The effects of CREB-mediated BDNF expression on memory- and anxiety-related behaviours in the adult mouse

by

Matthew Mariusz Florczynski

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Abstract

Experience drives changes in gene expression that mold and reorganize neuronal circuits. In response to neuronal activity, the transcription factor CREB binds to a regulatory site on *Bdnf* promoter IV to modulate BDNF protein levels. CREB and BDNF are extensively implicated in animal behaviour, but the role of the interaction between these proteins has not been studied. I used transgenic mice carrying mutations at the CREB binding site of *Bdnf* promoter IV (CREmKI mutation) to specifically disrupt this interaction. F1 ($N = 52$) and F2 ($N = 69$) mice underwent a battery of behavioural tests. All mice showed normal motor learning and spatial memory. Critically, F1 mutants showed impaired auditory fear memory, while F2 mutants showed heightened anxiety. I suspect that differences in *Bdnf* expression and compensatory effects contributed to discrepancies between the two generations. My findings highlight the relevance of BDNF expression levels for memory- and anxiety-related behaviours.

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5-HT	5-hydroxytryptamine; serotonin
AAV	adeno-associated virus
AB	accessory basal amygdala
AC	adenylyl cyclase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP-1	activating protein 1
Arc	activity-regulated cytoskeleton-associated gene; Arg3.1
ATF	activating transcription factor
Bcl-2	B-cell lymphoma protein-2
BDNF	brain derived neurotrophic factor
BLa	basolateral nucleus of the amygdala
BLA	basolateral complex of the amygdala
BMe	basomedial nucleus of the amygdala
BNST	bed nucleus of the stria terminalis
bp	base pair
bZIP	basic leucine zipper
C	carboxyl
C/EBP	CCAAT enhancer binding protein
CA	cornu ammonis
CAD	constitutively active domain; Q2
cAMP	cyclic adenosine 3,5-monophosphate
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CaRE	calcium response element
CBP	CREB binding protein
CeA	central amygdala
CeA_L	lateral nuclei of the CeA
CeA_M	medial nuclei of the CeA
CICR	calcium-induced calcium release
CMS	chronic mild stress
CNS	central nervous system
CpG	cytosine-guanine pairs
CRE	CREB recognition element
CREB	cAMP response element-binding protein
CREM	cAMP response element modulator
CREmKI	CaRE-3/CRE binding site knock-in mutation
CRF	corticosterone-releasing factor
CRTC	CREB regulated transcriptional coactivator
CS	conditioned stimulus
CTA	conditioned taste aversion
DAPI	4,6-diamidino-2-phenylindole
DAT	dopamine transporter
DG	dentate gyrus

dox	doxycycline
DRG	dorsal root ganglia
E	embryonic day (or early, in E-LTP)
EC	entorhinal cortex
EPM	elevated plus maze
ERK	extracellular signal-related kinase
fEPSP	field evoked post-synaptic potential
FPS	fear-potentiated startle
FRET	fluorescence-resonance based energy transfer
GABA	gamma-aminobutyric acid
GDNF	glial cell-line derived neurotrophic factor
GFP	green fluorescent protein
GluR	glutamate receptor
GPCR	G-protein coupled receptor
H1a	homer1a
HAT	histone acetyltransferase
HDAC	histone deacetylase
HFS	high frequency stimulation
HMT	histone methyltransferase
HPA	hypothalamic-pituitary-adrenal
HSV	herpes simplex virus
IA	inhibitory avoidance
ICER	inducible cAMP early repressor
i.c.v.	intracerebroventricular
IEG	immediate early gene
i.p.	intraperitoneal
ISI	inter-stimulus interval
ITI	inter-trial interval
KID	kinase-inducible domain
L	layer (or late, in L-LTP)
La	lateral nucleus of the amygdala
LA	lateral amygdala (same as lateral nucleus of the amygdala; La)
LBD	ligand-binding domain
LC	locus coeruleus
LGN	lateral geniculate nucleus
LiCl	lithium chloride
LTD	long-term depression
LTM	long-term memory
LTP	long-term potentiation
LV	lentivirus
MAPK	mitogen-activated protein kinase
MD	monocular deprivation
MeCP2	methyl CpG binding protein 2
MEF2	myocyte enhancer factor 2
mIPSC	miniature inhibitory post-synaptic current
MRI	magnetic resonance imaging
MSK	mitogen- and stress- activated kinase
MSN	medium spiny neuron

MTL	medial temporal lobe
N	amino
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NMDA	*N*-methyl-*D*-aspartate
NP	neuronal pentraxin
NPAS4	neuronal PAS domain protein 4
NSE-tTa	neuron-specific enolase-tetracycline transcriptional activator
NT	neurotrophin
OF	open field
OGN	oligonucleotide
P	postnatal day
p300	E1A binding protein
$p75^{NTR}$	pan-neurotrophin receptor
pA	polyadenylation
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PDE4	phosphodiesterase type IV
PI-3K	phosphoinositide-3 kinase
PKA	Ca^{2+}-dependent protein kinase
PKC	protein kinase C
PNS	peripheral nervous system
PP	protein phosphatase
PPF	paired-pulse facilitation
PTSD	post-traumatic stress disorder
PRP	plasticity-related protein
PRV	pseudorabies virus
PSD	post-synaptic density
PTB	phosphotyrosine-binding domain
PTP	post-tetanic potentiation
PVN	periventricular nucleus
RE1	restrictive silencing element 1
REST	RE1 silencing transcription factor
RPM	rotations per minute
RSK	ribosomal S6 kinase
Ser	serine
SB	subiculum
SH-2	src-homology-2 domain
Shc	SH2-containing sequence
SN	substantia nigra
STM	short-term memory
TAM	tamoxifen
TBS	theta-burst stimulation
TetOp	tetracycline responsive element
TNF	tumour necrosis factor
TORC	transducer of regulated CREB
Trk	tropomyosin-related kinase
US	unconditioned stimulus

USF1/2	upstream stimulatory factor 1/2
UTR	untranslated region
VMAT2	vesicular monoamine transporter-2
vmPFC	ventromedial prefrontal cortex
VSCC	voltage-sensitive calcium channel
VTA	ventral tegmental area

Chapter 1
Introduction

Science is ever striving towards a fundamental understanding of natural processes. If centuries of formal scientific study are any indicator, the most compelling and relevant of natural phenomena is the living organism. Understanding living things helps us understand ourselves – humans – and what physical qualities, personal attributes and underlying processes shape us, making us human.

Our knowledge might, perhaps, have progressed more quickly were it not for philosophical confusion that hindered the study of the human brain. In the seventeenth century, René Descartes proposed a theory of dualism that effectively separated the body from the mind (Hart, 1996). The behavioural psychology movement of the early 1900s stipulated that inner workings of the mind, which could not be observed, could not be studied (Watson, 1987). Scientific inquiry into the workings of the brain might also have caught on earlier if not for the misgivings of attempts to explain brain function. In the 1800s, Franz Joseph Gall and other phrenologists attempted to map the human mental faculties, which were themselves arbitrarily selected emotional and temperamental personality characteristics, onto distinct parts of the brain by feeling the outside of subjects' skulls (Fodor, 1983). Nineteenth century introspectionists tried to gain access to the human mind by asking subjects to reflect on their own mental processes, drawing conclusions that were speculative and inappropriately generalized (Wilson & Dunn, 2004). It would take important developments in scientific methodologies for the study of an organ as obscure as the brain to take off.

