (Angulo et al., 1991) as well as reports of increases in preproenkephalin in the hypothalamus (Iglesias et al., 1992). The changes noted in the hypothalamus were specific to a period of 2-3 weeks of isolation and were not seen with 6 weeks of isolation (Iglesias et al., 1992). Changes in circadian rhythms induced by social isolation are of particular notice. For example, altered electroencephalograms (EEGs) have been reported, reflecting changes in sleep and eating patterns in isolated animals (Ehlers et al., 1993) as well as circadian shifts in serotonin, tryptophan and 5-HIAA levels, which are reversed by imipramine (a tricyclic antidepressant) administration (Greco et al., 1990).

Despite these various studies, the literature is highly unsatisfying because of numerous inconsistencies in the experimental findings reported as well as the virtually complete lack of experimental evidence which directly relates a biochemical finding to correlated behavioral abnormalities. The various studies utilize different experimental methods,

with different strains of rodents, different ages at which the animals were isolated, different lengths of isolation periods, and different techniques by which the results were obtained. The effects of dominance or submissiveness in the group housed control animals also produces varying effects in regards to a stressor. Although not as apparent as nonhuman primates, dominant patterns are also noted in rodent models (Bartolomucci et al., 2005). One study observing isolated primates noted, after rehousing, levels of D2 dopamine receptors were increased only in dominant males but stayed the same in subordinate males (Morgan et al., 2002). They also showed that only the subordinate males found cocaine rewarding in a self-administration paradigm, suggesting that subordinate males undergoing an environmental stress are predisposed to drug abuse behavior. Regarding differences observed in corticosterone levels, the time in which the samples are taken could greatly affect results. Depending on the time in which samples are taken, at the onset of active phase or 4 hours later, results varied in the corticosterone differences between isolated or group housed animals, and the differences in levels after stressful stimuli (Stranahan et al., 2006). Overall, it can be concluded nevertheless that adulthood social isolation is a model which induces anxiety-like behavior and modulations in reward behavior (Hall, 1998). In addition, social isolation induces a wide variety of changes in neurophysiology, with differing results depending on the methods used. Moreover, it can be concluded that little is known about the neurobiological mechanisms underlying these behavioral abnormalities.

Social Isolation, Anxiety and Natural Reward Behavior

The observations that social isolation produces changes in anxiety-like and reward-related behavior has suggested that social isolation may also modulate the motivation for natural rewards, such as food and sex. And, as mentioned earlier, deficits in natural reward behavior are a hallmark of human depression. It is this overlap of anxiety, depression and reward-related behaviors, and the interface between them, that is of interest to motivational research. Sexual behavior represents one of the strongest natural rewards (Agmo et al., 2004), and the investigation of how depression and anxiety affect this natural reward are limited. Previous work has found social isolation producing changes in sexual behavior (Brotto et al., 1998), but the molecular mechanisms have never been elucidated. Although the medial preoptic area (MPOA) of hypothalamus is the region most associated with sexual behavior (Hull and Dominguez, 2007), it does receive projections from the NAc (Simerly and Swanson, 1986). In addition, whereas the MPOA mediates the consummatory aspects of sexual behavior, the mesocortical dopamine projections from the VTA to the NAc are known to be important for the appetitive and reward-related aspects of sexual behavior (Everitt, 1990; Hull and Dominguez, 2007). Also, following sexual experience or exposure, various regions of the limbic system show induction of c-Fos and related proteins (Balfour et al., 2004).

ORGANIZING HYPOTHESIS OF THIS DISSERTATION

The already established role of CREB in the spectrum of emotional processes provides a foundation to base the hypothesis of CREB playing a role in social isolation and its effects on anxiety and reward-related behaviors. The work presented in this dissertation

investigates the behavioral phenotypes produced by social isolation in adult Sprague-Dawley male rats and the contribution of CREB in the NAc to these phenotypes. These studies provide evidence that among the many relevant target genes for CREB in regulating the phenotypes, are altered expression of K^+ channels in the NAc. These channels are particularly interesting targets, since they would be expected to alter the excitability of NAc neurons. Indeed, direct evidence is provided connecting CREB, and regulation of specific K^+ channels to certain aspects of the social isolation behavioral phenotype.

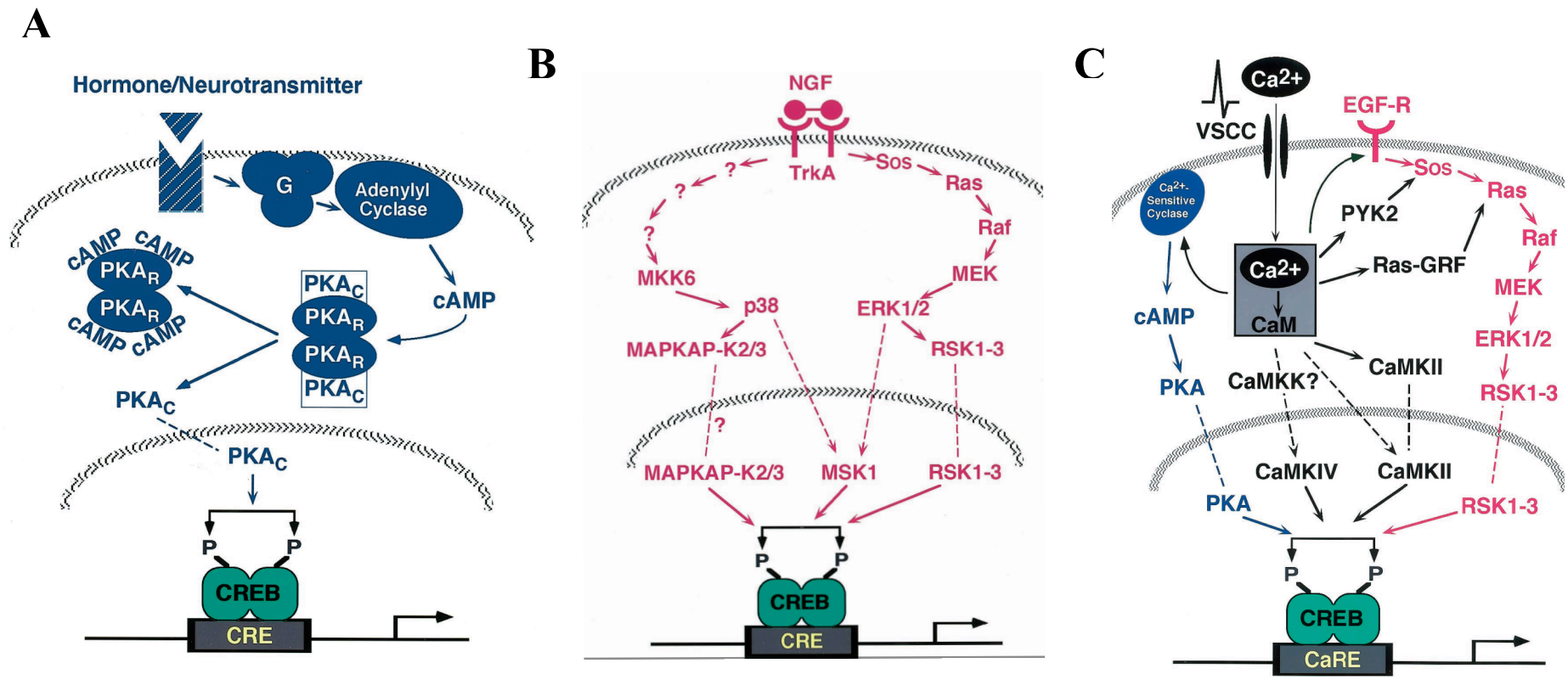
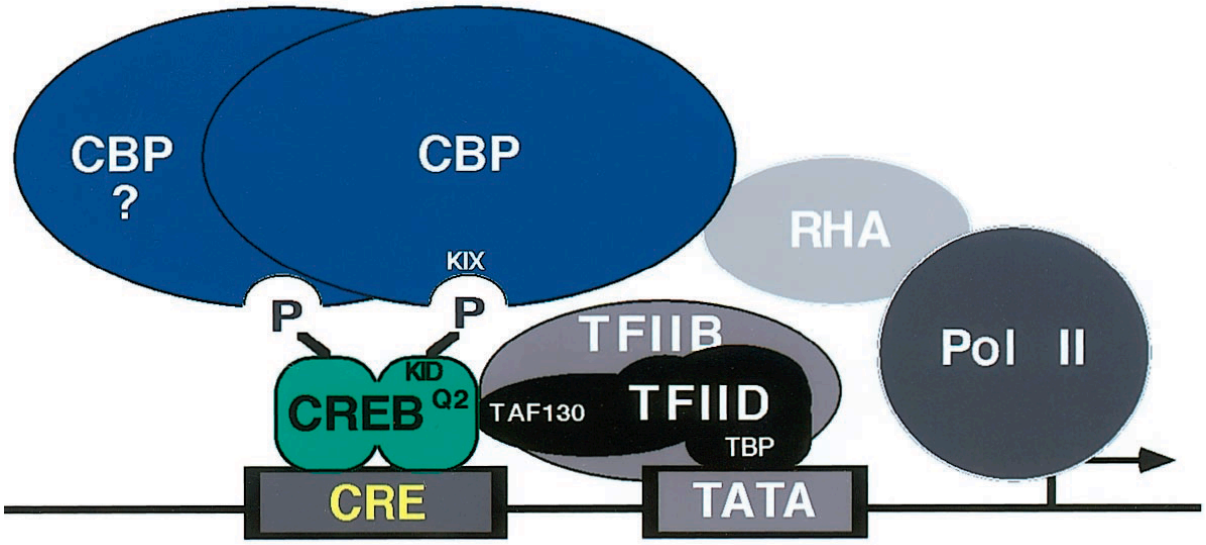


Figure 1.1: CREB signaling in the nucleus is mediated by several pathways. **A)** cAMP is increased in response to neurotransmitters and hormones which then activate AC via G-proteins, leading to the the activation of PKA. **B)** TrkA signaling phosphorlates CREB via two pathways: the Ras/Raf/MEK/Erk and the p38/MAPKAP-K2/3. **C)** CaMKs are activated in response to elevated levels in Ca2+, which can also affect the other signaling pathways of CREB. Activation of PKA, MAPKAP-K2/3, MSK1, RSK1-3 and CAMKII and IV all phosphorylate CREB at Ser 133, driving CRE-mediated gene transcription.



Shaywitz and Greenberg, 1999

Figure 1.2: CREB binding protein (CBP) KIX domain interacts with CREB KID domain, allowing binding to the TATA box downstream of CRE sequences. TFIID and TFIIB are basal transcription factors which interact with CREB via the Q2 domain of CREB. RHA (RNA helicase A) associates CBP with polymerase II (Pol II) which mediates stabilization and transcription of CRE genes. It is unclear whether CBP binds as dimers to CREB.

Table 1: Examples of CRE-target genes and genes with CRE Sequences

Neurotransmission and Peptides

Acetylcholinesterase, α1-GABAA receptor, β1and2 adrenergic receptors, calcitonin gene-related peptide, cholecystokinin (CCK), Corticotropin-releasing hormone, Dopamine β-hydroxylase, enkephalin, galanin, neurotensin, norepinephrine transporter (NET), prodynorphin, proenkephalin, somatostatin, substance P receptor, synapsin I, Vasopressin,
Vesicular monoamine transporter (VMAT), VGF

Growth Factors/Hormones

Brain Derived Neurotrophic Factor (BDNF), Insulin-like growth factor I (IGF-I), Leptin,
Transforming growth factor-β2 (TGF-β2), TrkB

Channels/Transporters

Glucose transporter 2 (GLUT2), Kv3.1 K+ channel, Na+/K+-ATPase α

Cellular Metabolism

Bcl-2, Cyclooxygenase-2 (COX-2), Cytochrome c, Glutamine synthetase (GS), Neuron
Specific enolase (NSE), superoxide dismutase (SOD2), Uncoupling protein 1-3 (UCP1-3)

Transcription

Activating Transcritpion Factor-3 (ATF-3), c-fos, CREB, ICER, JunD, mPer1-2

Modified from Lonze and Ginty, 2002

CHAPTER TWO

REGULATION OF ANXIETY AND INITIATION OF SEXUAL BEHAVIOR BY CREB IN THE NUCLEUS ACCUMBENS

Summary

Sexual deficits and other behavioral disturbances such as anxiety-like behaviors can be observed in animals that have undergone social isolation, especially in species having important social interactions. Using a model of social isolation in adult rats, an increase in anxiety-like and deficits in both the latency to initiate sexual behavior and the latency to ejaculate are observed. Using transgenic cAMP response element (CRE)-LacZ reporter mice, it is also shown that social isolation reduces CRE-dependent transcription within the NAc. This decrease in CRE-dependent transcription can be mimicked in nonisolated animals by local viral gene transfer of a dominant negative mutant of CRE-binding protein (CREB). Previously, it has been shown that this manipulation increases anxiety-like behavior. Here, it is shown that this manipulation also impairs initiation of sexual behavior in non-isolated animals, a deficit that can be corrected by anxiolytic drug treatment. This local reduction in CREB activity, however, has no influence on ejaculation parameters. The viral transgenic approach was also used to overexpress CREB in the NAc of isolated animals, and this local increase in CREB activity completely rescued the anxiety phenotype of the isolated animals as well as the deficit in initiating sexual behavior, but failed to rescue the deficit in ejaculation. This data suggests a role for the NAc in anxiety responses and in specific aspects of sexual

behavior. These results also provide insight into the molecular mechanisms by which social interactions affect brain plasticity and behavior.

Introduction

Within the central nervous system, changes in the activity of the transcription factor cAMP response element (CRE)-binding protein, (CREB) (Lonze and Ginty, 2002) have been related to many adaptive processes, such as learning and memory (Bourtchuladze et al., 1994; Yin and Tully, 1996; Impey et al., 1998; Mayford and Kandel, 1999; Silva and Murphy, 1999; Josselyn et al., 2001), antidepressant effects (Nibuya et al., 1996; Chen et al., 2001; Nestler et al., 2002a; Newton et al., 2002), and drug addiction (Konradi et al., 1994; Maldonado et al., 1996; Carlezon et al., 1998; Misra et al., 2001; Nestler, 2001; Pliakas et al., 2001; Walters and Blendy, 2001; Barrot et al., 2002; Shaw-Lutchman et al., 2002; Brunzell et al., 2003; McClung and Nestler, 2003; Shaw-Lutchman et al., 2003). Such changes have been identified in several discrete brain areas, among them the NAc, a forebrain structure critical for reward and motivation (Mogenson et al., 1980; Robbins and Everitt, 1996; Wise, 1998; Berke and Hyman, 2000; Koob and Le Moal, 2001; Kelley, 2004). Exposure to several forms of stress (Pliakas et al., 2001; Barrot et al., 2002; Shaw-Lutchman et al., 2002) or to drugs of abuse, either psychostimulants (Shaw-Lutchman et al., 2003) or opiates (Barrot et al., 2002; Shaw-Lutchman et al., 2002) increases CRE-dependent transcription in the NAc. The use of viral-mediated gene transfer to manipulate CREB activity specifically within this brain region demonstrated that local CREB overexpression reduces the rewarding effects of psychostimulants and

opiates (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002) which indicates that activation of this transcription factor may counteract adaptations that intensify drug reward. Previous findings with stress responses suggested that, more generally, increased CREB activity in the NAc reduces an animal's behavioral responses to a wide range of emotional stimuli, whether rewarding, anxiogenic or nociceptive (Barrot et al., 2002).

Although a causal link has not yet been established with certainty, experimental data strongly suggest that not only the activation of CREB but also its inhibition could be instrumental in some adaptive mechanisms. For example, chronic alcohol or nicotine treatment can decrease levels of phosphorylated CREB, the active form of the protein in the NAc (Misra et al., 2001; Brunzell et al., 2003; Pluzarev and Pandey, 2004). Moreover, viral-mediated expression of a dominant mutant of CREB revealed that local inhibition of CREB activity in the NAc potentiates behavioral responses to emotional stimuli, such as the rewarding effects of drugs of abuse (Carlezon et al., 1998; Barrot et al., 2002) but also increases anxiety-like behavior (Barrot et al., 2002). Transgenic mice deficient in CREB expression in the central nervous system also exhibit an increased anxiety phenotype (Valverde et al., 2004).

The NAc has been proposed to be a key area not only for responses to drugs of abuse but also for the behavioral response to natural rewards (Mogenson et al., 1980; Robbins and Everitt, 1996; Wise, 1998; Berke and Hyman, 2000; Koob and Le Moal, 2001; Kelley, 2004), such as food and water intake or sexual behavior. In this Chapter, local changes of CREB activity in the NAc was investigated to determine how changes might affect

sexual behavior in both naïve and sexually experienced males. Microinjections of a herpes simplex virus (HSV) vector allowed local overexpression of either CREB itself or the dominant negative mutant mCREB, in which mutation of serine-133 to alanine prevents its own activation and renders it an inhibitor of endogenous CREB and CRE-dependent transcription (Gonzalez and Montminy, 1989; Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002). This study focuses on the sexual behavior of male rats after the local manipulation of CREB activity in non-isolated animals. The functional changes observed with the manipulation of CREB are then linked to the consequences of an identified physiological situation, protracted social isolation (see Introduction). Chronic single housing in adulthood, which is associated with deprivation of environmental stimulation, decreases CRE-dependent transcription in the NAc, increases anxiety-like behavior, and induces sexual behavior deficits. Lastly, the anxiety-like behavioral deficits associated with social isolation can be rescued by restoring CREB activity in the NAc of isolated animals.

Material and Methods

Procedures were approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center at Dallas.

Viral-Mediated Gene Transfer

Surgery was performed on male Sprague-Dawley rats (Charles River Breeding Laboratories) (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002). Herpes Simplex Virus (HSV) vectors were injected bilaterally (1.5 μl per side), over 7.5 min, into the NAc shell (Sh) (relative to bregma: rat, anterior-posterior = +1.8, lateral = ±2.4, dorsal-ventral = -6.7 mm below dura, with a 10 degree angle) as previously described (Barrot et al., 2002). At the end of the experiment, the animals used in behavior were perfused (Barrot et al., 2002), and the injection placements were evaluated for each animal on 40-μm cresyl-violet stained coronal sections. Only animals with correct bilateral placements were used for analysis; less than 10% of all animals had to be excluded for incorrect placements. The injected viruses were HSV-LacZ, encoding the control protein or β-gal; HSV-CREB, encoding the wildtype CREB and HSV-mCREB, a dominant-negative CREB in which phosphorylation at Ser133 is inhibited by an alanine mutation (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002). These vectors have been previously validated for their effect on CREB activity, and the lack of effect of HSV-LacZ, by injecting the vectors into the NAc of CRE-LacZ reporter mice (Barrot et al., 2002) and by examining their effects on expression of an endogenous CREB-

regulated gene (Carlezon et al., 1998). Based on the time-course of transgene expression within the NAcSh (Carlezon et al., 1998; Barrot et al., 2002) animals were tested for behavior on day 3 post-injection.

Sexual Behavior

Rats arriving at 7-8 weeks old were placed in a room maintained on a shifted 12-hour light/dark cycle (lights on between midnight and 12 noon). They were either housed 2 per cage or isolated 1 per cage. Testing of animals was conducted after 10-14 weeks of isolation. Male sexual behavior (Bolanos et al., 2003) was assessed under red light conditions between 1PM and 6PM in circular arenas (60cm) containing wood chips on the floor. Males were given a 5-min acclimation period to the testing arena and testing began thereafter by the introduction of a receptive female to the arena. Behaviors recorded were: mounts, intromissions and ejaculation as described (Mogenson et al., 1980; Bolanos et al., 2003). More specifically, the following behaviors were measured: mount latency (from the start of the test to the first mount), intromission latency (from the start of the test to the first intromission), ejaculation latency (calculated from the first intromission until ejaculation), the number of mounts and intromissions to reach ejaculation, and the post-ejaculation interval (PEI) (the time from ejaculation until first following intromission). The observer scoring the behavior was blind to the experimental conditions of the males. Sprague-Dawley ovariectomized female rats (Charles River Breeding Laboratories) were used in these experiments. Receptivity of the female was induced by estradiol benzoate (50 mg, subcutaneous (s.c.) and progesterone (500 mg, s.c.) 48 and 4-6 hours before testing, respectively. One week before the experiments, the

females were trained for one copulatory series with an experienced male. Before testing on the experimental day, female receptivity was verified by exhibition of lordosis and accepted intromission by an experienced male. Each female was then only used to test one experimental male. For the first experiment, the behavior was studied over 4 consecutive copulatory series. As the behavioral effect of CREB manipulation was seen on the first copulatory series only, the following experiments focused on this first series and the first post-ejaculatory interval.

The influence of novelty and sexual inexperience on the behavioral phenotypes observed was tested by pre-exposing the males to receptive females. In the case of viral transgenic experiments, 3 repeated exposures at 48-hr intervals and lasting until completion of 1 copulatory series were performed prior to the injection of the HSV vectors. For the isolated animals, 3 repeated exposures at 48-hr intervals were performed to test the consequence of sexual experience.

Elevated Plus-Maze

Rats were tested for the time spent in the open and closed arms of an elevated plus-maze (55 cm from the floor, 12-cm x 50-cm arms) over 5 min as described (Barrot et al., 2002; Bolanos et al., 2003). The walls of the two closed arms were 40 cm tall. Testing was carried out under controlled light conditions (~90 lux) and scored blindly in regards to housing conditions or viral injection conditions.

Diazepam Injections

Diazepam or saline was given subcutaneously 20 min prior to the sexual behavior testing. First, on separate sets of animals, it was noted that the dose of 0.75 mg/kg of diazepam did not significantly alter sexual behavior (intromission latency: 221 ± 30 sec vs. 210 ± 59 sec in controls; ejaculation latency: 578 ± 118 sec vs. 607 ± 68 sec in controls), but that the same dose significantly increased the time spent in the open arms of an elevated plus-maze (120 ± 19 sec vs. 67 ± 14 sec in controls, $P<0.05$).

CRE-LacZ Activity in Reporter Mice

CRE-LacZ mice were used in which β-galactosidase (β-gal) is expressed under the control of CREs (Barrot et al., 2002; Brodie et al., 2004). The construct, which contains seven CRE consensus sequences in tandem upstream of a minimal somatostatin promoter and the *LacZ* gene, is flanked by 5' insulator sequences (Brodie et al., 2004). Adult mice were either group housed or single housed (socially isolated) for 10-12weeks. Perfusion of the animals, brain sectioning (40 μm sections), and immunostaining were performed using published procedures (Barrot et al., 2002). β-gal immunostaining was carried out using a goat anti-β-gal antibody (1:5000, Biogenesis, Brentwood, NH), a biotinylated rabbit anti-goat secondary antibody (1:200), and the biotin-streptavidin technique (ABC kit; Vector Laboratories) with 3,3'-diaminobenzidine as chromagen. Density of positive nuclei was determined bilaterally in the NAcSh over 3 sections separated by 240 μm for each animal. The positive cells were counted blindly with regard to the housing conditions of the animals.

Plasma corticosterone

Tail blood samples were obtained at 7:30 AM from rats that were single- or double-housed in the room with the shifted light/dark cycle. Collection of each blood sample was obtained within 4 min from the time the cage was taken from the animal room. The animals were restrained in plastic restraint devices that allowed the animals to breathe, and blood was taken from the tail artery at time 0, 30 min and 60 minutes. After 60 minutes of restraint, the animals were released and allowed to recover. After, thirty minutes of recovery, a final blood sample was taken from the tail artery. Blood was collected in ice-cold heparin coated tubes and centrifuged (1000 x g, 15 min, 4°C). Aliquots of plasma were stored at -20°C until assayed. Plasma corticosterone levels were determined by competitive enzyme immunoassay according to manufacturer' specifications (ALPCO Diagnostics, Windham, New Hampshire) (Bolanos et al., 2003).

Statistical Analysis

Two group comparisons were performed by *t*-tests. Multiple group comparisons on experiments using HSV vectors were accomplished by ANOVA followed by a Duncan post-hoc analysis. Data are expressed as mean ± SEM.

Results

Inhibition of CREB and sexual behavior: Increased latency for approach

The inhibition of CREB activity by local expression of mCREB profoundly disrupted the initiation of sexual behavior in naive males, measured either as latency to the first mount (Fig. 1C) or as latency to the first intromission (Fig. 1D). As a direct consequence of this delayed initiation of the behavior, only 18% of the animals with reduced CREB activity in the NAc completed a copulatory series within 15 min after their first contact with a receptive female, whereas 68% of control males achieved this goal. This deficit, however, specifically concerned the initiation of the first copulatory series. Neither the total number of mounts necessary to reach ejaculation (Fig. 1F), the latency to ejaculation (Fig. IE), nor the latency to initiate new copulatory series after completion of the first one (Fig. 1C) was affected by inhibition of CREB activity in the NAc.

Increased anxiety on approach behavior: Influence of anxiolytics or sexual experience

Injection of 0.75 mg/kg diazepam 20 min before testing reversed the deficit in initiating sexual behavior in naive animals with decreased CREB activity in the NAc (Fig. 2). This same dose of diazepam had no effect in control animals and did not significantly alter the latencies to initiate a second copulatory series in either control or mCREB-treated animals (Fig. 2). In a separate experiment, males were trained three times for sexual behavior in the week before the viral injection (either HSV-mCREB or HSV-LacZ). This training procedure also prevented the deficit in initiating sexual behavior normally

observed after expression of mCREB (data not shown, HSV-mCREB, $n = 9$, vs. HSV-LacZ, $n = 12$; mount latency: $P > 0.25$; intromission latency: $P > 0.75$). Interestingly, in experienced mCREB animals, a difference was observed in PEI (HSV-mCREB: 311.9 ± 12.3; HSV-LacZ: 267.1 ± 13.5; $p<0.03$, n=9-12), although before viral-expression there was no difference.

Social Isolation: Decreases in CRE-dependent transcription

The next study investigated whether protracted social isolation alters CREB activity in the NAc. Indeed, the density of β-gal-positive cells in the CRE-LacZ mice was reduced by 34% in the shell part of the NAc after 10-12 wk of social isolation (Fig. 3).

Social Isolation: Effects on sexual behavior, anxiety and corticosterone responses

Protracted social isolation of adults causes increased anxiety-like behavior, as shown by reduced time spent in the open arms of an elevated plus-maze, a behavioral test widely used to study anxiety (Fig. 4A). It also leads to a major deficit of sexual behavior in naive socially isolated males, increasing both the latency to initiate the behavior (Fig. 4 B and C) and the latency to reach ejaculation during the first intercourse (Fig. 4D). Once the intercourse was completed, the latency to initiate a second intercourse was normal in isolated animals (Fig. 4 B and C). If animals were trained three times for sexual behavior, the deficit in initiating the behavior disappeared (data not shown; single-housed, n = 10, vs. double-housed, n = 9; mount latency: $P > 0.15$; intromission latency: $P > 0.1$), whereas the deficit in latency to reach ejaculation remained ($P < 0.02$). Interestingly,

protracted social isolation had no effect on plasma corticosterone levels in response to restraint stress at 30 min ($P>0.25$), 60 min ($P>0.18$) or at recovery (30 min after 1 hour of restraint stress, $P>0.38$, data not shown). However, isolated animals did have a decrease in basal levels of corticosterone (DH: 39.82 ± 8.04ng/μl, SH: 20.52 ± 3.49ng/μl, $P<0.039$).

CREB overexpression in isolated animals: Reversal of anxiety-like phenotype

Using injections of HSV-CREB, CRE-dependent transcription was increased in the NAc of sexually naive isolated animals and then tested to examine whether this would rescue the behavioral phenotypes. It was previously shown that overexpression of WT CREB within NAc shell increased local CRE-dependent transcription (Barrot et al., 2002). HSV-CREB injection in the NAc of isolated animals corrected the anxiety phenotype of the animals in the elevated plus-maze (Fig. 4E) as well as their deficit in the latency to initiate the first sexual intercourse (Fig. 4 F and G), indicating that these deficits were the result of the local decrease in CRE-dependent transcription (see Fig. 3). However, despite the reversal of these two phenotypes, the increased latency to first ejaculation was still present (Fig. 4H), which suggests that this latter deficit is mediated by a different mechanism.

Discussion

In the present study it is shown that inhibition of the transcription factor CREB in the NAc leads to a deficit in initiating sexual behavior that is associated with an anxiety-like phenotype. Additional data also show that a decrease in CRE-mediated transcription in the NAc can physiologically result from protracted social isolation, a condition that also causes deficits in initiating sexual behavior and increased anxiety-like behavior. Finally, this study shows the anxiety phenotypes in isolated animals can be reversed by experimentally restoring CREB activity in the NAc.

Using virus-mediated gene transfer (Carlezon and Neve, 2003), it was found that CREB overexpression in the NAc shell had no significant effect on male sexual behavior, whereas CREB inhibition profoundly disrupted the initiation of sexual behavior in naive animals. This latter effect involved only the initiation of the first copulatory series, without affecting ejaculation parameters or the initiation of subsequent copulatory series. These findings suggest that a local decrease in CREB activity in the NAc affects only a specific aspect of sexual behavior. Previous work has shown that CREB inhibition in this brain region increases the rewarding effects of cocaine (Carlezon et al., 1998; Pliakas et al., 2001), morphine (Barrot et al., 2002), and even sucrose (Barrot et al., 2002). Thus, the present results, in which CREB inhibition induced a deficit in sexual behavior, are unlikely to be due to reduced rewarding aspects of sex per se. Because previously it has also been shown that decreased CREB activity in the NAc can also increase anxiety-like

behavior (Barrot et al., 2002), sexually naive HSV-mCREB animals were treated with the anxiolytic drug diazepam and this reversed the deficit in initiating sexual behavior without significantly affecting control animals or other parameters of sexual behavior. This finding suggests that the deficit in initiating sexual behavior is likely to be a consequence of increased anxiety in animals with decreased CREB activity in the NAcSh. The lack of effect of CREB overexpression per se, and the absence of effect of diazepam in control animals, reflect the likelihood that the experimental procedure (test within the animal room and under red light conditions) is nonanxiogenic for normal animals.

Control of anxiety is not viewed as a main function of the NAc. However, previous results (Barrot et al., 2002) and the present data show that this brain area can exert significant influence on anxiety-related behaviors. Moreover, a recent study of deep brain stimulation in human patients with severe anxiety disorders and obsessive-compulsive disorders showed significant reduction in severity of symptoms by targeting the shell of the NAc (Sturm et al., 2003). These results probably relate to the key position of the NAcSh within specific brain circuits. The NAcSh receives massive glutamatergic inputs from limbic areas such as prefrontal cortex, ventral subicullum of the hippocampus, and basolateral amygdala (Brog et al., 1993), all of which have been associated with processing stressful or anxiogenic stimuli (Charney and Deutch, 1996; Charney, 2003; Bannerman et al., 2004). The NAcSh also receives major inputs from several midline thalamic nuclei, including paraventricular inputs rich in neuropeptide Y, α-melanocyte-stimulating hormone, and catecholamines, which could also provide stress-related

information (Freedman and Cassell, 1994). In addition, the shell subregion receives inputs from multiple other brain areas (Brog et al., 1993) that have been shown to influence stress and anxiety-like responses (Picciotto et al., 2002; Walker et al., 2003; Aston-Jones and Harris, 2004), such as lateral septum, lateral habenula, extended amygdala (which includes the bed nucleus of the stria terminalis), lateral hypothalamus, and monoaminergic nuclei such as ventral tegmental area, dorsal raphe, and locus coeruleus. The fact that the NAc integrates information from all of the above structures and is an interface to action (Mogenson et al., 1980) probably explains its potential role in the behavioral expression of stress and anxiety.

A specific feature of the anxiety-like phenotype associated with initiation of sexual behavior must be noted: the anxiety phenotype was observed only in animals naive for sexual behavior. Once the first copulatory series has been completed, or if the animal was previously trained for sexual behavior, the deficit in again initiating the behavior disappeared. The possibility that the NAcSh might be specifically involved in processing emotional information with a novelty component has been raised by other studies. During novelty exposure, there is a transient increase in dopaminergic activity in the NAcSh (Rebec et al., 1997; Noguchi et al., 2001). It was also previously shown that manipulating and injecting an animal induces dopamine and Fos responses in the NAcSh, whereas the same manipulation repeated 2 hr later has no effect (Barrot et al., 1999). Similarly, an unfamiliar appetitive stimulus such as sweet chocolate taste induces a dopamine response in the NAcSh that habituates after a single preexposure (Bassareo et al., 2002). The present results might indicate that CREB inhibition in the NAcSh affects reactive anxiety

only in response to novelty, rather than inducing a constitutive anxiety state. It is, however, important to raise the alternative possibility that sexual experience in itself could be anxiolytic and thereby modify subsequent behavior. Further experiments are needed to understand the precise role the NAc might play in anxiety and novelty responses.

CREB activity in the NAc appears to be controlled by environmental information. In the present study, transgenic mice were used with a CRE-LacZ reporter gene (Barrot et al., 2002; Brodie et al., 2004) to show that protracted social isolation reduces CRE-dependent transcription in the shell of the NAc. Many stimuli have been previously shown to increase CREB phosphorylation or activity within this brain area. This is the case with drugs of abuse such as cocaine, amphetamine (Konradi et al., 1994; Shaw-Lutchman et al., 2003), or opiates (Barrot et al., 2002; Shaw-Lutchman et al., 2002). Different physical stressors, such as forced-swim stress (Pliakas et al., 2001), foot-shock, restraint stress, social stress, or repeated unpredictable stress (Barrot et al., 2002) also increase CREB activity in the NAcSh. The fact that social isolation decreases CREB activation, the opposite of what is found with several active stressors (e.g., foot-shock and swim stress), is probably due to the nature of the stimulus. Indeed, the NAc integrates sensory and limbic inputs, making social isolation likely to be processed differently from active stressors because social isolation involves the removal of environmental sensory stimuli instead of the addition or imposing of external stimuli. The difference between social isolation and other forms of stress is also reinforced by the lack of effect the isolation procedure on stress-induced changes in corticosterone levels. This later finding is in

marked contrast to the active stressors, all of which increase circulating corticosterone levels. Interestingly, decreased levels of phosphorylated CREB have also been observed in the NAc after chronic treatment with alcohol (Misra et al., 2001) or nicotine (Brunzell et al., 2003; Pluzarev and Pandey, 2004). Decreased CREB activity in the amygdala has been proposed to contribute to the anxiety resulting from alcohol withdrawal (Pandey, 2003). The results presented here raise the possibility that alcohol- or nicotine-induced decreases in CREB activity within the NAc could also be one mechanism underlying the increased anxiety observed during withdrawal from these drugs.