Nevertheless, our intellectual curiosity in ourselves and in the world around us eventually got the better of us – for the better. It was a matter of technological advances. Camillo Golgi and Ramon Santiago y Cajal discovered ways to visualize the basic building blocks of the nervous system, neurons (Cajal, 1894). John Eccles, Andrew Huxley and Alan Hodgkin helped characterize electrical properties of the synapse, and Eccles and Bernard Katz later discovered the neurotransmitter acetylcholine (Südhof & Malenka, 2008). Discoveries by Rosalind Franklin, James Watson and Francis Crick helped elucidate the double helical structure of DNA (Watson & Crick, 1974). These discoveries paved the way for an integrated understanding of electrical, chemical, and genetic processes in the brain. Nor can we overlook advances in the

understanding of global brain functions that came from the medical and neuroscientific fields. Wilder Penfield put together a functional map of the sensory and motor cortices based on observations made while electrically stimulating the brains of patients with epilepsy (Rasmussen, 1977). Alan Turing and other pioneering cognitive psychologists began characterizing computational processes in the brain (Turing, 1936). Early brain lesion studies in animals by Karl Lashley (Lashley, 1950), and studies in brain damaged patients such as Henry Molaison (HM) played a major role in understanding human memory and amnesia (Scoville & Milner, 1957). What was a series of bold, independent efforts to understand the basic, global and theoretical functioning of the brain, we can now see was actually one massive, interdisciplinary endeavour towards an integrated understanding. Basic genetic and physiological processes in the brain underlie variations and changes in behaviour. Understanding these processes can allow us to understand something as complex as the human mind and illnesses that affect it.

The investigation carried out in this thesis lies at the very intersection of genetic and molecular processes of the brain and their behavioural effects. One other factor is pertinent to my study: the influence of the environment and personal experience. We do not simply develop into adults by virtue of a carefully regulated genetic program, as is obvious from an abundance of observations of severe developmental effects after infantile sensory deprivation. Our genetic make-up is continuously responding to our environment and shaping the nervous system through epigenetic changes, the strengthening of synaptic connections, and the formation of new ones. These changes then modulate the ways in which we interact with the outside world under familiar and novel circumstances in the future. Even in adulthood, everyday experiences trigger changes in gene expression in the brain that mold and reorganize the neuronal circuits that underlie learning, memory and behaviour. My ensuing discussion will focus on two of these genes and how they respond to the outside world to modulate behaviour.

Two of the most extensively studied genes modulated by experience-dependent neuronal activity in the brain are cyclic adenosine 3,5-monophosphate (cAMP) response element-binding protein (*Creb*) and brain derived neurotrophic factor (*Bdnf*). CREB responds to increases in calcium at synapses by modulating the expression of genes, leading to the synthesis of new proteins. One of its effector sites is the calcium-response element-3/CREB recognition element (CaRE-3/CRE) regulatory region that controls the transcription of *Bdnf* promoter IV. The newly expressed *Bdnf* mRNA is translated into the BDNF protein, which induces long-term changes in

synaptic plasticity. Both CREB and BDNF have been extensively implicated in nervous system development, learning, memory and other behavioural processes, and in human cognitive disorders. However, the roles of the CREB-BDNF interaction in behaviour, and the effects of interfering with it, are yet to be studied systematically.

The investigation that follows attempted to characterize, for the first time, the behavioural profile of animals in which the ability of CREB to interact with BDNF in response to environmental stimuli had been disrupted. Understanding the role of the CREB-BDNF interaction will help us understand, at a fundamental level, how neural processes are able to respond to experiences in order to promote adaptive behaviour in the future.

Chapter 2
Literature Review

2.1 Key molecular players

At any point in time, a human cell expresses between 10 000 and 20 000 of ~30 000 genes in the genome (Alberini, 2009). At least 300 genes are turned on and off by neuronal activity resulting from events that induce neurotransmitter release, growth factor signaling, and neuronal stress (Greer & Greenberg, 2008; Lin *et al.*, 2008). The expression of these genes is typically subject to many layers of regulatory processes, allowing for tissue-specific expression patterns and precise temporal control. Two of the most extensively studied neuronal activity-dependent genes are *Creb* and *Bdnf*. While I was primarily interested in a specific interaction between these proteins, I begin by reviewing the structure and molecular interactions of each protein. In the proceeding sections, I will describe the role of each protein at the synapse and in the context of animal behaviour. Throughout the review I will also attempt to piece together plausible roles for the CREB-BDNF interaction in experience-dependent synaptic and behavioural changes before introducing the rationale for my experiments.

2.1.1 The cAMP response element binding protein

The transcription factor CREB is perhaps the most important modulator of cellular changes in the nervous system (reviewed in Lonze & Ginty, 2002). CREB has over 100 target genes (Impey *et al.,* 2004; Mayr & Montminy, 2001). It is implicated in processes underlying neuronal survival during development (Bonni *et al.*, 1999; Riccio *et al.*, 1999), circadian rhythms (Kornhauser *et al*., 1996), neuroprotection from disease (Deak *et al.*, 1998; Iordanov *et al.*, 1997; Tan *et al.*, 1996; Wiggin *et al.*, 2002), addiction (McClung & Nestler, 2003; Carlezon *et al.*, 1998), depression (Berton *et al.*, 2006; Duman & Monteggia, 2006; Duman *et al.* 1997), synaptic plasticity (Bailey *et al.*, 2000; Kandel, 2001), and learning and memory (Alberini, 2009; Josselyn, 2010; Lonze & Ginty, 2002; Silva *et al*., 1998; Won & Silva, 2008).

2.1.1.1 CREB family structure

The role of CREB in experience-dependent physiological processes was discovered by Montminy and colleagues while attempting to identify a nuclear protein activated by the cAMP

pathway *in vitro* (Gonzalez & Montminy, 1989; Montminy & Bilezikjian, 1987; Montminy *et al.,* 1990). cAMP, which is itself a second messenger implicated in immediate physiological responses to cellular stimulation (Brunelli *et al.*, 1976; Schacher *et al.*, 1988), was shown to phosphorylate CREB and induce the transcription of the *somatostatin* gene (Montminy *et al.*, 1986; Montminy & Bilezikjian, 1987). It was discovered that CREB bound to the *somatostatin* gene at a palindromic octanucleotide binding sequence (5'- TGACGTCA -3'), called the CREB recognition element (CRE). *Creb* was the first gene discovered in a family that consists of at least 10 genes that bind to CRE regulatory sites (Macho & Sassone-Corsi, 2003; Silva *et al.*, 1998). The CREB family is part of a larger superfamily of transcription factors, which includes activating protein 1 (AP-1), CCAAT enhancer binding protein (C/EBP), Fos, and Jun, all of which contain a basic-region leucine zipper (bZIP) domain structural motif at the carboxyl (C) terminus (reviewed in Alberini, 2009).

The *Creb* family includes the *Creb*, cAMP response element modulator (*Crem*), and activating transcription factor-1 (*Atf*-1) groups of transcription factors (for reviews see Alberini, 2009; Lonze & Ginty, 2002). CREB and ATF-1 are ubiquitously expressed, while CREM is confined mostly to the neuroendocrine system (Alberini, 2009). These groups of transcription factors differ somewhat in their molecular structure, which helps explain the functional differences between them (Fig. 2.1). At the C-terminus, the *Creb* gene contains a basic region, which allows it to bind to DNA, and a leucine zipper that allows CREB to form homo- or heterodimers with other bZIP transcription factors, contributing to the repertoire of CREB-mediated regulatory processes (reviewed in Hai & Hartman, 2001; Mayr & Montminy, 2001). At the amino (N) terminus, CREB has a bipartite transactivation domain consisting of glutamine rich regions Q1 and Q2/CAD (constitutively active domain) separated by a kinase-inducible domain (KID, also called P-box; reviewed in Lonze & Ginty, 2002; Silva *et al.*, 1998). Q1 and Q2/CAD are responsible for recruiting the basal transcriptional machinery, including several transcription factors and RNA polymerase II (Ferreri *et al.*, 1994; Xing & Quinn, 1994; reviewed in Quinn, 2002). The KID is needed for the activation of CREB via phosphorylation (Gonzalez & Montminy, 1989). CREM and ATF-1 lack the Q- and KID domains of CREB and are not activated by the factors that phosphorylate CREB (Lonze & Ginty, 2002).

CREB has two major isoforms generated by alternative splicing of the *Creb* gene, $CREB^{\alpha}$ and $CREB^{\delta}$, which differ only with respect to the presence of an α-domain separating Q1 and

KID (Lonze & Ginty, 2002). A third CREB isoform, $CREB^{\beta}$, differs in the structure of Q1 and is normally expressed at lower levels than $CREB^{\alpha}$ and $CREB^{\delta}$. However, $CREB^{\beta}$ is upregulated by compensatory mechanisms in mice lacking the two main CREB isoforms ($CREB^{\alpha\delta-/-}$ mice; Blendy *et al.*, 1996; Hummler *et al.*, 1994). During transcription, the *Crem* gene also undergoes alternative splicing to generate four possible isoforms of CREM, α, β, γ, or inducible cAMP early repressor (ICER), which repress CREB by competing for CRE sites (Ding *et al.*, 2005; Foulkes *et al.*, 1991). ICER contains multiple CRE sites and is actually upregulated when CREB is active, perhaps serving as a neuronal activity-dependent feedback mechanism of CREB signaling (reviewed in Won & Silva, 2008). Therefore, at the structural level, the regulation of CREB expression is influenced by dimerization with other bZIP family members, alternative splicing of the *Creb* gene, and repression by CREM.

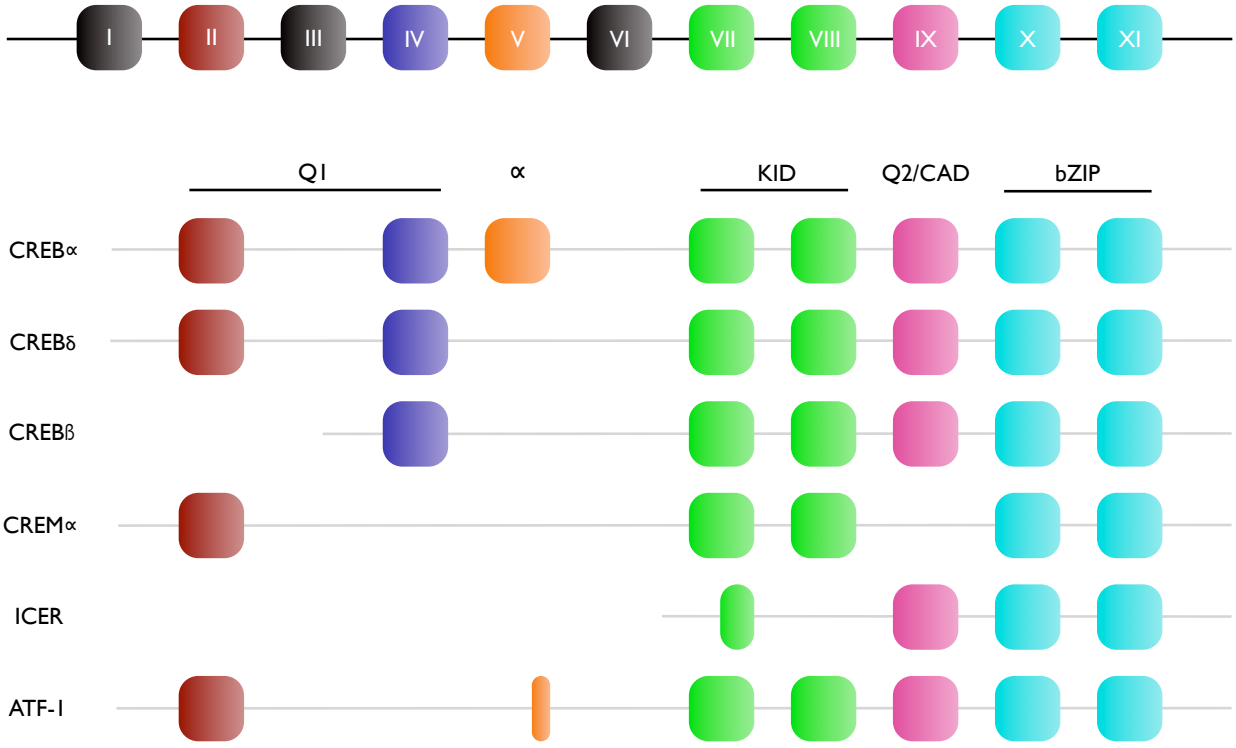

Figure 2.1: The CREB family of transcription factors has a characteristic gene domain structure
The CREB family of transcription factors includes the three isoforms of CREB (α, δ, and β), CREM, ICER and ATF-1. Each CREB family protein contains a bipartite transactivation domain at the N-terminus (*right*) consisting of glutamate-rich regions (Q1 and Q2/CAD) that recruit the basal transcriptional machinery, separated by a kinase-inducible domain (KID) necessary for phosphorylation. The C-terminus (*left*) forms a basic leucine zipper (bZIP) motif that allows DNA binding and dimerization with other CREB family members. Adapted from Lonze and Ginty (2002).

2.1.1.2 Regulation of CREB activity

Many competing and cooperating factors regulate CREB transcriptional activity. There are over 6000 binding sites for CREB in the genome (Impey *et al.*, 2004). Many of these sites contain the 8-nucleotide palindromic CRE sequence (5'- TGACGTCA -3'), but other CREB binding elements consist of only a subset of the palindromic nucleotides (5'- TGACG -3') or have multiple substitutions in the CRE site (Mayr & Montminy, 2001). The DNA binding domain of CREB ensures that it is constitutively bound to its target genes, and the Q2/CAD domain keeps the basal machinery needed for transcription assembled at the CRE site (Lonze & Ginty, 2002). Activation of CREB is stimulus-dependent and requires phosphorylation of serine residue 133 (Ser-133) of the KID, which can be induced by a variety of nuclear kinases (Gonzalez & Montminy, 1989).

Ca^{2+} is an important mediator of synaptic signaling cascades that lead to CREB activation (Sheng *et al.*, 1991; reviewed in Gallin & Greenberg, 1995; Greer & Greenberg, 2008; Shaywitz & Greenberg, 1999). Ca^{2+} can enter a neuron via several routes in response to stimulation. Depolarization induced by the neurotransmitter glutamate at an excitatory synapse triggers Ca^{2+} influx into the post-synaptic cell through L- and N-type voltage-sensitive calcium channels (VSCCs; Balkowiec & Katz, 2002; Ghosh *et al.*, 1994). Ca^{2+} ions also enter through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor channels containing the glutamate receptor 1 (GluR1) subunit (GluR2-containing AMPA receptors are not permeable to Ca^{2+}) shortly after the induction of depolarization (Batchelor & Garthwaite, 1997). A sufficient level of glutamate binding and depolarization unblocks the *N*-methyl-*D*-aspartate (NMDA) receptor by displacing Mg^{2+} from its ion channel, enabling Ca^{2+} to enter the cell (Morris *et al.*, 1990). Rising levels of intracellular Ca^{2+} can further trigger the release of Ca^{2+} from intracellular stores (calcium-induced calcium release; CICR) through caffeine-ryanodine sensitive receptors (Blöchl & Thoenen, 1995; reviewed in Berridge, 1998).

Experience-dependent neuronal responses depend on the magnitude of increase in Ca^{2+} concentration as well as the route of Ca^{2+} entry (reviewed in Greer & Greenberg, 2008). Ca^{2+} influx through L-type VSCCs, generally located close to the cell soma, causes an elevation in Ca^{2+} concentration close enough to the nucleus to allow entry of Ca^{2+} directly into the nucleus (reviewed in Catterall, 2000). Nuclear Ca^{2+} interacts with calmodulin and Ca^{2+}/calmodulin-

dependent protein kinase kinase (CaMKK) to activate CaMKIV, which phosphorylates CREB at Ser-133 (Bito et al., 1996; Ho et al., 2000; Kang et al., 2001). Ca^{2+} influx through NMDA receptors activates cytoplasmic CaMKII, which can interact with NMDA receptors to further potentiate Ca^{2+} entry (reviewed in Chin & Means, 2000), or translocate directly to the nucleus and phosphorylate CREB (Hardingham *et al.*, 2001). NMDA receptor-mediated increases in intracellular Ca^{2+} also activate cAMP, which is detected by adenylyl cyclase (AC) and promotes dissociation of the inhibitory and catalytic subunits of Ca^{2+}-dependent protein kinase (PKA; Huang & Kandel, 1994; reviewed in Kandel, 2001). Newly activated PKA can then translocate into the nucleus to phosphorylate CREB at Ser-133 (Gonzalez & Montminy, 1989). cAMP levels are also modulated by metabotropic glutamate receptors (mGluRs) and activation of AC through transmembrane receptors, such as the dopamine D1 receptor, leading to activation of PKA (Dash *et al.*, 1991). Finally, Ca^{2+} binding to calmodulin activates Ras, which leads to the phosphorylation of mitogen-activated protein kinase (MAPK). MAPK phosphorylates ribosomal S6 kinases (RSKs) 1, 2 and 3, which can phosphorylate CREB at Ser-133 (Xing *et al.,* 1996, 1998).