Social isolation, in species having important social interactions, can lead to behavioral disturbances (Gerall et al., 1967; Wright et al., 1991; Brotto et al., 1998; Hall, 1998), including anxiety and sexual deficits as mentioned earlier (see Introduction). Most studies of rodents have examined the effect of social isolation on young or adolescent animals, when isolation can affect brain maturation (Hall, 1998). However, deficits can also follow isolation in adult animals (Brotto et al., 1998; Hall, 1998). Here, it is shown that protracted social isolation of adults causes increased anxiety-like behavior, as shown by reduced time spent in open arms of an elevated plus-maze. It also leads to a major deficit of sexual behavior, increasing both the latency to initiate the behavior and the latency to reach ejaculation during the first intercourse. Although the experiments with mCREB expression indicate that a local decrease in CREB activity within the NAc increases anxiety-like behavior and delays initiation of sexual behavior, no effect was observed on the ejaculation parameters. In isolated animals, the results show that restoring CREB activity in the NAcSh, by use of virus-mediated gene transfer, rescues the anxiety

phenotype as well as the deficit in the latency to initiate sexual behavior. This finding suggests that these deficits can be caused by the local decrease in CRE-dependent transcription within this brain region, but it does not exclude a critical role of other brain areas or other molecular changes in regulation of these behaviors. In contrast, the increased latency to first ejaculation is still present after CREB is overexpressed in the NAc of isolated rats. Also, socially isolated animals that are sexually experienced do not have deficits in initiation of sexual behavior, but the deficits in ejaculation remain. In contrast, experienced non-isolated animals with decreased CREB activity have no deficits in initiation of sexual behavior or deficits in ejaculation. This observation confirms that the ejaculation deficit is probably mediated by a different molecular mechanism or brain area and is independent from the anxiety phenotype induced by social isolation.

The present findings raise the question of the molecular changes, downstream of CREB, responsible for the behavioral changes observed. As a transcription factor, CREB itself is unlikely to be the molecule acutely affecting behavior. Rather, modification of CREB activity alters expression of specific gene products that then alter neuronal responses to subsequent stimuli. Dynorphin has been identified as one of the target genes of CREB in the NAc (Carlezon et al., 1998), which mediates some of the behavioral effects of local changes in CREB activity (Carlezon et al., 1998; Pliakas et al., 2001). Dynorphin is, however, unlikely to be the target gene responsible for the observed sexual behavior phenotype. The injection of a κ- opioid antagonist into the NAc, which would antagonize dynorphin action, both increases female-directed behavior and prevents the deficit in copulation latencies induced by a systemic agonist (Leyton and Stewart, 1992), whereas

in the case with mCREB (which would be expected to reduce dynorphin expression) or social isolation, both result in deficits in these behaviors. A DNA microarray study in inducible CREB- or mCREB-overexpressing mice revealed many other target genes for this transcription factor in the NAc (McClung and Nestler, 2003). Further studies will investigate some of the target genes in which CREB may play a critical role in the sexual and anxiety phenotype demonstrated in this study. This will be addressed directly in the next Chapter.

In conclusion, these findings show that decreased CRE-mediated transcription within the NAc is one of the possible mechanisms from which key behavioral consequences of protracted social isolation may result. The present data reveal that anxiety (in particular anxiety to novelty), and related deficits in initiating sexual behavior, could be associated with a decrease in CRE-mediated transcription in the NAc, and that increasing CRE activity in this region of socially isolated animals can rescue their anxiety-like phenotype. These findings further explicate the role of CREB-related plasticity within the NAc in the control of complex behavior and provide insight into the neurobiological mechanisms underlying behavioral pathology caused by social isolation.

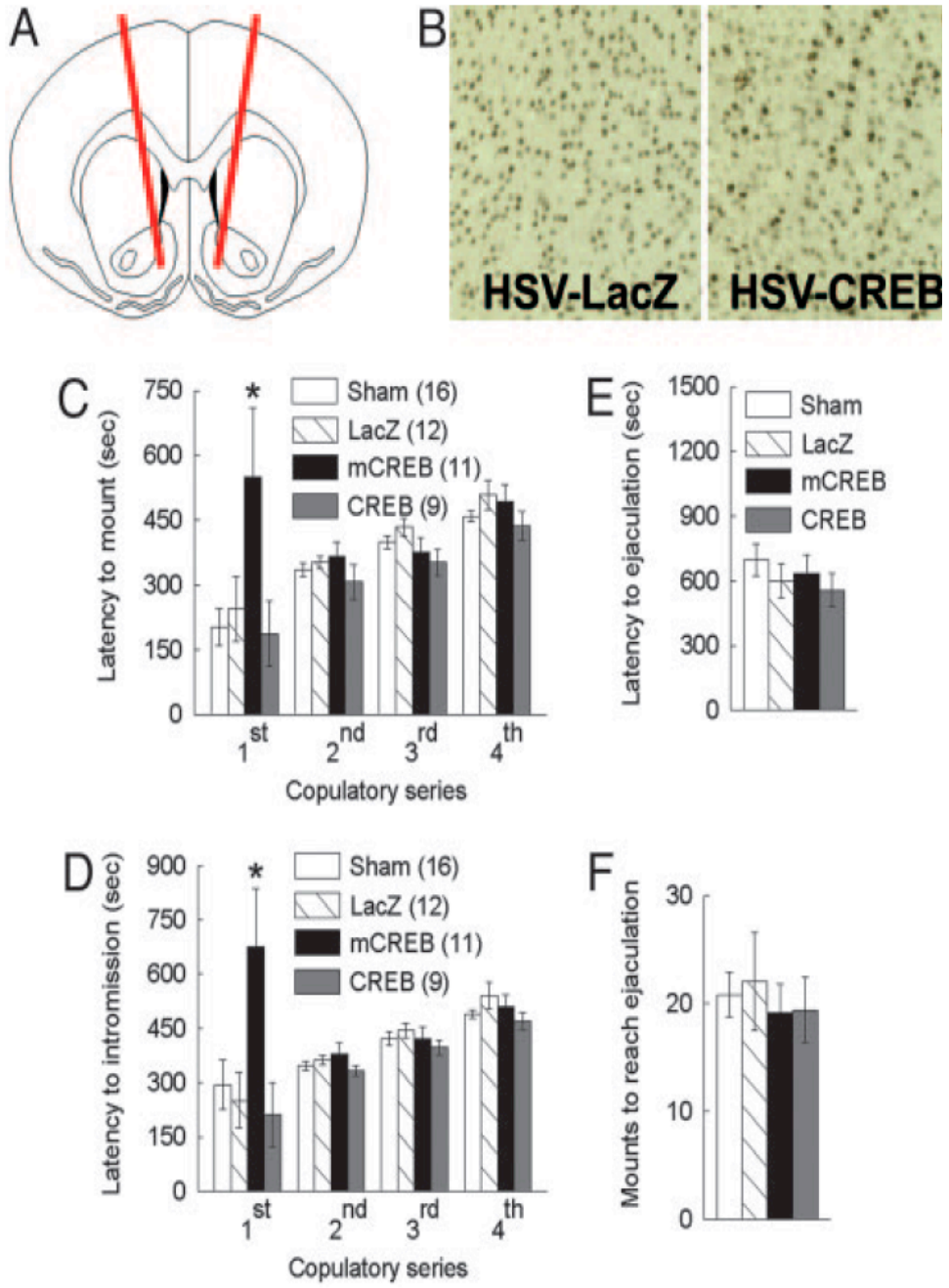


Figure 2.1: CREB and sexual behavior in double-housed males.

(A) Schematic of bilateral cannula placement for nucleus accumbens shell injections.
(B) CREB immunohistochemistry in nucleus accumbens shell after HSV-LacZ (Left) or HSV-CREB (Right) injections.
(C) The initiation of sexual behavior with a receptive female is impaired with microinjection of HSV-mCREB, but not HSV-CREB in the NAcSh, *P<0.05 in latency to first mount and
(D) latency to first intromsission.
(E) The delay between first intromission and first ejaculation is not affected by CREB manipulation.
(F) The total number of mounts necessary to reach ejaculation is not affected by CREB manipulation.

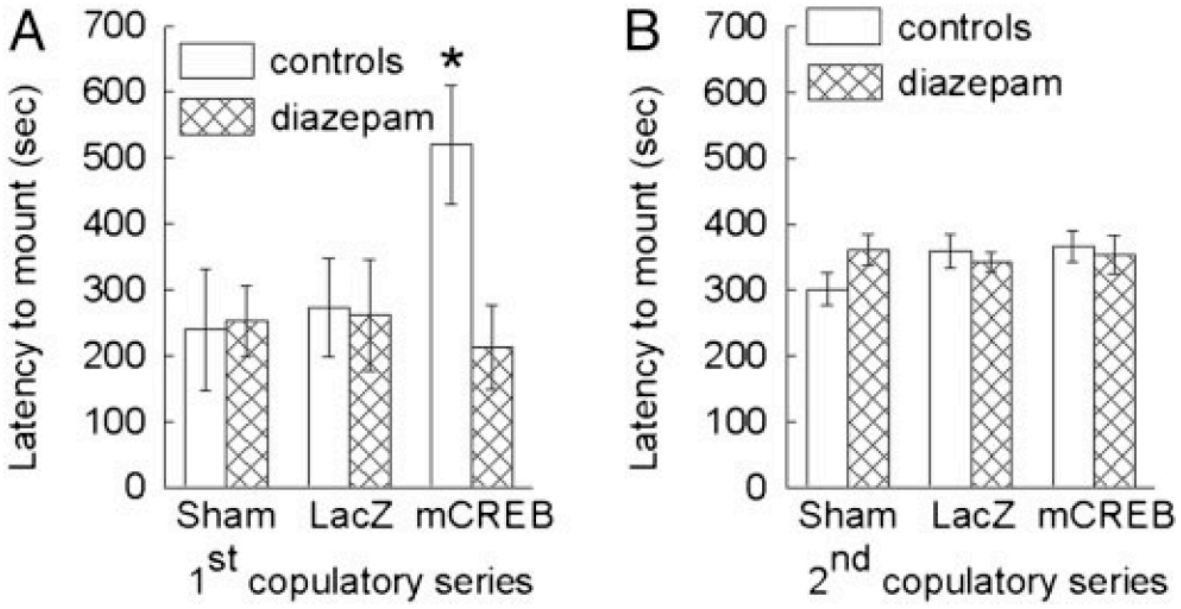


Figure 2.2: Diazepam reversal of HSV-mCREB phenotype.

(A) The increased delay to initiate sexual behavior, observed after HSV-mCREB microinjection, is corrected by a low dose of diazepam (0.75mg/kg, s.c.) $P<0.05$.
(B) The initiation of the second copulatory series is not affected by the diazepam injection.

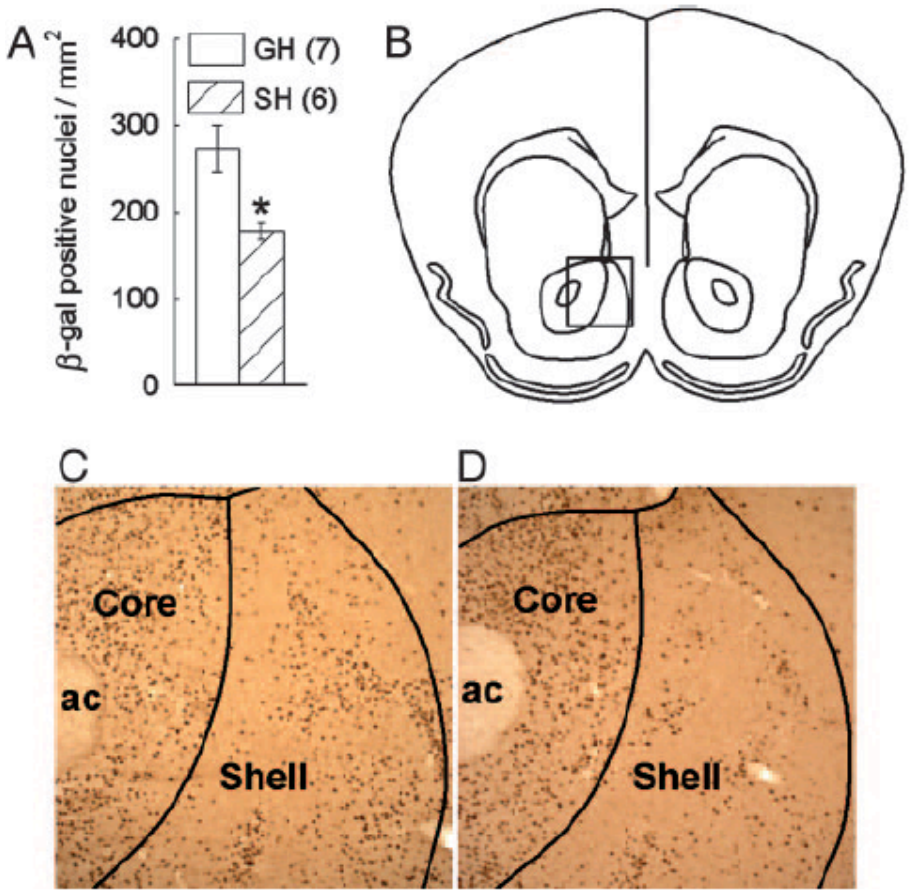


Figure 2.3: Influence of social isolation on CRE-dependent transcription.

(A) CRE-LacZ mice isolated for 10-12 weeks show decreased density of β-gal-positive nuclei within the shell of the NAc ($P<0.02$). GH, group-housed controls; SH, adult animals isolated for 10-12 weeks.
(B) Schematic of a coronal brain section at the level of the NAc. (Inset) Field view in C and D.
(C) β-gal immunostaining in the NAc of a group-housed CRE-LacZ mouse.
(D) β-gal immunostaining in the NAc of a adult CRE-LacZ mouse isolated for 10-12 week, which shows a reduction in the shell region.

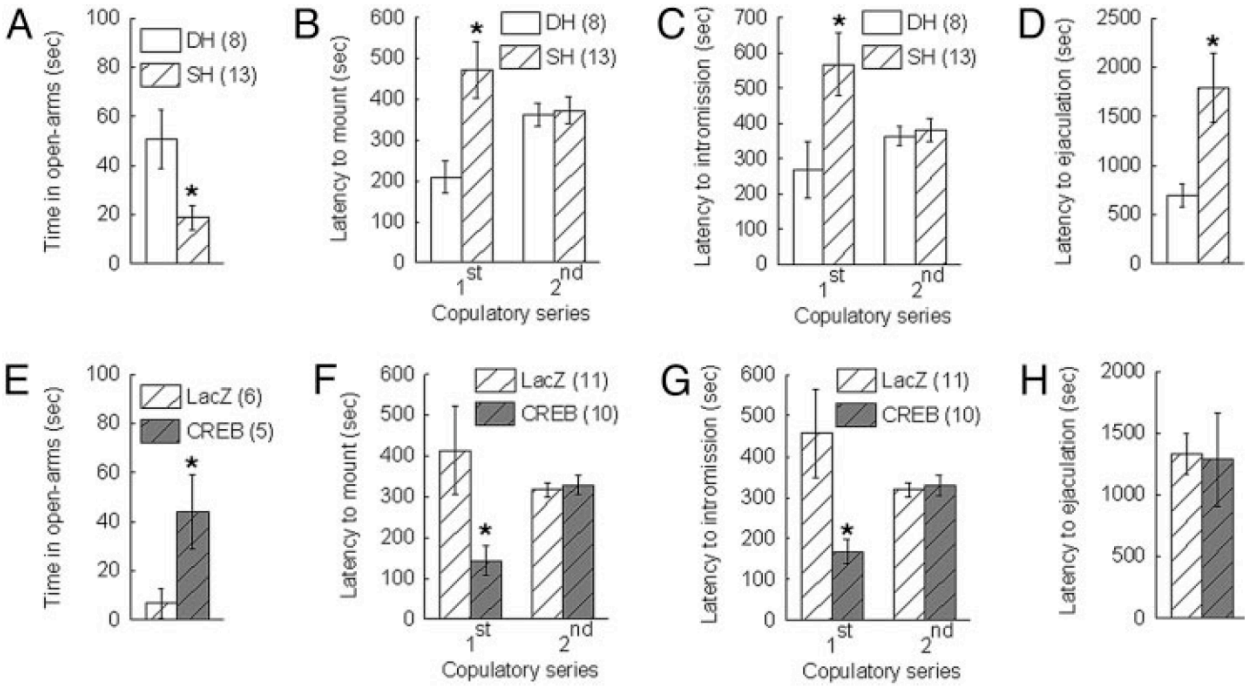


Figure 2.4: Influence of social isolation on sexual behavior and anxiety.

(A) Isolated animals spend less time in the open arms of an elevated plus maze (*P<0.015). DH, double-housed controls; SH, animals isolated 10-12 weeks.
(B-D) Both latency to initiate sexual behavior (*P<0.035) (B and C) and latency to ejaculate after intromission (*P<0.025) (D) are delayed in isolated animals.
(E) In isolated animals, the deficit in the time spent in open arms of an elevated plus-maze is corrected by local microinjection of HSV-CREB.
(F-H) In isolated animals, the microinjection of HSV-CREB in the NAc shell corrects the increased latency to initiate sexual behavior (*P<0.04) (F and G), but it does not correct the increased latency to ejaculate (H).