Two additional routes of CREB activation are initiated by growth factor binding to membrane tyrosine receptor kinases and by responses to stress. Growth factors activate the Ras-MAPK pathway, leading to activation of RSKs and structurally related mitogen- and stress-activated kinase (MSK) 1 and MSK2, which phosphorylate CREB (Deak *et al.*, 1998; Wiggin *et al.,* 2002; Xing *et al.*, 1996). Growth factor signaling also leads to the activation of CREB through the phosphoinositide-3 kinase (PI-3K) pathway (Lin *et al.,* 2001; reviewed in Cantley, 2002). The stress-induced kinase, SAPK2/p38MAPK, activates several downstream targets that phosphorylate CREB, including MAPKAP K2, MSK1, and MSK2 (Deak *et al.,* 1998; Tan *et al.,* 1996), in response to stress induced by hypoxia, stroke, or neuronal injury (reviewed in Lonze & Ginty, 2002).

CREB is also *deactivated* in an activity-dependent manner. Ca^{2+} influx into the neuron promotes protein phosphatase (PP) 1 and PP2A- mediated dephosphorylation of CREB at Ser-133, inhibiting CREB-mediated gene transcription (Bito *et al.*, 1996; Genoux *et al.*, 2002; Hagiwara *et al.*, 1994). Nuclear CaMKII and CaMKIV (Wu & McMurray, 2002; reviewed in Ghosh & Greenberg, 1995) can phosphorylate CREB at Ser-142 and Ser-143, which leads to the dissociation of CREB dimers. CREB activation can also be inhibited indirectly by

phosphodiesterase type IV (PDE4), which degrades cAMP (reviewed in Berton *et al.*, 2006). Thus numerous homeostatic mechanisms operate within the neuron to maintain an appropriate balance of CREB-mediated gene expression.

2.1.1.3 CREB-interacting proteins

Before phosphorylated CREB can initiate gene transcription, it must associate with co-activating proteins. The stimulus-dependent activation of CREB via Ser-133 phosphorylation facilitates the association of CREB with CREB binding protein (CBP; Chrivia *et al.*, 1993; Kwok *et al.*, 1994) and its paralog, E1A binding protein (p300; Eckner *et al.*, 1994). CBP and p300 have intrinsic histone acetyltransferase (HAT) activity, promoting bond formation between acetyl groups and the CREB target gene, which leads to conformational changes that make the gene accessible to transcriptional machinery (Oliveira *et al.* 2006). Binding of CBP to CREB appears to be a necessary step in CREB-mediated gene transcription. Preventing CBP from binding to CREB by mutation of Ser-133 to an alanine residue (S133A mutation) abolishes CREB-mediated transcription of the *somatostatin* gene (Gonzalez & Montminy, 1989).

Phosphorylation at Ser-133 does not always recruit CBP (Kornhauser *et al.*, 2002; Wu & McMurray, 2001; reviewed in Lonze & Ginty, 2002), and additional modifications to CBP, such as phosphorylation at Ser-301 by CaMKIV, may be necessary to activate CREB (Impey *et al.*, 2002). In fact, Kornhauser *et al.* (2002) demonstrated that maximal CREB-mediated gene expression was achieved when CREB was phosphorylated at Ser-133, 142, and 143, despite the fact that phosphorylation at Ser-142 and 143 prevents CBP from associating with CREB. This evidence raises the possibility that some CREB-dependent genes may be expressed independently of CBP (Lonze & Ginty, 2002). Indeed, it appears that CREB can be activated in a phosphorylation- and stimulus-independent manner through heterodimerization with CREB regulated transcriptional coactivators (CRTCs; previously referred to as transducers of regulated CREB or TORCs; Bittinger *et al.*, 2004; Conkright *et al.,* 2003; Iourgenko *et al.,* 2003).

2.1.2 Brain-derived neurotrophic factor

As in the case of CREB, the activity of BDNF is regulated by complex transcriptional mechanisms and interactions with other proteins. Through association with the high affinity receptor tropomyosin-related kinase (Trk) B and the non-specific pan-neurotrophin receptor

$p75^{NTR}$, BDNF executes a vast repertoire of functions (Barbacid, 1994; Binder & Scharfman, 2004; Poo, 2001). During development, BDNF is involved in neuronal survival (Ernfors *et al.*, 1994), axonal path-finding (Hu *et al.*, 2005), and formation of inhibitory synapses in the brain (Hong *et al.*, 2008; Kohara *et al.*, 2007). In adulthood, BDNF is extensively implicated in synaptic and structural plasticity (Hu *et al.*, 2011; Korte *et al.*, 1995, 1996), neuroprotective effects (Santarelli *et al.*, 2003; Saylor & McGinty, 2008), anxiety (Chen *et al.*, 2006; Nibuya *et al.*, 1995), addiction (reviewed in Bolanos & Nestler, 2004), and learning and memory (Egan *et al.*, 2003; reviewed in Mahan & Ressler, 2011; Tyler *et al.*, 2002). Abnormalities in BDNF expression in the brain also underly neurodegenerative (Baker *et al.*, 2005) and neuropsychiatric diseases (Berton *et al.,* 2006; Duman & Monteggia, 2006).

2.1.2.1 The neurotrophin family

The neurotrophin (NT) family consists of at least four soluble growth factors restricted in expression to the nervous system (for reviews, see Huang & Reichardt, 2001; Poo, 2001). The NTs have garnered a lot of interest because of their crucial roles in development, elucidated in pioneering studies that led to the discovery of nerve growth factor (NGF; Levi-Montalcini & Hamburger, 1951). Rita Levi-Montalcini, Victor Hamburger, Stanley Cohen and colleagues isolated NGF after discovering that a mouse sarcoma in close proximity, but not in direct contact, with a chick embryo promoted the growth of nerve fibers from the embryo via the diffusible factor, NGF, passing through connecting blood vessels. The same growth-promoting effect was achieved with a transplant of normal mouse tissue, use of snake venom, and a number of other tissues and agents, all of which contained NGF (reviewed in Levi-Montalcini, 1964, 1982, 1987). Small quantities of NGF were needed for embryonic neurons to survive (Johnson *et al.*, 1980; Levi-Montalcini & Angeletti, 1963), and NGF synthesized by target tissues could be taken up retrogradely by afferent nerve fibers (Sröckel *et al.,* 1974; Hamburger *et al.*, 1981). It was also demonstrated that NGF released from a micropipette *in vitro* could direct the growth of neurites in the direction of the source of NGF release (Gundersen & Barrett, 1979). This and other evidence led to the postulation, known as the *neurotrophic factor hypothesis*, that developing neurites compete for NTs released by target tissues in order to survive (Thoenen & Barde, 1980).

Subsequent research led to the discovery of the other members of the NT family, BDNF (Barde *et al.*, 1982), NT-3 (Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990), and NT4/5 (Berkemeier *et al.*, 1991; Ip *et al.*, 1992)[1]. The NTs share considerable (~50%) amino acid sequence homology (Binder & Scharfman, 2004) and contain characteristic cysteine residues and disulfide bridges (Leibrock *et al.*, 1989). Each NT is synthesized in a pro-NT form containing an N-linked glycosylation site that undergoes extracellular cleavage (Chao & Bothwell, 2002). Mature NTs are noncovalently-1 linked homodimers consisting of a start codon and signaling peptide (reviewed in Binder & Scharfman, 2004). They have a distinct three-dimensional structure with two pairs of antiparallel beta-strands and cysteine residues that form a cystine knot motif. It appears that every population of neurons is innervated by at least one neurotrophic factor (reviewed in Huang & Reichardt, 2001; Lewin & Barde, 1996).

2.1.2.2 Neurotrophin receptors

The functional effects of NTs are carried out by NT-receptor complexes. Structural differences among NTs account for their differential ability to bind with high-affinity, membrane-bound Trk receptors (reviewed in Huang & Reichardt, 2001; Teng & Hempstead, 2004). NGF binds specifically to TrkA, BDNF and NT-4/5 bind to TrkB, and NT-3 binds to TrkC. Thus differential expression of Trk receptors among neuronal populations can result in brain region-specific effects of different NTs. TrkA, B and C mRNAs also undergo alternative splicing, which can affect their binding properties and cellular function. In the case of TrkB, the expression of different isoforms may not overlap, suggesting that alternative splicing of TrkB is a mechanism by which NTs modulate different functions in different regions of the nervous system. Neurons can be innervated by more than one NT (Fan *et al.*, 2000; Kuruvilla *et al.*, 2004), express more than one Trk receptor activated by the same NT (Clary & Reichardt, 1994), or require an activity-dependent signal, such as cAMP or Ca^{2+}, to incorporate a Trk receptor into the cellular membrane (Meyer-Franke *et al.*, 1998), further contributing to variations in function. While the functional significance of Trk receptor binding by NTs is incompletely understood,

[1] NT-6 and NT-7 genes have also been identified in fish, but are not found in mammals or birds. In fish, NT-6 and NT-7 act on the same populations of neurons as NGF (see Huang & Reichardt, 2001)

binding to Trk receptors appears to be necessary for the induction of most the responses that are mediated by NTs (Huang & Reichardt, 2001).

Structural similarities among NTs allow them to bind non-specifically to the low affinity, membrane-bound, pan-NT receptor, $p75^{NTR}$ (reviewed in Dechant & Barde, 2002; Frade & Barde, 1998). $P75^{NTR}$ is a member of the tumour necrosis factor (TNF) superfamily of receptors and plays a role in determining which cells survive during development. Binding of NTs to the extracellular domain of $p75^{NTR}$ induces apoptosis by activating p53 through the Jun kinase signaling pathway (Aloyz *et al.*, 1998; Casaccia-Bonnefil *et al*., 1996; Frade *et al.*, 1996). $p75^{NTR}$ also appears to have survival-promoting effects by enhancing retrograde transport of NTs and promoting neurite outgrowth (Curtis *et al.*, 1995; Harrison *et al.*, 2000). In the presence of Trk receptor binding, $p75^{NTR}$ is less effective at inducing apoptosis and may actually enhance survival-promoting effects of low concentrations of NTs (Yoon *et al.*, 1998; reviewed in Mamidipudi & Wooten, 2002). Therefore, in conjunction with Trk receptors, $p75^{NTR}$ expression helps mediate the cellular effects of NTs.

2.1.2.3 The Bdnf gene

The *Bdnf* gene consists of at least eight distinct promoters (exons I to VIII) that can initiate transcription to produce distinct transcripts, each with a 5' exon that is alternatively spliced to a common 3' coding region (Fig. 2.2; Aid *et al.*, 2007). The *Bdnf* gene also contains a neuron restrictive silencing element 1 (RE1) between exons I and II bound by the RE1 silencing transcription factor (REST) repressor complex in non-neuronal cells, limiting the transcription of *Bdnf* to neurons (Abuhatzira *et al.*, 2007). The use of alternative promoters, alternative splicing, and polyadenlyation sites allows the *Bdnf* gene to code for at least 18 distinct transcripts, which, remarkably, are all translated to an identical BDNF protein (Aid *et al.*, 2007). The BDNF protein consists of 252 amino acids and is a small molecule, with molecular mass of approximately 14 000 (Leibrock *et al.*, 1989). The regulatory mechanisms that induce distinct *Bdnf* transcripts are still poorly characterized. However, it is suspected that the differential transcription of the *Bdnf* gene is under many levels of cell- or brain region-specific and temporal control (Hong *et al.*, 2008; Greer & Greenberg, 2008).

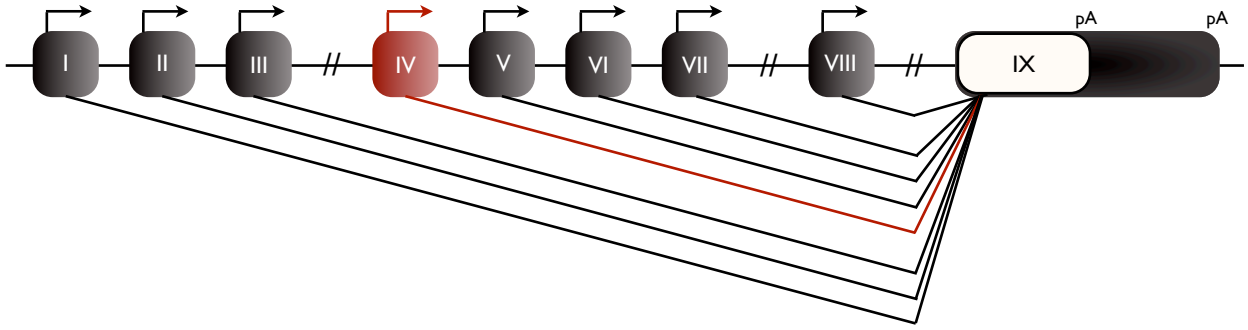

Figure 2.2: The *Bdnf* gene contains 8 distinct promoters that code for 18 different transcripts of the BDNF protein
Promoters on exons I to VIII are alternatively spliced to a common coding exon (exon IX) that has two alternative polyadenylation (pA) sites. *Bdnf* promoter IV (*red*) is the most responsive to neuronal activity. Adapted from Hong *et al.* (2008).

2.1.2.4 Regulation of BDNF expression

Both basal and activity-dependent mechanisms of BDNF expression contribute to its cellular and behavioural effects (for reviews see Bramham & Messaoudi, 2005; Poo, 2001; Thoenen, 1995). Basal levels of BDNF are thought to have *permissive* roles, allowing cells to carry out important physiological processes, while activity-dependent changes in BDNF expression are thought to have *instructive* roles, triggering cellular responses to physiologically relevant events.

In rats and mice, *Bdnf* mRNA levels are detectable during embryonic development and peak between post-natal days (P) 10-14, decreasing thereafter (Aid *et al.*, 2007). BDNF is detectable at high levels in the hippocampus, cerebral cortex, cerebellum, striatum, and spinal cord (Ernfors *et al.,* 1990; Hofer *et al.,* 1990; Patterson *et al.*, 1992; Kolbeck, *et al.*, 1999), but there is considerable variability in patterns of expression mediated by different promoters (Aid *et al.*, 2007). Timmunsk and colleagues isolated *Bdnf* mRNA from rats and mice at different stages of development and adulthood to characterize the brain-wide expression of the various *Bdnf* transcripts (Aid *et al.*, 2007; Timmunsk *et al.*, 1993, 1995). All of the *Bdnf* exons were detectable during embryonic and early post-natal development, although only exon IV mRNA was detectable as early as embryonic day (E) 13 (Aid *et al.*, 2007). In adult rodents, basal levels

of mRNA from exons I, III, IV, VI, VIII, and IXA[2] were expressed at high levels in all 10 brain regions sampled, while expression of exons IIA-C, V, and VII was more variable, though generally most robust in the hippocampus. Timmunsk and colleagues also infused kainic acid into the rat hippocampus to determine which promoters were most sensitive to neuronal activity. While mRNA from several exons was upregulated 3-6 hours after stimulation, only exon IV mRNA was upregulated robustly 24 hours after kainic acid treatment. Hong *et al.* (2008) have since shown that exon I and IV mRNA are most robustly upregulated in the mouse visual cortex after sensory stimulation or kainic acid treatment. Unlike the activity-dependent expression of *Bdnf* exon I mRNA, which requires protein synthesis, *Bdnf* exon IV behaves more like an immediate early gene (IEG; Castrén *et al*., 1998; Lauterborn *et al*., 1996). Therefore, promoter IV-driven expression of *Bdnf* has the capacity to mediate both acute and long-term instructive responses to changes in neuronal activity.