CHAPTER THREE

CREB REGULATION OF NUCLEUS ACCUMBENS EXCITABILITY MEDIATES SOCIAL ISOLATION-INDUCED BEHAVIORAL DEFICITS

Summary

Previously it was demonstrated that prolonged social isolation induces an anxiety-like phenotype via decreased levels of CREB activity in the nucleus accumbens shell (NAcSh). This present study shows that social isolation also induces anhedonia-like symptoms, which are not mediated by CREB. However, both the anxiety and anhedonia symptoms, as well as the deficit in CREB activity in the NAcSh, are reversed by chronic, but not acute, antidepressant treatment. Furthermore, decreased CREB activity in this region is found to cause anxiety-like symptoms by inducing expression of certain K^+ channels and reducing the electrical excitability of NAc neurons. Together, these studies establish a selective role of decreased CREB activity in the NAcSh in anxiety-like behavior, and provide a novel mechanism by which antidepressant treatments regulate anxiety symptoms after social isolation.

Introduction

Depression and anxiety are common forms of mental illness in the general population. While they are classified as distinct syndromes by the *Diagnostic and Statistical Manual* (American Psychiatric Association), symptoms of depression and anxiety often co-occur,

and to widely varying extents in different subtypes of the illnesses. Despite the importance of these clinical phenomena, very little is known about distinctions between depression- and anxiety-like symptoms in animal models (Nestler et al., 2002a). Models of "active" stress, such as foot shock, restraint stress, social defeat, and learned helplessness, produce depressive- and anxiety-like phenotypes; the molecular mechanisms of these models have been extensively studied, although specific molecular mediators of depression versus anxiety symptoms have not yet been described (Mendelson and McEwen, 1991, 1992; Shaw-Lutchman et al., 2002; Perrotti et al., 2004; Pawlak et al., 2005; Berton et al., 2006). Even less well studied, however, is an "inactive" model of stress, social isolation in adulthood, which like active stress models mimics aspects of human depression and anxiety (Hall, 1998) (see Introduction). This lack of attention is unfortunate, since social isolation would appear particularly relevant to subtypes of human depression and anxiety disorders, including social isolation induced in the elderly, homebound adults, obese adolescents and individuals with hearing loss (Arlinger, 2003; Sjoberg et al., 2005; Robinson, 2006; Choi and McDougall, 2007; Owen, 2007).

Social isolation has long been studied, however, most models to date have focused on isolation early in life, either as pups or adolescents (Wright et al., 1991; Genaro and Schmidek, 2002; Ruedi-Bettschen et al., 2004). These models study the effects of isolation during developmental stages on later behavior as adults, a very different model than adulthood social isolation (Hall, 1998). Reports of adulthood isolation indicate a strong anxiety-like phenotype (Jankowska et al., 1991; Vasar et al., 1993; Ahmed et al.,

1995), increased alcohol intake (Parker and Radow, 1974; Ehrman, 1990; Schenk et al., 1990; Ehlers et al., 2007), modulation of responses to rewarding stimuli (Alexander et al., 1978; Bozarth et al., 1989; Deroche et al., 1994; Ahmed et al., 1995), changes in circadian rhythms (Greco et al., 1990) and changes in running-induced neurogenesis (Stranahan et al., 2006). Although reports on changes in neurochemistry are often conflicting (Hall, 1998), there appears to be decreased serotonergic and noradrenergic function and metabolism in various regions of the brain (Segal et al., 1973; Petkov et al., 1987; Miachon et al., 1993), although these changes have not been linked to associated behavioral abnormalities. In addition, reports on corticosterone levels have produced varying results, however, it is believed that isolated animals may have a greater sensitivity to corticosterone levels in response to stressful activities (Plaut and Grota, 1971; Miachon et al., 1993; Stranahan et al., 2006).

Previous work demonstrated that activity of the transcription factor, CREB (cAMP response element binding protein), in the nucleus accumbens shell subregion (NAcSh) is a key regulator of an animal's responses to emotional stimuli (Pliakas et al., 2001; Barrot et al., 2002; Newton et al., 2002). Also, the previous chapter provided evidence that prolonged social isolation of adult mice and rats induces a state of profound anxiety, which is mediated via decreased activity of CREB, in the NAcSh: the anxiety symptoms induced by social isolation are blocked by viral-mediated overexpression of CREB in the NAcSh, while overexpression of mCREB (a dominant negative form of CREB) mimics these symptoms in normal animals (Barrot et al., 2005). Activation of CREB activity in the NAcSh in response to active stress or to drugs of abuse dampens an individual's

responses to rewarding as well as aversive stimuli (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002; Newton et al., 2002; Pandey et al., 2005). This has led to the suggestion that CREB induction in this region may mediate a state of emotional numbing, and contribute to depression-like symptoms such as anhedonia (decreased ability to experience pleasure) (Carlezon et al., 2005).

In this Chapter, the behavioral phenotype induced by adult social isolation is more fully characterized. Here it is shown that this form of inactive stress produced by isolation also induces symptoms of depression, specifically, anhedonia, despite a decrease in CREB activity in the NAcSh. Interestingly, chronic, but not acute administration of standard antidepressant medications reverses both the anxiety- and depression-like symptoms seen after social isolation. Further, chronic antidepressant administration also reverses the reduction in CREB activity in the NAcSh which is a feature of social isolation. However, only the anxiety-like symptoms appear to be truly mediated by CREB in this brain region. While the depressive-like symptoms and imipramine treatment correlate with CREB changes, CREB alone is not sufficient to reverse depressive-like symptoms.

In addition, DNA expression arrays are used to gain insight into the molecular mechanisms by which social isolation, antidepressants, and CREB regulate NAcSh function. Here it is shown that decreased CREB activity in the NAcSh depresses the electrical excitability of NAc neurons by upregulating several K^+ channels, and that this molecular adaptation specifically mediates anxiety-like symptoms induced by social isolation.

Together, studies described in this Chapter provide fundamentally new information concerning the molecular mechanisms underlying the behavioral consequences of adulthood social isolation and offer new insight into the generation of anxiety symptoms seen in affective syndromes.

Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas Southwestern Medical Center at Dallas.

Social Isolation and Imipramine Treatment

Animals arrived at 7-8 weeks of age and were place in a 12-hr light/12-hr dark cycle room (lights between 7 AM and 7 PM). They were housed either two per cage or isolated one per cage. Testing of the isolated animals began after 10-12 weeks of isolation. In the case of acute imipramine treatment, after 10 weeks of isolation, animals received 5 days of oral imipramine (10 mg/kg daily) in drinking water; for chronic imipramine treatment, animals were isolated for 6 weeks and then given imipramine in the drinking water for 4 weeks. Trunk blood levels were taken and measured by HPLC to ensure clinically relevant levels of the drug. For the active metabolite, desipramine, levels from blood samples of drinking double-housed and single-housed rats were comparable to injected rats (Drinking Levels: 117.5± 11.2; Injected: 116.5 ± 6.5; n=2-8; $P>0.97$). There were no differences between double-housed and single-housed for desipramine levels, $P>0.18$).

Sexual Behavior

Sexual behavior of adult male Sprague-Dawley rats (Charles River Breeding Laboratories) was conducted as described (Barrot et al., 2005) under red light conditions between 8PM and 2AM in circular arenas (60cm) containing wood chips on the floor.

Males were given a 5-min acclimation period to the testing arena and testing began thereafter by the introduction of a receptive female to the arena. Behaviors recorded were: mounts, intromissions and ejaculation as described (Sodersten and Ahlenius, 1972; Bolanos et al., 2003). More specifically, we measured: mount latency (from the start of the test to the first mount), intromission latency (from the start of the test to the first intromission), ejaculation latency (calculated from the first intromission until ejaculation), the number of mounts and intromissions to reach ejaculation, and the post-ejaculation interval (PEI) (the time from ejaculation until first subsequent intromission). The behavior was videotaped and scored by an observer blind to the experimental conditions of the males. Sprague-Dawley ovariectomized female rats (Charles River Breeding Laboratories) were used in these experiments. Receptivity of the female was induced by estradiol benzoate (50 mg, s.c.) and progesterone (500 mg, s.c.) 48 and 4-6 hours before testing, respectively. One week before the experiments, the females were trained for one copulatory series with an experienced male. Before testing on the experimental day, female receptivity was verified by exhibition of lordosis and accepted intromission by an experienced male. Each female was then only used to test one experimental male. The first series of sexual behavior and the first PEI were then measured.

Elevated Plus-Maze

Rats were tested for the time spent in the open and closed arms of an elevated plus-maze (55cm from the floor, 12-cm x 50-cm arms) over 5 min as described (Barrot et al., 2002). The walls of the two closed arms were 40 cm tall. Testing was carried out under

controlled light conditions (15-20 lux) and scored blindly with regards to housing condition, imipramine treatment, or viral injection conditions.

Sucrose Preference

The sucrose preference test consisted of a two-bottle choice paradigm as described previously (Bolanos et al., 2003), in which the animals were given the choice between water versus sucrose. Before testing, animals were habituated to drink from two bottles of water for 5 days. At the start of the experiment, animals were allowed unlimited total access to water in one bottle and ascending concentrations of sucrose (0, 0.125, 0.25, 0.5, 1 and 2% solutions) in the other bottle, for two days at each concentration. Water and sucrose consumption were measured daily at 6 PM, and then placement of the bottles was switched to ensure that animals did not have a drinking side preference, left versus right. For animals under isolation and imipramine treatment, to ensure imipramine was not masking the sucrose taste, an accelerated schedule was introduced. Imipramine animals were taken off imipramine for a 4 day period of sucrose testing, and allowed water or sucrose without imipramine; 0.0, 0.5, 1 and 2% sucrose solutions in one bottle or water in the other. In this case, bottles were measured every 6 hours and changed positions, and each concentration was only introduced for a 24 hour period. In the case of viral gene transfer experiments, where transgene expression lasts only 3 days, a shorter sucrose preference test was used (Barrot et al., 2002). Briefly, prior to surgery animals were given a 3-day exposure to two 1% solution bottles followed by a 30 min pre-test with one bottle of 1% and one bottle of water. Two days after surgery, the animals were then given a 30 min post-test, similar to pre-test.

Viral-Mediated Gene Transfer

Surgery was performed on male Sprague-Dawley rats. AAV or HSV vectors were injected bilaterally (1.5 μl per side), over 7.5 min, into the NAcSh (relative to bregma: rat, anterior-posterior = +1.8, lateral = ±2.4, dorsal-ventral = -6.7 mm below dura, with a 10 degree angle) as previously described . At the end of the experiment, the animals used in behavior were perfused, and the injection placements were evaluated for each animal on 40-μm cresyl-violet stained coronal sections. Only animals with correct bilateral placements were used for analysis; less than 10% of all animals had to be excluded for incorrect placements. The injected viruses were HSV (herpes simplex virus)-mCREB, a dominant-negative CREB in which phosphorylation at Ser133 is inhibited by an alanine mutation (Carlezon et al., 2000; Barrot et al., 2002; Carlezon and Neve 2003; Barrot et al., 2005); HSV-GFP or HSV-LacZ, encoding the control protein GFP or b-gal; HSV-Kir2.1, a wildtype inward rectifying potassium channel (Dong et al., 2006); AAV (adeno-associated virus)-CREB; and AAV-GFP. Based on the time-course of transgene expression within the NAcSh, animals were tested for behavior between days 2-4 after injection of HSV vectors, or 3-5 weeks after injection of AAV vectors.

CRE-LacZ Activity in Reporter Mice

CRE-LacZ mice were used in which β-gal is expressed under the control of CREs (Barrot et al., 2002; Brodie et al., 2004). The construct, which contains seven CRE consensus sequences in tandem upstream of a minimal somatostatin promoter and the LacZ gene, is flanked by 5' insulator sequences (Brodie et al., 2004). Adult mice were either group housed or single housed (socially isolated) for 6 weeks, and then injected daily with

saline or 10 mg/kg of imipramine intraperitoneal (i.p.) for 4 weeks. Perfusion occurred 4 hours post-injection for all experiments. After perfusions, brain sectioning (40 μm sections) and immunostaining were performed using published procedures (Shaw-Lutchman et al., 2002; Barrot et al., 2005). β-gal immunostaining was carried out using a goat anti-β-gal antibody (1:5000, Biogenesis, Brentwood, NH), a biotinylated rabbit anti-goat secondary antibody (1:200), and the biotin-streptavidin technique (ABC kit; Vector Laboratories) with 3,3'-diaminobenzidine as chromagen. Density of positive nuclei was determined bilaterally in the NAcSh over 6 sections separated by 120 μm for each animal using the unbiased stereology program Stereoinvestigator. The positive cells were counted blindly with regard to the experimental conditions of the animals.

Electrophysiological Recordings

All recordings were performed blind to the experimental conditions of the animals. To minimize possible stress effects on the recordings, rats were anesthetized and perfused immediately for 40-60 sec with ice-cold artificial CSF (aCSF), which contained (mM): 128 NaCl, 3 KCl, 1.25 NaH_2PO_4, 10 D-glucose, 24 $NaHCO_3$, 2 $CaCl_2$, and 2 $MgCl_2$ (oxygenated with 95% O_2 and 5% CO_2, pH 7.35, 295-305 mOsm). Acute brain slices containing NAcSh were cut using microslicer (DTK-1000, Ted Pella Inc., Redding, California) in sucrose-aCSF, which was derived by fully replacing NaCl with 254 mM sucrose, and saturated by 95% O_2 and 5% CO_2. Slices were maintained in the holding chamber for 1 hr at 37°C. Recording external solution at a flow rate 2.5 ml/min was aCSF without NaH_2PO_4, to which was added by 1 mM kynurenic acid and 100 mM picrotoxin to block ionotropic glutamate and $GABA_A$ receptors. In experiments for K^+

channel-mediated responses, 1 mM tetrodotoxin and 200 mM Cd^{2+} were used to block Na^{+} and Ca^{2+} channels, respectively. Whole-cell current clamp recordings, as described previously (Dong et al., 2006), were performed at 34°C. Recordings were obtained from medium spiny neurons of NAcSh. Patch pipettes (3-5 MΩ) were filled with an internal solution containing (mM): 115 potassium gluconate, 20 KCl, 1.5 $MgCl_2$, 10 phosphocreatine, 10 Hepes, 2 ATP-Mg and 0.5 mM GTP (pH 7.2, 285 mOsm). Whole-cell recordings were made under continuous single-electrode voltage clamp mode (AxoClamp 2B, Axon Instruments), then converted to Bridge mode for current clamping. Data acquisition was made using DigiData 1322A and pClamp 8 (Axon Instruments). Series resistance was less than 20 MΩ and Bridge was compensated. Resting membrane potential (RMP) was measured immediately after whole-cell current clamp mode was made in the absence of current injection. Then, membrane potential was maintained at -70 mV in all later experiments. Current-voltage relationship in normal aCSF (in the absence of Na^{+} and Ca^{2+} channel blockers) was obtained by measuring voltage responses for ~300 ms. K^{+} current-voltage relationship was obtained by measuring voltage responses between 1.0 and 1.2 sec.

Microarray Analysis

Animals were housed 6 weeks for social isolation experiments, followed by AAV-CREB or AAV-GFP surgeries, or imipramine/control water treatment 4 weeks before tissue collection. After rapid decapitation and brain extraction, punches of NAcSh were taken using a 14-gauge needle and quickly frozen on dry ice until RNA was extracted. Bilateral punches were pooled for two animals, with 3 independent tissue samples used

per group. RNA was reversed transcribed into cDNA and labeled for microarray analysis as described (Berton et al., 2006). Genes were analyzed by GeneSpring program, and determined significant if regulated by at least a 20% difference and p value less than 0.05 adjusted for multiple comparisons.

There is a very high degree of confidence in these array findings for several reasons. First, on Affymetrix chips, each gene is represented multiple times. Second, highly rigorous statistical analyses are used to identify regulated genes. Third, for each array, the RNA used is pooled from NAc from 2 animals, which decreases differences attributable to individual variability and increases the statistical power of these experiments (Peng et al., 2003). Each treatment group is compared directly with its controls, which are handled, treated, and killed at the same time, and under the same conditions. As well, the RNA is isolated and labeled, and arrays are run at the same time, for each experiment. Statistical analysis of our array data has indicated a consistent false discovery rate of ~0.1 for our arrays, and control experiments have shown that an extremely small number of transcripts (0.035%) might be regulated by external factors, such as differences between animals, their environment, or technical deviations (McClung et al., 2005). Fourth, all arrays are performed in triplicate using separate groups of animals. Under these conditions, when noise (i.e., "absent" genes) is excluded, Affymetrix arrays have been shown to achieve strong statistical power (Shippy et al., 2004). Fifth, genes identified on arrays are routinely verified by use of real-time PCR (qPCR or RT-PCR) on independent tissue samples; typically ~90% of genes that show

significant regulation on the arrays replicate with qPCR, which again indicates a low false positive rate (see (McClung et al., 2005; Berton et al., 2006).

Real-Time Polymerase Chain Reaction (RT-PCR)

HSV-mCREB or HSV-LacZ was injected into the NAcSh as described previously(Barrot et al., 2002; Barrot et al., 2005), and 3-4 days later, RNA was extraction from NAcSh punches and cDNA was prepared. Primer sets for *kcns2, kcnd3* and *kcnj2* were validated, and then used on the cDNA generated from NAcSh tissue. All values were normalized to control LacZ samples.

Statistical Analysis

For two group comparisons, student t-tests were used. ANOVA was used for experiments that compared effects of housing conditions and drug treatment. Data are expressed as mean ± SEM.