2.1.2.5 BDNF transport and signaling

BDNF can undergo retrograde or anterograde transport and is involved in short- and long-range signaling (Fig. 2.3). For the most part, BDNF and TrkB are synthesized in the soma and packaged into secretory vesicles that are transported to pre- or post-synaptic terminals of the neuron (reviewed in Poo, 2001). BDNF and its receptor may also be synthesized locally in synaptic terminals that contain the necessary translational machinery (Grigston *et al.*, 2005; Kang & Schuman, 1996; Tiedge & Brosius, 1996). TrkB receptors are then internalized in the synaptic membrane, while BDNF is secreted into the synaptic cleft. As is characteristic of other neurotrophins (Sröckel *et al.,* 1974; Hamburger *et al.*, 1981), BDNF can be secreted from the dendritic terminals and initiate a retrograde signaling cascade by binding to the extracellular domains of membrane-bound receptors of the pre-synaptic neuron. It can also bind to receptors on the synaptic terminal from which it was just released.

BDNF can also undergo anterograde transport when it is released from the axon terminal (Altar & DiStefano, 1998; Altar *et al.*, 1997). The transynaptic transmission of BDNF to a post-synaptic neuron was demonstrated directly by transfecting the nuclei of cultured cortical neurons

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[2] Exon IXA is a 5' extension of the coding region of *Bdnf* that may undergo alternative splicing but is not a promoter.

with cDNA plasmids coding for BDNF fused with green fluorescent protein (GFP) and visualizing the cells at a later time point (Kohara *et al.*, 2001). After 48 hours, the fluorescent signal had spread to the soma of the post-synaptic neuron, indicating that BDNF had been transported from the axon terminal and across the synapse. Blocking spontaneous neuronal activity with tetrodotoxin prevented the fluorescent signal from spreading to the post-synaptic neuron, while enhancing synaptic transmission with picrotoxin enhanced the post-synaptic signal. These findings suggested that the anterograde transport of BDNF is dependent on neuronal activity. They are also consistent with reports of synaptic vesicles containing BDNF (Moller *et al.*, 1998). An anterograde transport mechanism may explain how BDNF transmits long-range signals that result in its widespread distribution throughout the brain, including brain regions that do not contain *Bdnf* mRNA (Altar *et al.*, 1997; Poo, 2001).

BDNF initiates most of its cellular functions by binding with the extracellular domain of TrkB (Huang & Reichardt, 2001). Binding of the BDNF ligand induces TrkB receptor dimerization, which activates the intracellular kinase domains of TrkB and triggers autophophorylation of its tyrosine residues (Barbacid, 1994; reviewed in Patapoutian & Reichardt, 2001). Phosphorylation of the tyrosine residues activates the BDNF-TrkB complex and also creates binding sites for proteins containing phosphotyrosine-binding (PTB) or src-homology-2 (SH-2) domains (reviewed in Huang & Reichardt, 2001; Pawson & Nash, 2000). Once activated, the BDNF-TrkB complex is internalized in an endocytic vesicle and directed to other parts of the cell by active transport (Poo, 2001). Activated BDNF-TrkB complexes appear to carry out cellular functions as they are being shuttled to other parts of the cell. This is supported by evidence that the transport of BDNF alone after inactivation of TrkB in axons of the isthmo-optic nucleus of chick embryos did not induce the survival-promoting effects of BDNF (von Bartheld *et al.*, 1996). Many important signaling cascades carried out by BDNF-TrkB complexes are induced through activation of the Ras-ERK (extracellular signal-related kinase) pathway (reviewed in Binder & Scharfman, 2004; Haung & Reichardt, 2001). This signaling cascade begins with the binding of phosphorylated TrkB to SH2-containing sequence (Shc), which is itself phosphorylated by TrkB (Stephens *et al.*, 1994). Newly activated Shc then recruits the adapter proteins Grb-2 and SOS, which activate Ras (Haung & Reichardt, 2001). While Ras activation can occur prior to endocytosis of a NT-Trk receptor complex, the signaling cascades carried out by Ras only occur after the complex has been internalized (York *et al.*,

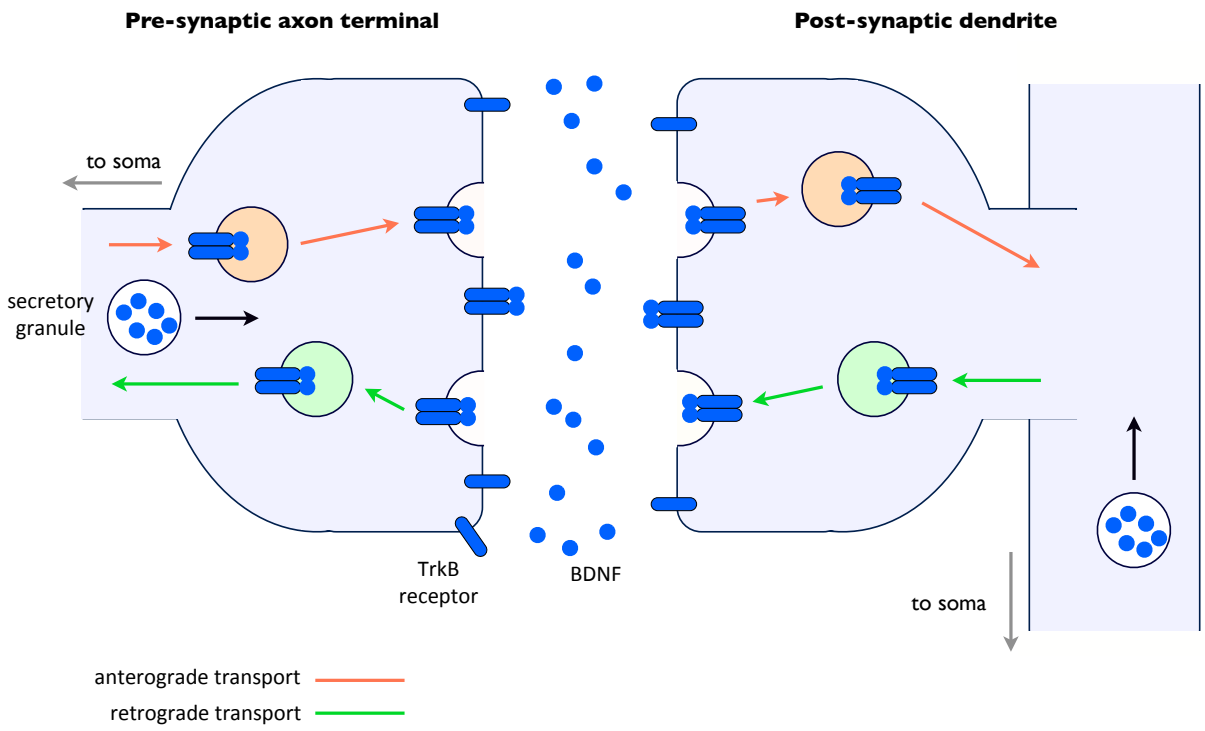

Figure 2.3: BDNF-TrkB complexes transmit retrograde and anterograde signals
BDNF is synthesized primarily in the soma and transported retrogradely to dendritic terminals in secretory granules. Once released, BDNF can diffuse across the synaptic cleft to the pre-synaptic neuron, where it binds to membrane-bound TrkB receptors. BDNF induces TrkB receptor dimerization and autophosphorylation of intracellular tyrosine residues. Activated BDNF-TrkB complexes are then endocytosed and transported retrogradely to the soma, where they execute various cellular functions. Alternatively, newly synthesized BDNF can be transported to the axon terminal and diffuse across the synaptic cleft to initiate TrkB receptor activation at the post-synaptic cell, resulting in anterograde signaling. Adapted from Poo (2001).

2000). One of the important effectors activated by Ras is PI-3K. PI-3K facilitates transport of NTs and is a major mediator of survival-promoting effects (Kuruvilla *et al.*, 2000; York *et al.*, 2000). Ras also activates ERK kinases, leading to activation of RSK kinases and MAP kinases, which phosphorylate CREB (Xing *et al.*, 1998).

BDNF can also interact with $p75^{NTR}$ by binding to it directly or through the association of an activated BDNF-TrkB complex with $p75^{NTR}$ (reviewed in Dechant & Barde, 2002; Frade & Barde, 1998). NT binding to $p75^{NTR}$ leads to activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), which promotes neuronal survival (Middleton *et al.*, 2000). Activation of $p75^{NTR}$ can also induce apoptosis by activating the Jun kinase pathway (Aloyz *et*

al., 1999; Frade *et al.*, 1996) and by promoting sphingolipid turnover, which generates the apoptosis-inducing factor ceramide (Casaccia-Bonnefil *et al*., 1996; Dobrowsky *et al.*, 1994).

In summary, BDNF and other NTs can influence biological activity via a variety of mechanisms. The effects of BDNF are likely to vary in different populations of cells expressing different concentrations of signaling molecules and combinations of receptors. Together with the array of factors controlling *Bdnf* transcription, this paints a complicated, and still poorly understood picture of the mechanisms by which BDNF mediates neural processes.

2.1.3 The CREB-BDNF interaction

CREB and BDNF are each implicated in a variety of critical cellular functions in the developing and adult brain. Thus any neural pathway involving both CREB and BDNF is likely to mediate important cellular processes. For the remainder of this review, I will focus on the interaction between CREB and BDNF and its effects on behaviour. I was particularly interested in promoter IV-driven *Bdnf* expression because of the ability of this promoter to respond directly to neuronal activity and to induce synaptic changes that last long after the causal event. Tao *et al.* (1998) showed that *Bdnf* exon IV has a CRE-like binding sequence located ~35 base pairs (bp) 5' of the promoter IV initiation site that binds CREB, inducing *Bdnf* transcription. Hong *et al.* (2008) subsequently generated a transgenic mouse line with a mutation in the CRE-like binding sequence that I investigated in my study.

The activity-dependent expression of BDNF is mediated by activation of excitatory synapses. Glutamate binding to NMDA and non-NMDA receptors (Zafra *et al.*, 1990), acetylcholine binding to muscarinic receptors (da Penha Berzaghi *et al.*, 1993), and stimulation with kainic acid (Rudge *et al.*, 1995) were initially found to up-regulate *Bdnf* mRNA in rat hippocampal cultures, while the inhibitory neurotransmitter γ-aminobutyric acid (GABA) was found to down-regulate *Bdnf* transcription (Zafra *et al.*, 1991, 1992). *Bdnf* promoter IV-mediated transcription is initiated by a neuronal activity-dependent increase in intracellular Ca^{2+} (reviewed in Greer & Greenberg, 2008). Glutamate binding at excitatory synapses induces depolarization, which opens L-type VSCCs. These channels are somatodendritically localized, providing a convenient means for Ca^{2+} entering the cell to transmit signals to the nucleus. The activation of PKA, CaMKs and the Ras-ERK pathway by Ca^{2+} can all lead to the

phosphorylation of CREB. However, other studies have found increases in BDNF expression induced by neuronal activity to be more dependent on Ca^{2+} influx through N-type VSCCs and from intracellular stores compared to L-type VSCCs (Balkowiec & Katz, 2000; 2002).

A series of coordinated signaling events involving a number of transcription factors is responsible for *Bdnf* promoter IV activation (Figure 2.4). In its inactive state, *Bdnf* exon IV constitutively binds to at least four transcription factors. These include CREB (Tao *et al.*, 1998) and methyl CpG binding protein 2 (MeCP2; Chen *et al.*, 2003a), which are inactive under basal conditions, and myocyte enhancer factor 2 (MEF2), which is phosphorylated and represses transcription (Flavell *et al.*, 2006). The role of the fourth transcription factor, upstream stimulatory factor 1/2 (USF1/2), is still unknown (Chen *et al.*, 2003b). The repression of *Bdnf* promoter IV is also ensured by the recruitment of histone deacetylases (HDACs) by MEF2. HDACs remove activating acetyl groups to keep the chromatin tightly wound around histones so that it is inaccessible to transcriptional machinery, while histone methyltransferases (HMTs) induce methylation of cytosine-guanine pairs (CpGs) in the chromatin to further repress transcription (reviewed in Bannister & Kouzarides, 2004, 2011; Cheung & Lau, 2005).

Within ~5 minutes of neurotransmitter release and Ca^{2+} influx through VSCCs, the cascade of events begins that will initiate transcription of exon IV (reviewed in Greer & Greenberg, 2008). These events include the dephosphorylation of MEF2 at Ser-408 by calcineurin, and, critically, the phosphorylation of CREB at Ser-133 that allows CBP to associate with CREB. Once dephosphorylated, MEF2 dissociates from HDACs, which appear to then be deactivated by CaMKII (Flavell *et al.*, 2006). CBP has intrinsic histone acetyltransferase (HAT) activity, resulting in the acetylation of histones and unwinding of chromatin containing the *Bdnf* gene (Bannister & Kouzarides, 1996; Martinez-Balbás *et al.*, 1998). CBP also recruits the basal transcriptional machinery, including RNA polymerase II. CRTCs may also contribute to the recruitment of RNA polymerase II (Bittinger *et al.*, 2004; Conkright *et al.*, 2003; Iourgenko *et al.*, 2003).

Although the signaling events that lead to the transcription of exon IV begin shortly after a synaptic event, there is a lag of ~15-30 minutes before transcription begins (Greer & Greenberg, 2008). Two other events that appear to occur within this time are the synthesis of neuronal PAS domain protein 4 (NPAS4), which also binds to *Bdnf* promoter IV (Lin *et al.*,

2008), and finally, the phosphorylation of MeCP2 at Ser-421 (Zhou *et al.*, 2006). The delayed phosphorylation of MeCP2 closely parallels the onset of *Bdnf* promoter IV transcription, suggesting that this is the final step needed to activate the promoter. The transcription of *Bdnf* promoter IV lasts for 6-24 hours and is eventually stopped by phosphatase-mediated dephosphorylation of CREB at Ser-133 and MeCP2 at Ser-421 (Aid *et al.*, 2007; Hong *et al.*, 2008). Phosphorylation of CREB at Ser-142 and 143, and phosphorylation of MEF2 at Ser-408 is also needed to repress transcription and return *Bdnf* promoter IV to its basal state (Gong *et al.*, 2003).

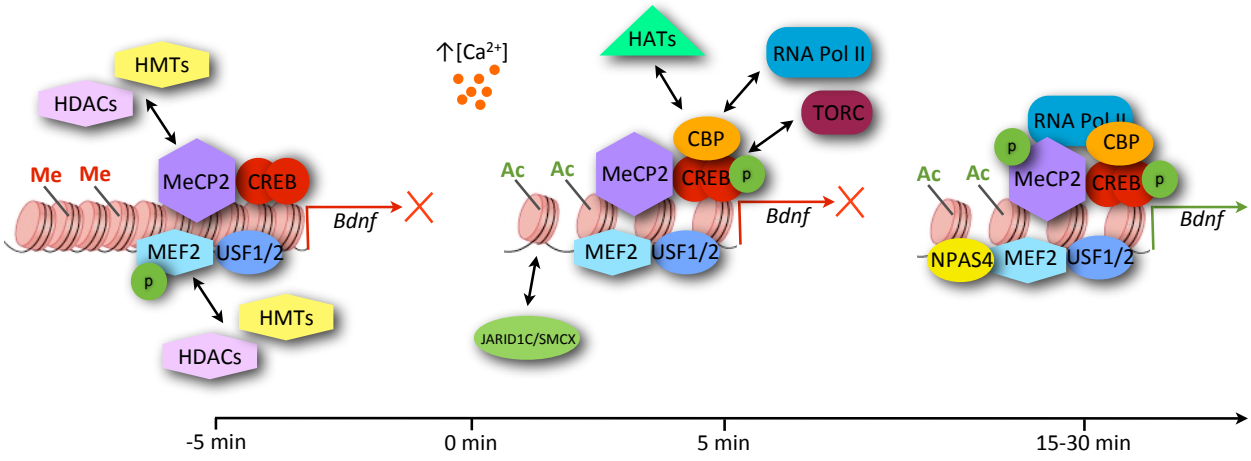

Figure 2.4: Activation of promoter IV-mediated *Bdnf* transcription depends on calcium influx triggered by neuronal activity