Results

Anhedonia-like symptoms induced by social isolation: Reversal by antidepressant treatment

In the previous chapter, it was shown that social isolation induces two deficits in sexual behavior compared to normal (double-housed) animals: increased latency to first sexual approach and increased latency for ejaculation (Barrot-Wallace et al., 2005). Here, these findings were replicated in addition to the finding that chronic (28-32 days) treatment with the standard antidepressant medication, imipramine (10 mg/kg/day), in the drinking water restored the ejaculation impairment in sexual behavior to normal levels (Fig. 1A) and the anxiety phenotype in the elevated plus maze (Fig. 1B). In this isolation study, imipramine was delivered in the drinking water, based on the notion that daily i.p. injections would dramatically disrupt the social isolation treatment, and confirmed clinically relevant blood levels with this approach (see Materials and Methods). However, while chronic imipramine improved the sexual performance of socially isolated animals, the drug dramatically impaired performance in normal (double-housed) animals (Fig. 1A). In contrast, acute administration of imipramine (in the drinking water for 5 days) did not influence sexual behavior in either socially isolated or double-housed animals in that the deficits remained for the isolated animals and the drug did not disrupt normal function in control animals (data not shown). These findings highlight the dramatically different effects of chronic imipramine in normal animals compared to those subjected to prolonged social isolation.

Increased latency to ejaculation has been reported in animal models with depressive-phenotypes, therefore this finding could represent a symptom of anhedonia induced by chronic social isolation (Yirmiya, 1996; Bolanos et al., 2003). It was therefore determined whether social isolation induces other anhedonia-like symptoms, namely, deficits in sucrose preference seen after many types of active stress (Papp et al., 1991; D'Aquila et al., 1997; Strekalova et al., 2004). As shown in Fig. 1C and D, social isolation decreased sucrose intake and preference, although there were no changes in total liquid intake (sucrose + water) for all concentrations, except at the 2% concentration where the DH animals sucrose intake makes the overall intake much higher (data not shown). The deficit in sucrose drinking was completely restored by chronic administration of imipramine (Fig. 1D and Fig. 1E), while acute administration had no effect and the groups of animals still displayed differences (data not shown).

Because sucrose intake and preference was decreased in isolated animals, indicating an anhedonia-like phenotype, the effects social isolation were explored in the forced swim test (Porsolt et al., 1978). As seen after several forms of active stress (Prince and Anisman, 1984; Consoli et al., 2005), isolation actually caused an increase in latency to immobility on day 1, but showed no differences between isolated and control groups on day 2 (data not shown).

Decreased CRE activity induced by social isolation: Reversal by antidepressant treatment

Given the prior evidence that prolonged social isolation during adulthood decreased CRE activity in the NAcSh compared to group-housed animals (Barrot-Wallace et al., 2005), it

was of interest to study whether imipramine treatment affected this phenomenon. As shown in Fig. 2, chronic administration of imipramine completely restored levels of CRE activity in the NAcSh to those seen in group-housed control animals. Interestingly, however, chronic imipramine treatment of group-housed animals dramatically reduced CRE activity in this brain region. These strikingly diverging effects of imipramine on CRE activity in isolated and control animals paralleled the drug's effects on sexual behavior.

Role of CREB in the NAcSh on anhedonia- and anxiety-like behavior

As discussed in the Introduction, levels of CREB activity in the NAcSh have been shown to regulate many types of emotional behavior in normal animals, however, its effects in the social isolation model have not been fully explored. The influence of CREB activity in this brain region was therefore studied on the full range of behavioral abnormalities seen after social isolation. It has been previously demonstrated that HSV-mediated overexpression of CREB in the NAcSh of isolated animals reversed the increase in anxiety-like behavior exhibited by these animals, but did not influence latency to ejaculation (Barrot-Wallace et al., 2005). However, this method overexpresses CREB for a few days only, while chronic imipramine, which reverses the ejaculation latency deficit, restores CREB levels over a much longer time frame. To test this possibility, AAV vectors were used to overexpress CREB in the NAcSh for 4 weeks. (It is known that AAV vectors express transgenes stably for at least 6 months [data not shown].) Similar to HSV-CREB overexpression, more prolonged AAV-CREB overexpression completely treated the anxiety phenotype found in isolated animals, as reflected in the elevated plus

maze (Fig. 3A) and the time to initiation of sexual behavior (Fig. 3B). However, AAV-CREB, like HSV-CREB, did not affect ejaculation latency in isolated animals (Fig. 3C), nor did it treat the deficit seen in sucrose preference under these conditions (Fig. 3D). As reported previously with HSV-CREB overexpression (Barrot et al., 2002), AAV-CREB overexpression in the NAcSh of non-isolated animals also decreased sucrose intake (Fig. 3D).

Regulation of gene expression in the NAcSh by social isolation: Effect of antidepressant treatment and CREB

To gain insight into the molecular basis by which prolonged social isolation alters NAcSh function, and how chronic imipramine treatment and levels of CREB in this region modify the sequelae of social isolation, gene expression in the NAcSh was characterized under these various conditions by use of DNA expression microarray analysis. The overall amount of gene regulation provided several interesting lessons (Table 1). First, social isolation upregulates 100-fold more genes than it downregulates in the NAcSh. Surprisingly, this is similar to active forms of stress, which upregulate 14 to 18-fold more genes, depending on the time period after the last stressor (Berton et al., 2006). Second, under both AAV-CREB and chronic imipramine conditions, the double-housed animals show nearly a two-fold increase in overall gene regulation compared with the social isolation condition. This suggests that there may be an overall dampening in gene regulation in the isolation condition. This possibility is supported by the upregulation of HDAC4 seen in this condition, an adaptation which would be expected to promote gene repression (Kumar et al., 2005; Tsankova et al., 2007). Third, the heatmaps shown in Fig. 4 highlight the very different effects that chronic imipramine induces in the NAcSh

of socially isolated versus double-housed animals. Overexpression of CREB in the NAcSh of double-housed animals induces a pattern of gene expression in general opposite to that induced by chronic imipramine, and vice versa. This is consistent with the ability of chronic imipramine to decrease CREB activity in the NAcSh and with the prior reports that CREB overexpression increases depression-like behavior (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002; Newton et al., 2002). In contrast, CREB's effects were very different in socially isolated animals. As seen in Fig. 5, the pattern of gene regulation in the isolated condition is generally opposite to that seen in isolation with imipramine, while isolation with CREB only partially reverses this pattern.

These gene sets were next analyzed to identify specific genes that could be responsible for the behavioral phenotypes found for social isolation, chronic imipramine, and CREB overexpression in the NAcSh. More specifically, it was hypothesized that genes regulated in common upon both CREB overexpression and chronic imipramine treatment in isolated animals could contribute to the reversal of anxiety-like symptoms seen under both conditions, while those genes regulated uniquely by imipramine treatment may play a role in reversing reward-related deficits found in the social isolation model (Table 1). Of the genes in common in the isolation CREB and isolation imipramine groups, glutamate receptor signaling is downregulated, as evidenced by reduced expression of the NMDA receptor subunits NR1 and NR2B genes (isolated group with CREB) and NR1A and the AMPA glutamate receptor subunit GluR2 (isolated group with imipramine). Under isolation conditions alone, PKC epsilon is upregulated, however in the isolated group with CREB it is downregulated. In addition, in the isolated condition HDAC4 is

upregulated, but in the isolated with CREB, several histone modifying genes are downregulated, including HDAC10, SWI/SNF4, Histone H2A, with upregulation of histone cell regulation defective interacting protein 5. Of the genes that are uniquely regulated in the NAc of the isolated group with imipramine are those downregulated consisting of: bone morphogenetic protein receptor1A, sertolin, diacylglycerol kinase, B cell lymphoma 2 (bcl2)-activating transcription factor, (an anit-apoptotic protein found to be upregulated with chronic antidepressants in the hippocampus (Murray and Hutson, 2007), and mitogen activated protein kinase kinase kinase 3 and 10; upregulated proteins consists of protein phosphatase 1 subunit 15, glutathione reductase, neuregulin 2 and spermatogenesis associated protein 7. Although these genes do not imply a direct anxiolytic or antidepressant mechanism, they do offer insight into the behavioral phenomenon produced by CREB or imipramine treatments.

Regulation of K^+ channels by social isolation

Among the genes prominently regulated by social isolation, compared to double-housed animals, are several subtypes of K^+ channels, which were upregulated in isolated conditions (Table 1). Certain of these channels also satisfied the criterion stated above in that their induction by social isolation appeared to be mediated via CREB. Regulation of these genes was noted in previous arrays of mCREB transgenic mice versus their control littermates (McClung and Nestler, 2003). Additionally, with CREB overexpression, several of these channels and related genes are downregulated.

Because K^+ channel regulation is a crucial determinant of a neuron's electrical excitability, the electrophysiological properties of medium spiny neurons in the NAcSh were examined in isolated versus control animals. NAc neurons in brain slices from socially isolated animals exhibited a small but statistically significant hyperpolarization of the neurons' resting membrane potential, consistent with an upregulation of K^+ channels (Fig. 6A). Social isolation had no effect on current-voltage relationships under normal recording conditions (Fig. 6B and C), however, when Na^+ and Ca^{2+} channels are blocked, decreased membrane resistance in K^+ channel-mediated responses became evident (Fig. 6D and E). This increase in K^+ channel current and the attendant reduction in NAc cell excitability seen after social isolation, when CREB activity is suppressed in the NAc, is consistent with a recent study, which showed directly that decreased CREB activity in the NAc causes similar effects on NAc neuronal excitability (Dong et al., 2006).

Regulation of anxiety- but not anhedonia-like symptoms by K^+ channels in the NAc: Role of CREB

Given the evidence that increased K^+ channel expression is induced in NAcSh after social isolation, it was important to explore if the behavioral phenotype of isolated animals could be mimicked in non-isolated animals by artificially overexpressing K^+ channels and reducing the excitability of NAc neurons. Because Kir2.1, an inwardly rectifying K^+ channel, strongly decreases NAc neuronal excitability and mimics the effects of decreased CREB activity on these neurons (Dong et al., 2006), the wildtype Kir2.1 channel in an HSV vector was overexpressed in the NAc of non-isolated animals. This manipulation caused an anxiety-like phenotype in the elevated plus maze, as well as in

initiation of sexual behavior (Fig. 7A and B). However, no effect of HSV-Kir2.1 was observed on anhedonia-related measures, that is, ejaculation latency and sucrose preference (Fig. 7C and D).

The selective effect of Kir2.1 overexpression on anxiety-like symptoms resembles the selective role of CREB in these behavioral sequelae of social isolation. The microarray data along with previous studies indicate that manipulation of CREB can alter potassium channel expression. To determine whether reductions in CREB activity are also sufficient for increasing K^+ channel expression, HSV vectors were used to overexpress mCREB or LacZ in the NAcSh of non-isolated animals. Using RT-PCR, the levels of *kcnj2* were measured and an increase was found with mCREB overexpression over LacZ expression (mCREB: 1.762 ± 0.248; LacZ: 1.018 ± 0.0746, p=0.012, n=8) as well as a trend in the increase of *kcnd3* (mCREB: 1.61±0.277; LacZ: 1.189 ±0.137, p=0.07, n=14-22). In addition, the social isolation condition showed a significant increase in *kcnd3* (SH: 1.203 ± 0.0859; DH: 1.001 ± 0.038, p=0.028, n=9-10) and trends in *kcnj2* (SH: 1.384 ± 0.209; DH: 1.019 ± 0.0662, p=0.065, n=9-10) and *kncs2* (SH: 1.258 ± 0.099; DH: 1.042 ± 0.086, p=0.062, n=9-10).

These findings, along with the selective anxiety-like phenotype induced by mCREB overexpression in NAcSh, provide further support for our hypothesis that social isolation increases anxiety-like behaviors via the downregulation of CREB and the subsequent upregulation in K^+ channels in this brain region.

Discussion

The present study shows that prolonged social isolation of adult animals causes reward-related deficits as well as an anxiety-like phenotype. Both abnormalities are reversed by chronic, but not acute, imipramine treatment. These findings thereby establish social isolation as a novel animal model of depression- and anxiety-like behavior that responds uniquely to chronic antidepressant administration. The generation of animal models of mood disorders that respond, like the human conditions, to chronic antidepressant treatment has been a major goal for the field (Nestler et al., 2002a; Duman and Monteggia, 2006). Recent research has shown that novelty-suppressed feeding (Dulawa and Hen, 2005) and social defeat (Berton et al., 2006) also show unique responses to chronic administration of antidepressants and, interestingly, both of these models involve a combination of depression- and anxiety-like symptoms. Social isolation, as described here, represents an additional model, however, one that is uniquely related to passive as opposed active stress and, therefore, potentially related to subtypes of human depression and anxiety syndromes related to isolation. Several studies have shown social isolation in obese adolescents, adults with hearing loss, and the elderly population, with symptoms of depression and anxiety (Costello and Kendrick, 2000; Arlinger, 2003; Sjoberg et al., 2005; Chou et al., 2006; Heinrich and Gullone, 2006; Robinson, 2006; Choi and McDougall, 2007; Owen, 2007). The present animal model begins to elucidate the mechanisms in depression and anxiety due to isolation, which may differ from the mechanisms underlying depression and anxiety produced by active stress. These

differences may lead to better therapeutics for the different subtypes of depression caused by active stress versus isolation.

Results of the present study especially provide unique information about the molecular basis of the anxiety-like symptoms induced by social isolation. Here it is shown that social isolation decreases CREB activity in the NAcSh, an effect reversed by chronic imipramine, and that reduced levels of CREB mediate the anxiety-like, but not anhedonia-like, behavior seen under these conditions. Overexpression of CREB in isolated animals restores only the anxiety-like phenotype and does not affect the deficits seen in reward-related behavior. Conversely, overexpression of the dominant negative mutant mCREB causes only anxiety-like behavior and does not result in anhedonia-like symptoms. In fact, previous work has shown that decreased CREB activity in the NAcSh, achieved via overexpression of mCREB, in non-isolated animals increases responses to rewarding stimuli, while increases in CREB, achieved via CREB overexpression, show decreased responses (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002). In addition, these overexpression systems modulate anxiety-like behavior in non-isolated animals, with mCREB increasing anxiety and CREB decreasing it (Barrot et al., 2002). Prior work has also shown that mCREB causes antidepressant-like responses in the forced swim and learned helplessness tests, with CREB causing pro-depression-like responses (Pliakas et al., 2001; Newton et al., 2002). Together, these findings support a model where social isolation decreases CREB activity in the NAcSh and this mediates the increase in anxiety-like behavior, while the restoration of CREB activity induced by antidepressant treatments reverses those anxiety symptoms. In

contrast, the anhedonia-like phenotype induced by social isolation, which is also reversed by antidepressant treatment, is not solely related to CREB activity in the NAcSh and is presumably mediated by other molecular pathways in this brain region or by other neural circuits.

Although the increase in ejaculation latency is interpreted here as a deficit in sexual reward, it has not been previously reported as a depressive phenotype per se. However, in other animals models which produce depressive-like phenotypes, (induced by LPS injections or methylphenidate treatment as pups), an increase in ejaculation latency is also reported (Yirmiya, 1996; Bolanos et al., 2003). Interestingly, chronic imipramine in non-isolated control animals causes an increase in ejaculation latency, similar to that seen in isolated animals. This is in agreement with human reports, as well as rodent findings, of sexual side effects of antidepressant medications (Rothschild, 2000; Ferguson, 2001). This finding dramatically parallels differences in antidepressant responses seen in normal humans and those with some stress-related disorders. Thus, antidepressants do not produce mood elevating or anxiolytic effects in normal individuals, and sexual deficits caused by depression or anxiety can be alleviated by these treatments in affected individuals. Likewise, chronic imipramine decreases CREB activity in the NAcSh of normal animals, but increases CREB activity in socially isolated animals. It will be interesting in future studies to understand the molecular basis by which chronic imipramine causes opposite effects on CREB activity in the NAcSh, depending on the behavioral history of the animal, since such information could provide novel insight into

the unique effects of long-term antidepressant administration in human patient populations.

Microarray analysis of NAcSh from single-housed versus double-housed animals revealed several genes of interest, such as upregulation of activating transcription factor 2 (ATF2), protein kinase C-epsilon (PKC epsilon) and Ca^{2+}/calmodulin-dependent protein kinases I and IV, (CaMKI and IV), all of which play important roles in CRE-mediated transcription (Shaywitz and Greenberg, 1999; Lonze and Ginty, 2002; Carlezon et al., 2005). It is also striking that while social isolation shows much more upregulation of genes, CREB and imipramine, both in the isolated and control groups, show predominately downregulation. The CREB condition also has a greater number of genes regulated than the imipramine condition in both groups of animals. In addition, it is interesting to note how the genes regulated by social isolation are changed by imipramine and CREB. Comparing gene regulation induced by isolation, imipramine in isolation nearly shows an opposite effect on regulation, while CREB only produces a subset of these changes (as seen in Fig. 5).

Most striking is the amount of overall gene regulation in the isolated compared to double-housed animals under CREB or imipramine differences. There are roughly half as many genes being regulated in the isolated condition with CREB or imipramine than there are in double-housed condition under the same treatments. This finding suggests an overall dampening of gene regulation in the isolated condition, which is consistent with upegulation of histone deacetylase 4 (HDAC4) after social isolation as revealed by the

microarray data. HDACs are known to inhibit gene transcription by removing acetyl groups from histones on DNA, thereby tightening DNA and making it inaccessible to transcription factors (Tsankova et al., 2007). HDAC4 is a major Class II HDAC in brain, which has been shown previously to block the rewarding effects of cocaine when overexpressed in the NAc (Kumar et al., 2005). These results raise the possibility that some of the behavioral symptoms induced by social isolation may be mediated by regulation of HDAC4 expression, or overall repression of gene transcription. Although regulation of HDAC4 is not specifically reversed by CREB overexpression in the SI condition, other HDAC related genes are downregulated, including HDAC10, Histone H2A and SWI/SNF 4 as shown by the microarrays. Further, the overall patterns of gene expression suggest that while CREB overexpression exerts generally similar effects on gene regulation in the NAcSh of socially isolated versus normal animals, the effects of chronic imipramine are very different. This further underscores the unique effects of imipramine in stressed individuals as noted above.

The downregulation of genes involved in glutamatergic neurotransmission, NR1, NR1A, NR2B and GluR2, as well as the upregulation of mGluR8, a Group III metabotropic glutamate receptor, seen in the array lists of isolated animals with CREB and imipramine treatment (Table 1) supports the hypothesis of glutamate antagonists producing alleviations to certain psychiatric conditions (Manji et al., 2003; Javitt, 2004). It has been shown that antagonists of NMDA receptors in certain brain regions can lead to antidepressant-like effects (Paul and Skolnick, 2003) and that antagonists of Group I mGluRs (Cosford et al., 2003) or agonists of Group III mGluRs (Palucha et al., 2004) can

produce anxiolytic effects. Perhaps CREB overexpression or imipramine treatment leads to regulation of these receptors, resulting in an anxiolytic-like phenotype.

The microarray findings also led to the investigation of the electrophysiological properties in the NAcSh after social isolation. Based on the upregulation of several K^+ channels in this region in socially isolated animals, and the reversal of this upregulation by either CREB or imipramine, it was hypothesized that dampening the electrical excitability of NAcSh neurons may be a crucial step in molecular events by which social isolation induces anxiety-like symptoms, but not anhedonia. Indeed, it was shown that social isolation increased the hyperpolarization of NAcSh neurons, consistent with an upregulation of K^+ channel expression, and that artificially mimicking this effect, via viral-mediated overexpression of a K^+ channel in this region in non-isolated animals, mimics the increase in anxiety-like symptoms, but not the anhedonia-like symptoms, induced by social isolation. It is shown further that downregulation of CREB activity in the NAcSh, via overexpression of mCREB, is sufficient to increase levels of some of these K^+ channels also upregulated by social isolation. Together, these findings define a specific molecular pathological pathway by which social isolation, via downregulation of CREB activity and upregulation of K^+ channel expression, induces anxiety symptoms. It must also be noted that in previous studies upregulation of K^+ channel expression also led to increased sensitivity to drug reward (Dong et al., 2006). While the present study shows a strong anxiety phenotype, but no differences in natural-reward behavior, perhaps a stronger stimulus such as cocaine would produce differences with K^+ channel overexpression.

Work of the present study provides new insight into the role of CREB in the NAcSh as a crucial regulator of responses to emotional stimuli (Fig. 8). Prior work has demonstrated that drugs of abuse and active stress induce CREB activity in this region and that this "high CREB" state is associated with a general blunting of emotional responding, including anhedonia-like symptoms and reduced anxiety-like behavior. In contrast, prolonged social isolation during adulthood decreases CREB activity in the NAcSh, and this "low CREB" state is associated with emotional hyper-reactivity, including profound anxiety. This role for CREB in the NAcSh as an "emotional rheostat" is consistent with CREB's role as an electrophysiological rheostat of NAc neuronal excitability, with increased CREB increasing NAc excitability and decreased CREB decreasing it, as shown by Dong et al. (2006) and in the present study as well. Future efforts will investigate if chronic administration of imipramine is able to normalize CREB activity at both extremes: preventing an increase in CREB activity from active stress, and as already demonstrated, increasing it when in the low state. In this way, we hypothesize that antidepressants may reverse anhedonia-like symptoms by preventing induction of CREB activity during periods of active stress, and reversing anxiety-like symptoms when CREB activity is low. Clearly, the situation is far more complex, with anxiety and depression symptoms being regulated by numerous molecular mediators not only in the NAc, but in several other brain regions as well (Brown et al., 1999; Walf and Frye, 2006; Warner-Schmidt and Duman, 2006). Nonetheless, the present findings further characterize the long-term sequelae of social isolation, and provide novel insight into the neural and

molecular mechanisms that distinguish anxiety and depression symptoms in a chronic stress model.

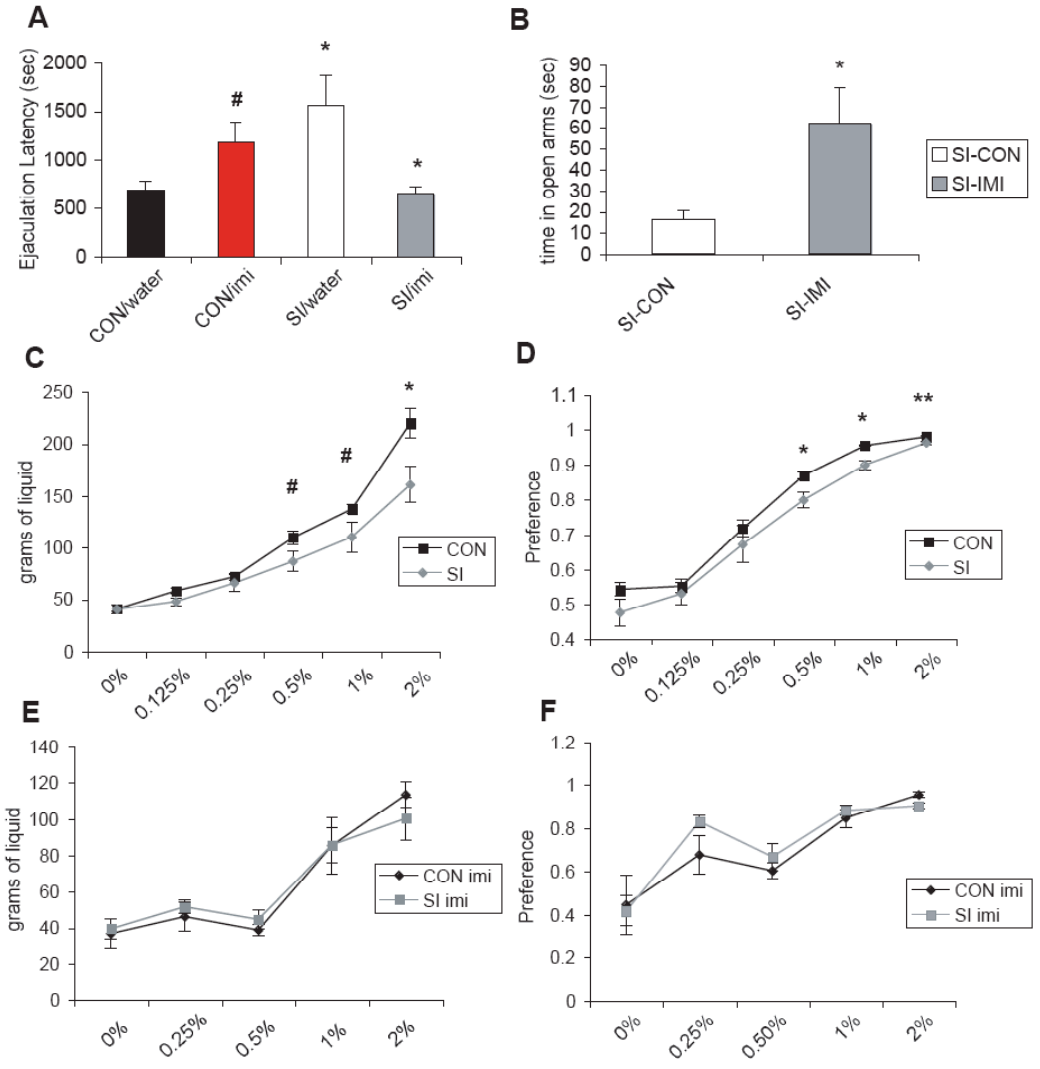


Figure 3.1: SI induces reward behavior deficits.

(A) An increase in ejaculation latency compared to CON/water animals is observed in CON/IMI and SI animals, however under imipramine administration, the sexual behavior deficit in SI animals (SI/IMI) is reversed (*$p<0.040$, n=5-8) while double housed imipramine (CON/imi) animals show a trend in increased ejaculation latency (#$p<0.11$, n=5-7).
(B) Anxiety-like phenotype in isolated animals is also reversed by imipramine (n=8, *$p<0.05$).
(C) Isolated animals (SI) show decreased sucrose intake (n=12-13, #$p<0.1$, *$p<0.01$) and
(D) sucrose preference (n=12-13, *$p<0.01$, **$p<0.002$) compared to control double housed (CON) animals. Total liquid consumed is not significantly different for all concentrations, except 2% where sucrose intake is much higher in CON animals.
(E) SI animals after chronic imipramine administration (SI IMI) do not show any differences in sucrose intake
(F) or sucrose preference compared to CON imipramine (CON IMI) animals ($p>0.2$; n=6-8).

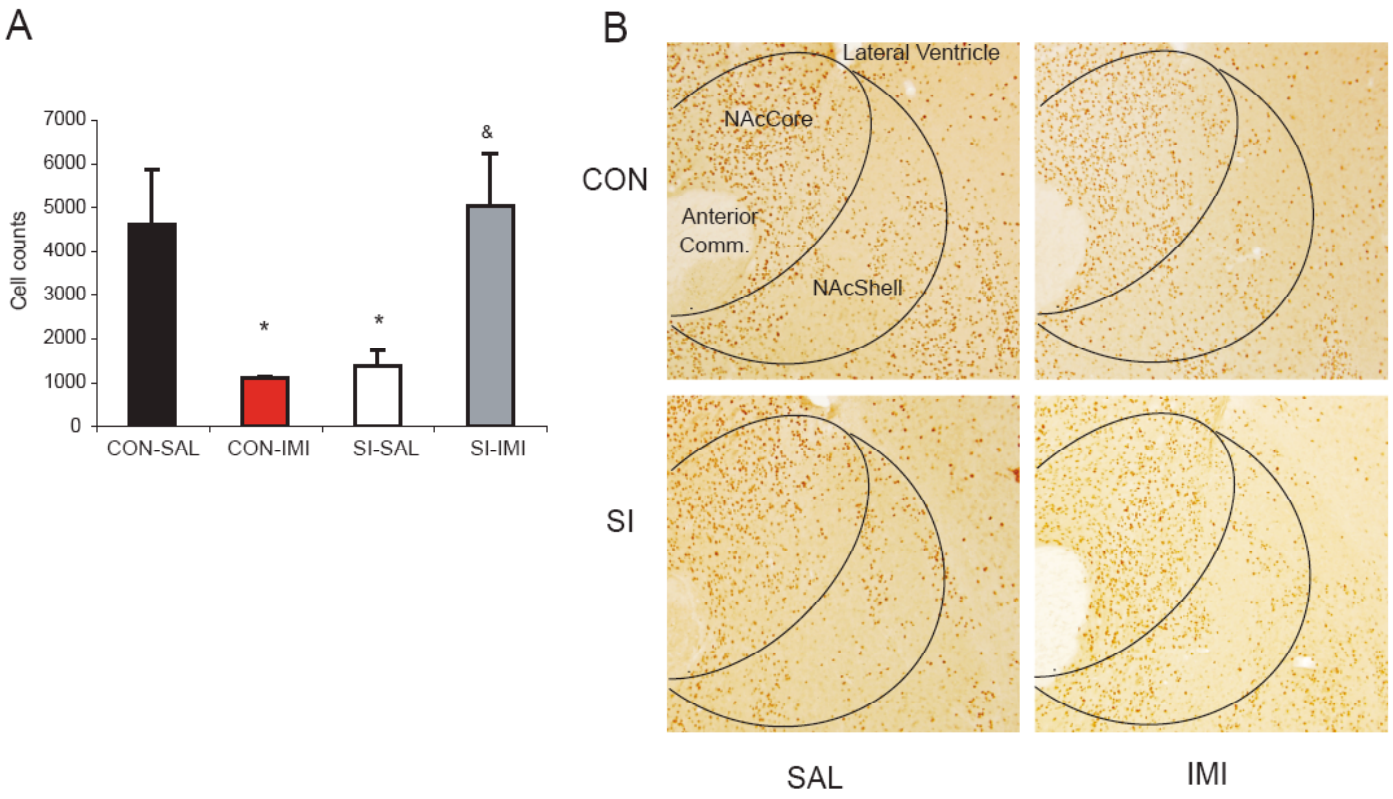


Figure 3.2: Social isolation and imipramine modulates CRE-Activity in the NAcSh.

(A) CON (group housed) animals under chronic imipramine administration (i.p. 10mg/kg) (n=3, *$p<0.05$), as well chronic social isolation (SI) (n=3, *$p<0.05$), decreases CRE-activity compared to CON-SAL, while chronic administration of imipramine in SI (n=3, $^{\&}p<0.05$) increases CRE-activity compared to SI-SAL. (B, top L to R) CON-SAL and CON-IMI (B, bottom L to R) SI-SAL and SI-IMI

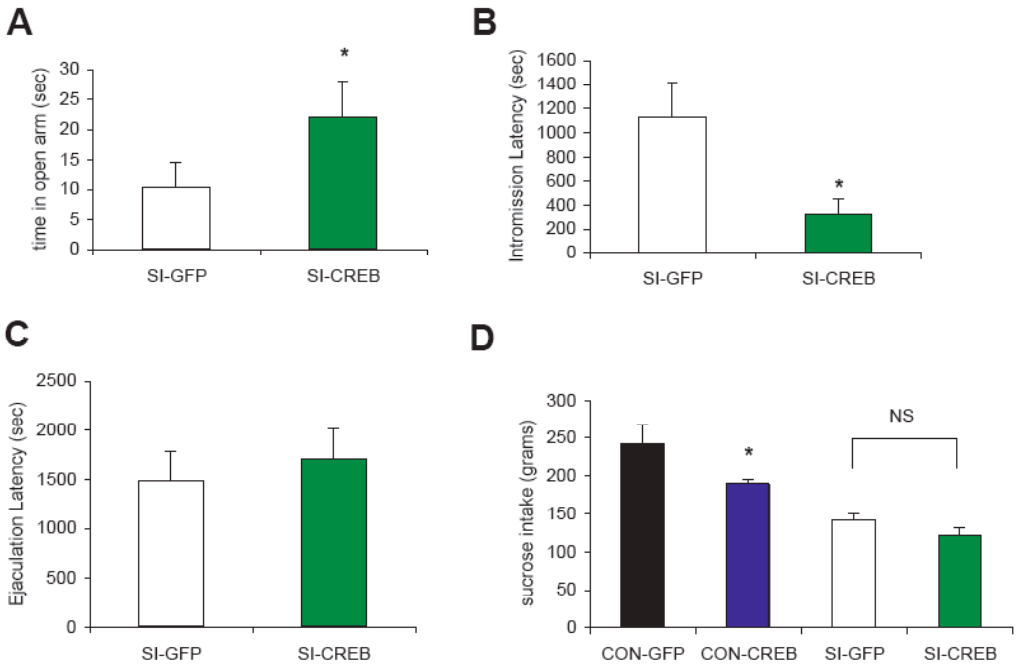


Figure 3.3: Increasing CREB in SI reverses anxiety-like behavior but not reward-related deficits.

(A) Increasing CREB in NAcSh by overexpression via AAV-viral mediated gene transfer decreases anxiety-like behavior in EPMZ (n=12-13, *p<0.05) and
(B) in naïve males initiation of sexual behavior (*p<0.015, n=7-8),
(C) but does not affect ejaculation latency (p>0.33, n=5-7) or
(D) sucrose intake (shown here at one percent); (SI:p>0.13, n=13).
Conversely, CREB overexpression in CON animals decreases sucrose intake
(CON-GFP vs CON-CREB:*p<0.045, n=4).

SI vs DH

up (303)

gene	p-value	fold Δ
ATF2	0.021	1.235
PKC, epsilon	0.050	1.284
CamK1	0.033	1.310
CamK4	0.001	1.368
adrenergic aR2c	0.009	1.325
casein k 1eps	0.041	1.394
cl channel 3	0.004	1.239
Kcns2	0.032	1.231
Kcnj4	0.017	1.283
Kcnma1	0.027	1.245
Kcnd3	0.028	1.246
Kcna1	0.044	1.261
Kcnq3	0.042	1.362
Hdac4	0.014	1.349
Jak2	0.005	1.238

down (3)

Calcineurin	0.038	1.502
neuronatin	0.004	1.294

SI CREB vs SI gfp

up (35)

gene	p-value	fold Δ
Grm8	0.040	1.555
cacng5	0.040	1.330

down (471)

PKC epsilon	0.014	1.300
Nts	0.014	1.830
Ache	0.023	1.396
IL gf	0.043	1.442
kcnc3	0.037	2.290
Cacna1a	0.012	1.370
Gabrg3	0.042	1.496
Histone H2a	0.045	1.755
HDAC10	0.0002	1.339
SWI/SNF 4	0.021	1.419
hsf1	0.024	1.431
Grina	0.039	1.305
Grin2b	0.008	1.305
COMTD	0.038	1.480
CART	0.024	1.495
VGF	0.034	1.650
GPR88	0.031	1.538
Interleukin13F	0.042	1.382

SI IMI vs SI control

up (25)

gene	p-value	fold Δ
PP1R15b	0.043	1.896
Gsr	0.025	1.439
neuroregulIn2	0.031	1.348
Spata7	0.041	1.319

down (125)

MapKKK10	0.037	1.670
Map3k3	0.025	1.397
Grinl1a	0.015	1.376
Gria2	0.047	1.324
Dgka	0.024	1.417
Gpr48	0.016	1.316
Bhlhb9	0.015	1.359
BCL2-atf1	0.014	1.495
sertolin	0.038	1.528
Bmpr1a	0.025	1.684

DH CREB vs DH gfp

up (70)

gene	p-value	fold Δ
IRS1	0.023	1.636
TGFB IEGR3	0.032	1.695
cacng5	0.007	1.420

down (932)

Htr4	0.038	2.030
TH	0.016	1.436
Adora2a	0.048	1.852
Prkch	0.044	1.740
Drd1a	0.032	1.852
Ppp1r1b	0.042	1.960
Pde10a	0.041	1.705
Pde1c	0.011	1.673
Camk4	0.045	1.559
Pde4b	0.045	1.550
AC 5	0.023	1.879
CART	0.044	1.825
JunB	0.029	1.801
kcnj4	0.024	1.588
K chmf1	0.013	1.313
kcnc3	0.016	1.377
kcna4	0.024	1.588
kcnab1	0.047	1.761
cacna2d3	0.016	1.755
Kcnip2	0.028	1.628
Grm5	0.006	1.393
Grm4	0.008	1.440
Cdk6	0.011	1.852
RGS2	0.007	1.544
RGS3	0.027	1.775
RGS8	0.037	1.634
RGS9	0.044	1.879
RGS10	0.028	1.476
GPR6	0.032	1.764
GPR88	0.039	1.889
GPR149	0.041	1.961
Gad1	0.041	1.702
Dgkb	0.049	1.557

DH IMI vs DH con

up (144)

gene	p-value	fold Δ
Htr3b	0.026	1.329
Htr4	0.022	2.801
Prkch	0.018	1.526
Ache	0.038	1.355
Ppp1r1a	0.029	1.375
Drd1a	0.049	1.467
PDE 1B	0.009	1.411
Adora2a	0.034	1.387
kcnh4	0.039	1.350
Ppp1r2	0.013	1.390
GPR149	0.020	1.684
Nts	0.021	1.763

down (253)

Adra2a	0.045	1.773
Gria3	0.032	1.671
kcnmb4	0.014	1.539
Homer1	0.040	1.494
TGFB-IEGr	0.031	1.450
GPR51	0.046	1.536
Calb1	0.037	1.512

Table 3.1: Differential gene regulation and potential gene targets of DH and SI animals with CREB and imipramine treatment.

Table 3.1 Definitions:

Ache: Acetylcholinesterase; **AC:** Adenylate cyclase; **Adora:** Adenosine A receptor; **Adr:** Adrenergic receptor; **ATF:** Activating Transcription Factor; **BCL2-atf:** BCL2-associated transcription factor; **Bhlhb:** basic helix-loop-helix domain; **Bmpr:** Bone morphogenetic protein receptor; **Cacna2d3:** Calcium channel, voltage-dependent, alpha2/delta3; **Cacng:** calcium channel, voltage-dependent, gamma; **Calb:** calbindin; **CamK:** Ca2+ Modulating Kinase; **CART:** cocaine and amphetamine regulated transcript; **cdk:** cyclin-dependent kinase; **Cl:** Chloride; **COMTD:** catechol-O-methyltransferase domain; **Dgk:** Diaceylglycerol kinase; **Drd:** Dopamine receptor; **Gabrg:** Gamma-aminobutyric acid (GABA) A receptor; **Gad:** glutamate decarboxylase; **Gria:** AMPA glutamate receptor; **Grina:** NMDA glutamate receptor; **GPR:** G-protein coupled receptor; **Grm:**metabotropic glutamate receptor; **Gsr:** Glutathione reductase **Hdac:** histone deacetylase; **Hsf:** heat shock transcription factor; **Htr:** 5-hydroxytrptamine (serotonin) receptor; **IL gf:** insulin like growth factor; **IRS:** insulin receptor substrate; **Jak:** Janus kinase; **JunB:** Jun-B oncogene; **kcna:** potassium voltage-gated, shaker-related; **kcnc:** potassium voltage-gated, Shaw related; **kcnd:** potassium voltage-gated channel, Shal-related; **kcnh:** potassium voltage-gated channel, subfamily H; **kcnip**: kv channel-interacting protein; **kcnj:** potassium inwardly-rectifying channel, subfamily J; **kcnm:** potassium large conductance calcium-activated channel, subfamily M; **kcnq:** potassium voltage-gated channel, subfamily Q; **kcns:** potassium channel (kv9.2); **MapKKK (Map3K)**: Mitogen activated protein kinase kinase kinase; **Nts;** Neurotensin; **Pde:** phosphodiesterase; **PKC:** Protein Kinase C; **Prkch:** Protein Kinase A, eta; **Ppp:** protein phosphatase; **RGS:** regulator of G-protein signaling; **spata:** spermatogenesis associated; **TGFB IEGR:** TGFB inducible early growth response; **TH:** tyrosine hydroxylase; **VGF:** VGF nerve growth factor

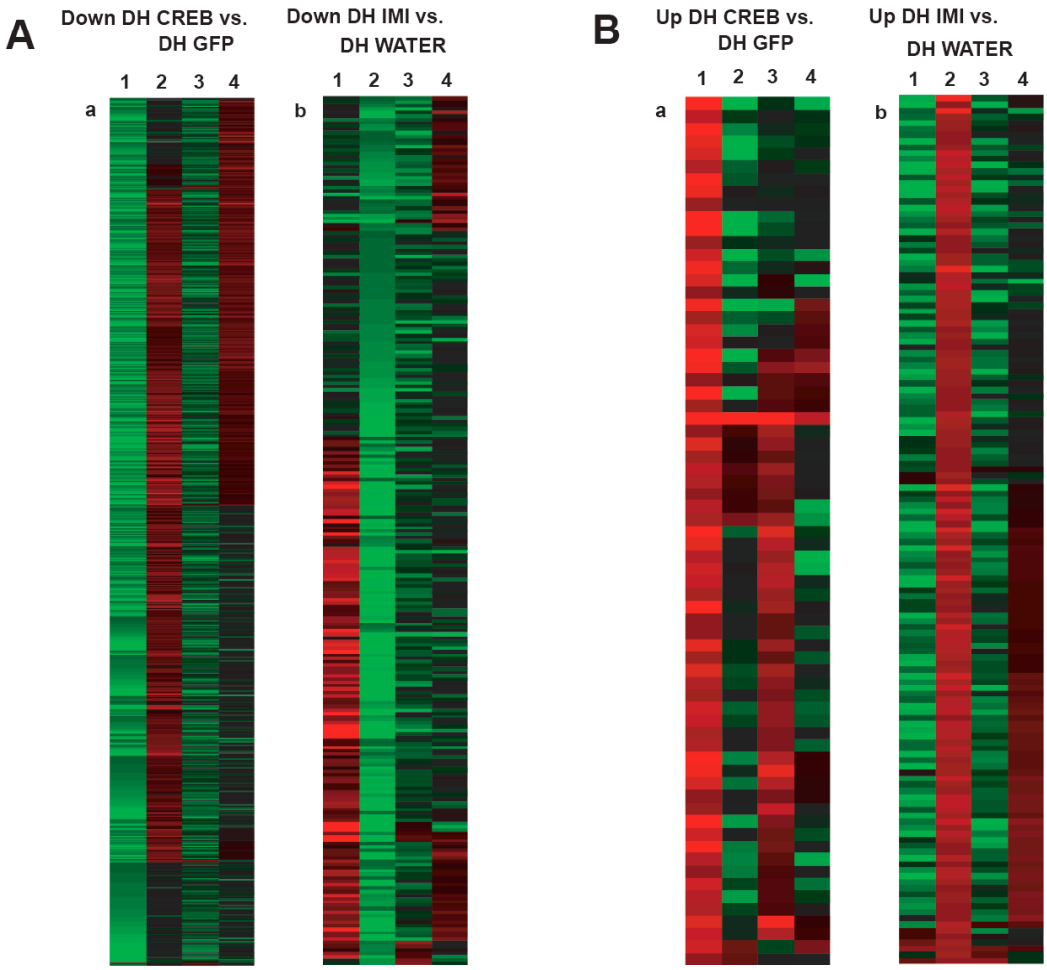


Figure 3.4: Differential gene regulation with CREB and Imipramine treatment in DH and SI rats.
(A) Genes that are downregulated in DH rats with a) CREB or b) Imipramine treatment.
(B) Genes that are upregulated in DH rats with a) CREB or b) Imipramine treatment.

Column 1) DH-CREB vs DH-GFP
Column 2) DH-IMI vs DH-WATER
Column 3) SI-CREB vs SI-GFP
Column 4) SI-IMI vs SI-WATER

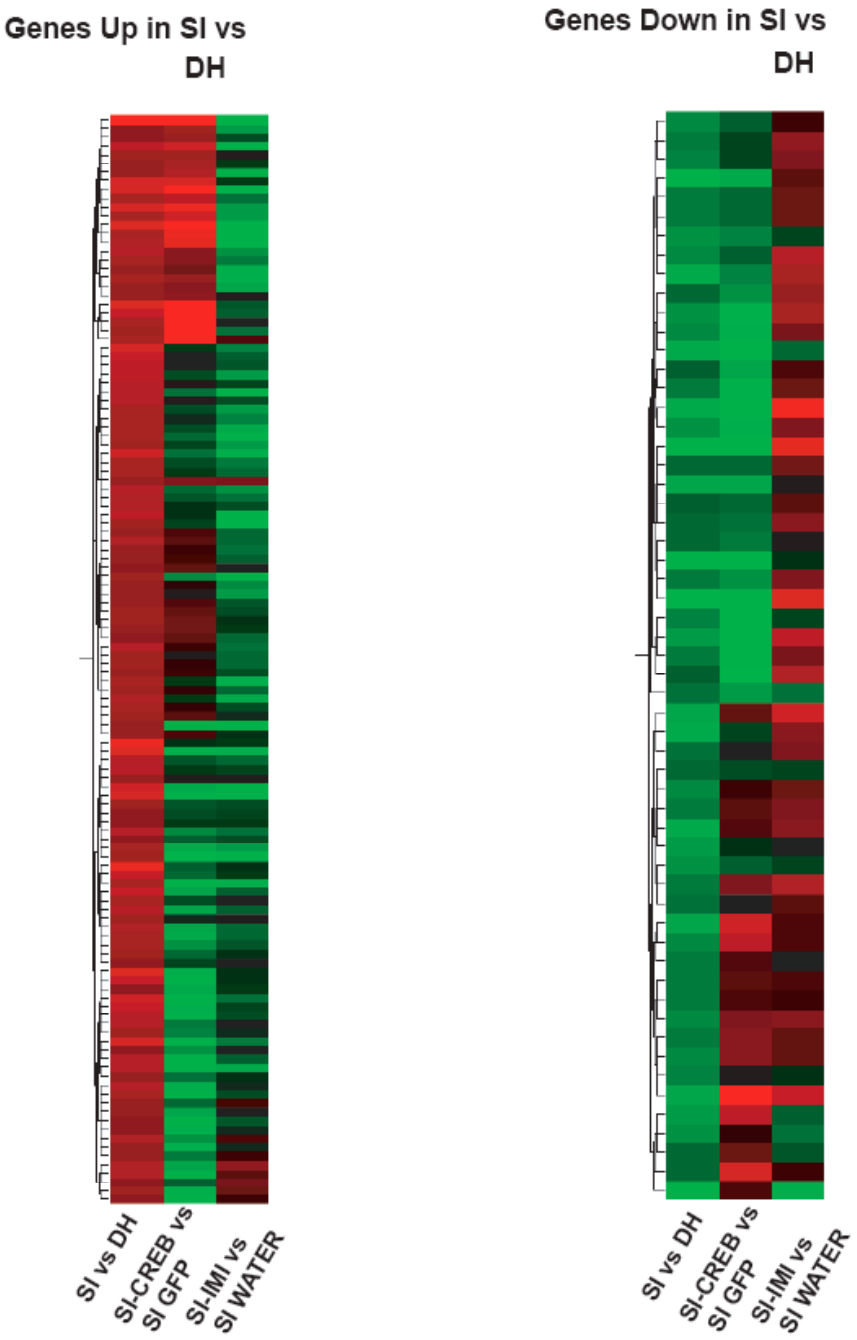


Figure 3.5: Patterns of gene regulation in SI vs DH animals.

Genes (A) upregulated or (B) downregulated under SI conditions are only partially reversed with CREB overexpression, but show much more overall reversal in regulation by imipramine treatment.

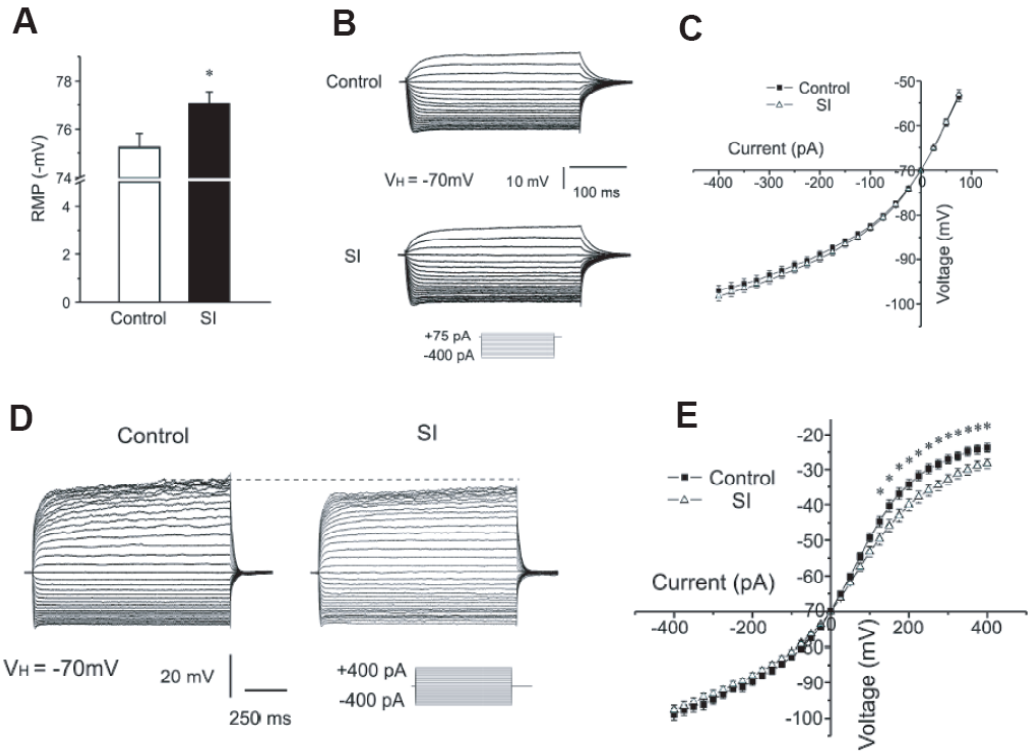


Figure 3.6: Effect of SI on resting membrane potential (RMP) and current-voltage relationship.

(A) SI significantly reduces RMP (n=21-25, *p<0.05).
(B) Sample traces obtained by step current injection.
(C) No differences are found in current-voltage relationship under normal conditions (n=21-24, p>0.4).
(D) Sample traces recorded from control and SI groups in the presence of Na+ and Ca2+ blockers, held at -70 mV.
(E) SI significantly decreases membrane input resistance (n=13-15, *P<0.01).

Recordings were performed using whole-cell current clamp configuration. RMP was measured under the condition of no current injection immediately after switching from voltage clamp to current clamp. Current-voltage curve was obtained in normal ACSF (A-C) or in presence of Na^{+} and Ca^{2+} channel blockers (D and E) when cell membrane was held at -70mV by current injection.

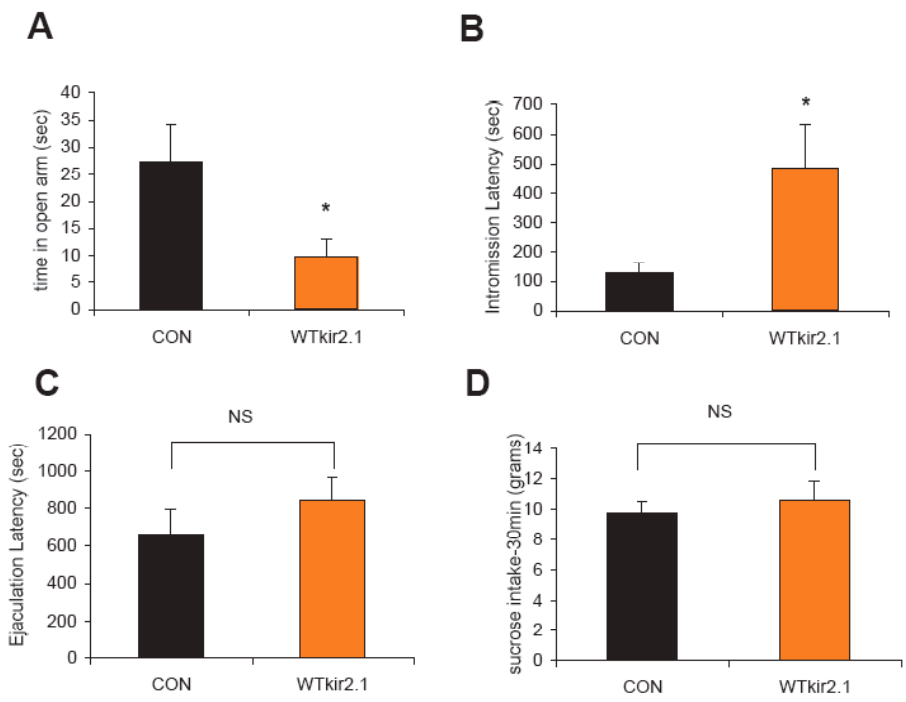


Figure 3.7: Overexpression of wildtype inward rectifying potassium channel kir2.1 in NAcSh of non-isolated animals leads to an anxiety-like phenotype.

(A) Anxiety phenotype is observed with kir2.1 overexpression in NAcSh of non-isolated animals in elevated plus maze (n=9-12, *$p<0.03$,) and
(B) increased latency for approach in sexual behavior (n=5, *$p<0.05$), but no differences in
(C) ejaculation latency ($p>0.33$, n=5) or
(D) sucrose intake ($p>0.8$, n=17-18).

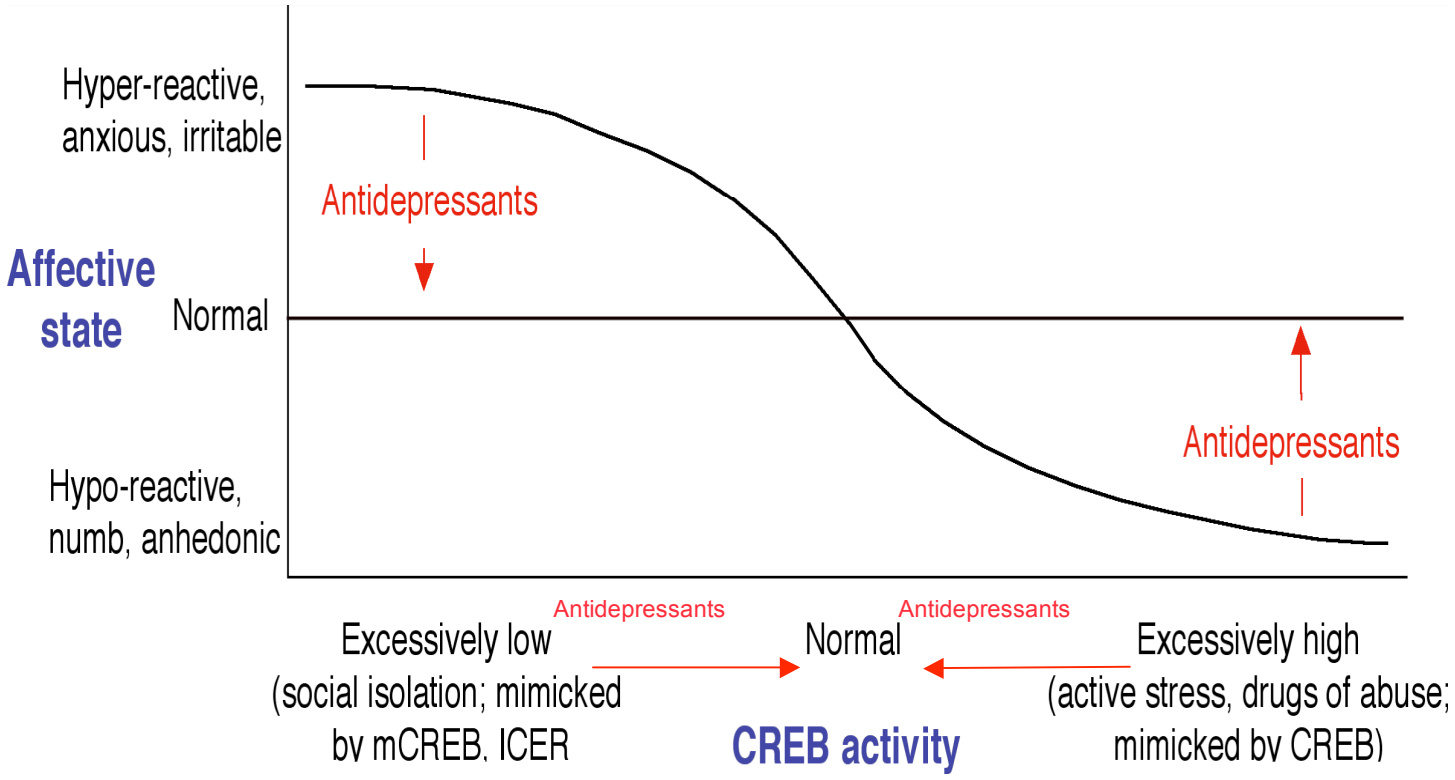


Figure 3.8: Model of CREB as a "rheostat of emotion" in the NAc.

Excessively low or excessively high states of CREB activity (x-axis) can lead to behavioral abnormalities (y-axis), which are restored to normal behavioral affective states with antidepressant treatment. In addition, excessively low CREB activity (as seen with SI) can be restored to normal by antidepressants and it is hypothesized that pretreatment with antidepressants can prevent the excessively high state of CREB seen with active stress.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

The role of CREB in complex behavior

Over approximately the past 30 years, the role of cAMP and CREB have been the focus of learning and memory research (Kandel, 2001). This research has led to the finding that CREB is induced in the hippocampus with learning, leads to the formation of long-term memory and can act to enhance memory as well increase synaptic strength and plasticity (Dash et al., 1990; Frey et al., 1993; Huang et al., 1994; Yin et al., 1994; Yin et al., 1995). More recently, CREB in the hippocampus was discovered to also play a role in antidepressant action, with increases seen in CREB after antidepressant administration, and the overexpression of CREB leading to antidepressant effects (Thome et al., 2000).

The role of CREB in NAc: Drugs of Abuse

Studies of the role of CREB in the NAc have produced many interesting findings. CREB overexpression in drug reward-related behavior as measured by CPP is decreased while mCREB increases the conditioned drug response (Carlezon et al., 1998). However, CREB overexpression in cocaine self-administration behavior produces different findings. Blocking CREB by using CREB antisense oligonucleotide infusions in the NAc produces downward shifts in self-administration of cocaine (Choi et al., 2006). This study also found CREB antisense oligonucleotides increased the threshold dose required for reinstatement (Choi et al., 2006). In addition, studies of self-administration using

PKA inhibitors produced similar results in reducing baseline cocaine self-administration but shifting the dose response curve to the left indicative of increased sensitivity to the drug (Self et al., 1998). The self-administration and CPP studies, when viewed together, indicate that CREB, while decreasing sensitivity to the rewarding effects of a drug, may actually augment cocaine intake by requiring more drug to obtain its rewarding effects. When CREB is blocked in the self-administration paradigm, the drug intake is overall decreased. However, blocking CREB in the CPP paradigm leads to an enhancement in drug reward. This may indicate that blocking CREB increases the satiety for the drug, producing less self-administration behavior because the animals do not need as much drug to feel the effects. It is interesting to note that, although CREB manipulation in the NAc does not drastically change sexual behavior in experienced male animals, an increase in the PEI (post-ejaculation interval) was observed with mCREB overexpression (see Chapter Two). This parameter indicates increased sexual satiety before the male is able to engage in additional copulation (Hull and Dominguez, 2007). It is interesting to compare this behavior with the self-administration behavior observed with CREB antisense oligonucleotide infusions, and the increased time between each injection (Choi et al., 2006).

Since CREB is also increased with drug exposure in the NAc (Barrot et al., 2002; Shaw-Lutchman et al., 2003), it is possible that the cycle of drug craving is reinforced by CREB, with drug intake leading to an increase in CREB, and increased CREB levels producing decreased sensitivity to the drug, requiring higher amounts to reach the behavioral rewarding effect. This cycle reinforces the addictive phenotype.

The role of CREB in NAc: Depression and anxiety-like behaviors

In addition to its role in drug reward, CREB in the NAc has also been shown to play a role in depressive-like behaviors. CREB is induced not only with drugs of abuse but also with stress-related paradigms (Barrot et al., 2002), and these stress paradigms can lead to behavioral phenotypes related to depression (Papp et al., 1991; Dalvi and Lucki, 1999; Nestler et al., 2002a; Duman and Monteggia, 2006). In addition, overexpression of CREB produces depressive-like phenotypes in decreased latency to immobility and overall decreased time swimming in the FST (Pliakas et al., 2001). Conversely, mCREB and ICER overexpression have the opposite effect (Barrot et al., 2002; Green et al., 2006). In relation to natural reward, CREB overexpression also decreases sucrose preference, while mCREB and ICER increase it (Barrot et al., 2002; Green et al., 2006). The reduced sucrose preference in CREB animals indicates anhedonia, a loss of interest in pleasurable stimuli, which is another hallmark of depression (American Psychiatric Association 2000). Transgenic animals lacking CREBαΔ also show modulation of depressive- and anxiety-like traits, with an increase in conditioned reward behavior to cocaine, antidepressant-like effects in the FST and TST behavioral tests, increased anxiety and increased alcohol intake (Walters and Blendy, 2001; Conti et al., 2002; Pandey et al., 2005; Blendy, 2006).

Another characteristic of CREB overexpression is reduced sensitivity to not only rewarding stimuli, but also aversive stimuli (Barrot et al., 2002). Overexpression of

CREB in the NAc leads to a decrease in anxiety-like behavior, however, only under anxiogenic producing circumstances. In contrast, mCREB and ICER enhance this anxiety (Barrot et al., 2002; Green et al., 2006). CREB modulation also produces differences in behavior to aversive stimuli, such as foot shock. CREB overexpressing animals require more intensity in the foot shock apparatus for a reaction to occur, while mCREB animals are much more sensitive to the aversive effects (Barrot et al., 2002).

The role of CREB in social isolation: The other side of CREB

Social Isolation and Anxiety

Previous studies, along with the novel findings presented here, have implicated social isolation as a model which induces anxiety-like phenotypes (Jankowska et al., 1991; Maisonnette et al., 1993; Vasar et al., 1993; Ahmed et al., 1995). Although the role of social isolation in depression-like behaviors has been unclear in the past, this present work also provides evidence for social isolation as a depression model. Reports on the changes in neurotransmitters and receptors have been variable as well in adulthood social isolation (Hall, 1998), but the current work shows clearly a reduction in CRE-mediated transcription in the NAc, with consistent results across multiple studies, using two lines of CRE-LacZ animals. Because previous work shows an anxiety phenotype induced with mCREB (Barrot et al., 2002), it was not surprising that socially isolated animals, with lower levels of CRE-activity, would be more anxious on the elevated plus maze. However, it was surprising that they would show an anxiety-like behavior in approach of a novel yet rewarding stimulus, such as an estrous female (Barrot-Wallace et al., 2005).

This anxiety was clearly CREB-mediated, due to the fact that mCREB in non-isolated animals produced the same anxiety-like behavior, and this anxiety was reversed by a low dose of diazepam. Also in isolated animals, with lower CREB activity, the anxiety-like behavior was reversed by wildtype CREB overexpression (Barrot-Wallace et al., 2005).

Isolation, Imipramine and Sexual Deficits

The most complexing phenotype however, was the increased latency for ejaculation observed in the isolated animals. Previous studies indicating depressive-like phenotypes induced by LPS injections or methylphenidate exposure as pups also led to increases in ejaculation latencies (Yirmiya, 1996; Bolanos et al., 2003). These findings suggested that perhaps the increase in ejaculation latency was a depressive-like phenotype observed in the isolated animals. The fact that imipramine restored the ejaculation latency in the isolated animals suggested that this may indeed be a depression-like behavioral deficit. In addition, in two-bottle choice tests, isolated animals consistently showed decreases in sucrose preference and sucrose intake, indicating a depressive-like phenotype. However, isolated animals on chronic imipramine administration drank comparable levels to their control, double-housed counterparts.

Imipramine and CREB activity

It was also interesting to note that in isolated animals, CRE-activity was reduced in the NAc, but with imipramine treatment, the CRE levels were restored. The hypothesis that excessively low levels of CREB in the NAc may lead not only to an anxiety phenotype,

but perhaps also a depression phenotype led to testing the effect of overexpressing CREB in the NAc. Although the previous study had showed no change in ejaculation behavior of isolated animals with or without CREB overexpression, this expression was short-acting due to the HSV temporal constraints (Barrot-Wallace et al., 2005). Using an AAV virus, we were able to express CREB for 4 weeks, the time it takes for imipramine to produce its effects in the social isolation and other animal models as well as in clinical populations (American Psychiatric Association 2000). However, AAV-CREB overexpression also showed no differences in ejaculation latency in the isolated animals. The isolated AAV-CREB animals did show faster approach behavior than the isolated AAV-GFP control virus animals, which replicates the finding that CREB restoration in isolated animals leads to reversal of anxiety-like effects. In addition, isolated animals on chronic imipramine had shown increases in CRE-activity in the NAc, as well as decreased anxiety on the elevated plus maze. Taken together, these studies indicated that decreased levels of CREB in the NAc induced by social isolation lead to a strong anxiety-like effect that is reversed by chronic imipramine and by overexpression of CREB. However, CREB overexpression was not able to reverse the depressive-like symptoms induced by prolonged social isolation, which indicates that this phenotype, while it correlates with CREB regulation in the NAc, is mediated via CREB-independent mechanisms.

Electrophysiology of Social Isolation

A pilot study of microarray analysis between control grouped housed animals or socially isolated single housed animals suggested possible mechanisms by which downregulation

of CREB may result in anxiety-like effects. Upregulation of several types of potassium channels in the NAc of isolated animals led to the testing of the electrophysiology of these neurons. An increase in hyperpolarization of the resting membrane potential was found in the isolated condition, indicating a stronger driving force required for neuronal firing. In addition, a decrease in membrane resistance was also found in the isolated condition, consistent with an increase in ionic conductance. Previous studies have shown the inward rectifying potassium channel *kcnj2*, (kir 2.1) to have a quiescent effect on NAc neurons, much like mCREB overexpression (Dong et al., 2006). *Kcnj2* is also a family member of a potassium channel gene found in the array pilot study upregulated by social isolation. Indeed, overexpressing kir2.1 in the NAc of non-isolated animals produced the same type of anxiety-deficits both in elevated plus maze and in approach for sexual behavior. However, no other behavioral deficits such as modulation of ejaculation, sucrose intake or FST were observed.

Decreases in CREB lead to Increases in Potassium Channels

Linking CREB and potassium channel regulation, overexpressing mCREB in the NAc led to an increase in various potassium channels, as did social isolation, tested by RT-PCR. This work shows that upregulation of potassium channels by downregulation of CREB in the NAc in a social isolation model leads to anxiety-like, but not depression-like, effects. It is interesting to note that CREB is almost exclusively thought of as a transcriptional activator, however, here it is shown that downregulation of CREB produces an increase in mRNA levels of certain genes. This may be explained by CREB's ability to activate repressors of gene transcription, such as ICER (Lonze and Ginty, 2002). Also, it has

been found that certain splice variants of CREB can function as repressors (Bartsch et al., 1998; Sakai et al., 1999; Shaywitz and Greenberg, 1999).

Social Isolation and Depressive-like phenotypes

This study was of particular importance because, although CREB activity was reduced in the NAc, a depressive-like phenotype was still present. This is in contrast to other reports showing selective reduction of CREB in the NAc leading to antidepressant effects (Thome et al., 2000; Pliakas et al., 2001; Barrot et al., 2002; Newton et al., 2002; Green et al., 2006). Although the depressive-like phenotype was not found to be CREB specific in the NAc, it still indicates that not every condition that reduces CREB in the NAc will lead to an antidepressant-like effect. While imipramine increases CREB in the NAc and restores depressive-related behaviors, this does not appear to be a contribution of CREB in the NAc alone, as CREB overexpression in the NAc of isolated animals did not produce this effect. It is speculated that social isolation produces a downregulation of CREB in several brain regions, possibly including the hippocampus, which has been shown to play a role in depression-like behavior (Carlezon et al., 2005; Gronli et al., 2006). In the isolation model, perhaps imipramine is also increasing CREB in the hippocampus, as it has been shown to in other studies (Thome et al., 2000). This probable effect, along with action in other brain regions, may thereby reverse the behavioral effects related to depression phenotypes.

Other brain regions involved in the depressive-phenotype of isolated animals are potential subjects of future investigation. However, this research is of particular interest

because the depressive phenotype observed in the isolated animals may represent a different subtype of depression than the subtype observed with increased CREB levels in the NAc. While CREB overexpression in the NAc of non-isolated animals produces "emotional blunting" (Barrot et al., 2002; Carlezon et al., 2005), the isolated animals appear hyper-sensitive to aversive stimuli while still presenting "depressive-like" behaviors. Perhaps there is a distinction in depression induced by social isolation compared with depression due to active stress and increased CREB in the NAc. This hypothesis may contribute to the subtypes of depression present in humans, from hyper-reactive and irritable (sleeping very little and agitation), compared with the emotional numbing depression behavior (characterized by increased sleep and dysphoria) (American Psychiatric Association 2000). Although this hypothesis is speculative, future experiments may be conducted to elucidate the underpinnings involved in the different subtypes of depression.

The current findings presented here do however provide a much-needed model for co-morbid depression and anxiety, in which the depressive-phenotype is sensitive to chronic but not acute antidepressant administration. Secondly, this work supports a role for CREB in the NAc in mediating anxiety, especially to novelty, by controlling the excitability of neurons through potassium channels, and also possibly provides a novel mechanism by which antidepressants act in alleviating anxiety.

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VITAE

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