Several transcription factors are constitutively bound to regulatory sites of *Bdnf* promoter IV. In the absence of Ca^{2+} these factors maintain promoter IV in an inactive state by recruiting HDACs and HMTs. The influx of Ca^{2+} into the post-synaptic neuron is triggered by neurotransmitter release in response to stimulation, resulting in the phosphorylation of CREB at Ser-133, allowing it to recruit CBP and the basal transcriptional machinery. Dephosphorylation of MEF2 further leads to inactivation of repressor complexes. The final step in the activation of *Bdnf* promoter IV is the phosphorylation of MeCP2, which occurs 15-30 minutes after the initial Ca^{2+} signaling event. Adapted from Greer and Greenberg (2008).

2.1.4 Summary

Above I have illustrated some of the key cellular mechanisms that regulate CREB and BDNF. Both proteins are subjected to many levels of cell population- and brain region-specific control, as well as temporal regulation. Although most of the *Bdnf* promoters are responsive to neuronal activity, promoter IV is most robustly induced in an activity-dependent manner in terms of both magnitude and duration of induction. CREB is directly involved in the activation of *Bdnf* promoter IV transcription, as are a number of other important transcription factors implicated in synaptic function, behaviour and disease. For example, MEF2 negatively regulates the formation of dendritic spines (Flavell *et al.*, 2006; Pulipparacharuvil *et al.*, 2008) and is implicated in learning and memory (Vetere *et al.*, 2011). Mutations of MEF2, as well as a number of genes regulated by MEF2, have been implicated in mental retardation, autism and epilepsy (Flavell *et al.*, 2008; Le Meur *et al.*, 2010). Similarly, MeCP2 interacts with *Bdnf* to modulate learning and memory (Im *et al.*, 2011; Li *et al.*, 2011) and mutations in MeCP2 cause Rett syndrome, a disorder characterized by severe mental retardation and progressive neurological symptoms (Amir *et al.*, 1999). Therefore, a number of factors that regulate *Bdnf* promoter IV are important for normal function, and investigating the role of this promoter in behaviour promises to shed useful insights about an important mechanism in the brain.

2.2 Introduction to behavioural neuroscience

Most of the basic and pre-clinical research in the field of neuroscience employs animals as model systems for human behaviour. While observations of animals cannot capture the full complexity of human behaviours, there is a strong belief that fundamental attributes of behaviour are demonstrated by all organisms with fully evolved nervous systems and have provided many insights into human brain function (Kandel, 2001). The use of model systems is crucial for answering scientific questions that can only be probed with invasive methodology in a carefully controlled environment. Mice are a formidable model system for such study because of their close genetic homology with humans and their capacity for learning, as well as the ease with which colonies can be bred and maintained in the lab. With training, mice can learn to efficiently navigate complex environments, associate objects in the environment with aversive or

appetitive experiences, and enact goal-directed behaviours in order to avoid pain or receive a reward. Mice also react in predictable and well-characterized ways to unfamiliar settings or stressful events, lending them to the study of anxiety-related behaviours. Most importantly, mice can be manipulated to under- or over-express genes implicated in human brain function and disease pathology.

2.2.1 Genetic mutations in mice

Mice with genetic mutations are commonly employed in behavioural neuroscience research. This approach involves generating a target DNA construct, which either lacks the gene of interest or contains a mutated version of the gene, and transfecting embryonic stem cells with the target construct (Rudolph *et al.*, 1998). Mutant embryonic stem cell lines can then be implanted into fertile female mice that, after a series of mating events, generate transgenic offspring.

The simplest types of genetically manipulated mice are those that lack both alleles of a target gene from the time of conception (constitutive knock-out mice). An advantage of this approach is that it permits the study of an animal that completely lacks the gene product of interest throughout its lifetime. However, mice that are null mutants for CREB ($CREB^{null}$ mice; Rudolph *et al.*, 1998) or BDNF ($BDNF^{-/-}$ mice; Korte *et al.*, 1995; 1996) die soon after birth, so they are not useful for behavioural studies. Alternatively, it is possible to generate mice that express one allele of a target gene or one or more of its isoforms. These mice are typically viable without gross phenotypic abnormalities. For instance, in $BDNF^{+/-}$ mice the gene product is reduced by a known proportion (~50%), so the magnitude of behavioural deficits can be informative about the function of the gene (Ernfors *et al.*, 1994). However, developmental compensatory mechanisms might exist for the loss of a gene. In $CREB^{\alpha\delta-/-}$ mice, expression of the β isoform of CREB and all isoforms of CREM are up-regulated, so the importance of specific CREB isoforms cannot be accurately studied using these mice (Balschun *et al.*, 2003; Gass *et al.*, 1998; Hummler *et al.*, 1994). An alternative method for knocking down gene expression is to generate a mouse with a knock-in mutation, which constitutively expresses a mutated form of the gene of interest (referred to as a dominant-negative mutation). This is a more physiologically relevant approach since it is more common for congenital genetic defects to arise from a mutation in a gene rather than the complete loss of a gene. However, since the mutation is

present from the time of conception, developmental compensatory mechanisms can still mask the effect of the mutation.

The use of conditional genetic mutations can provide brain-region specific or temporal control for the experimental manipulation, circumventing problems associated with compensatory mechanisms. Spatial and temporal control of genetic mutations can be achieved by using the phage-P1 derived Cre/LoxP recombination system (Capecchi, 1989; Sauer & Henderson, 1989; Tsien *et al*., 1996). Cre-recombinase is a member of the integrin family that promotes the head-to-tail joining of 34 bp loxP sequences; thus any gene of interest flanked by loxP sequences (referred to as a floxed gene) will be excised by Cre-recombinase. The excision of a target gene can be restricted to a particular brain region or neuronal population by controlling the expression of loxP with a desired viral promoter. Use of the Nestin promoter results in transgene expression throughout the brain early in embryonic development (Tronche *et al.*, 1999; Zimmerman *et al.*, 1994), while the αCaMKII promoter restricts transgene expression to post-mitotic excitatory neurons in regions of the forebrain, including the hippocampus, amygdala, cortex and striatum (Balschun *et al.*, 2003; Otto *et al.*, 2001; Tsien *et al.*, 1996). The induction of a genetic mutation can also be temporally controlled. Injection of the synthetic drug tamoxifen (TAM) can be used to induce expression of a transgene tagged with a mutant ligand-binding domain (LBD) of the human estrogen receptor (Josselyn *et al.*, 2004; Kellendonk *et al.*, 1999; Kida *et al.*, 2002). The transgene will not be expressed under normal conditions, but injection of mice with TAM activates the mutant LBD-transgene construct, leading to its expression. Reversible control of transgene expression can be achieved using the tetracycline responsive element (TetOp-Cre) and the neuron-specific enolase-tetracycline transcriptional activator (NSE-tTa) inducible system (Hensler *et al.*, 2007; Mayford *et al.*, 1996; Monteggia *et al.*, 2004). The tTa/TetOp-mediated activation of Cre-recombinase can be turned on and off by the removal or addition, respectively, of doxycycline (dox) in the diet of the mouse.

The same techniques that are used to constitutively or conditionally knock down the expression of genes can be used to induce the expression of a mutant gene that is more active than the wild-type gene (dominant-active mutation). Thus the function of a target gene can be effectively down-regulated or up-regulated in a spatially and temporally controlled manner using genetic manipulations. These approaches can be informative for understanding the role of a gene in a physiological or behavioural process.

2.2.2 Virus-mediated gene transduction

Virus-mediated gene transduction is a more easily controlled approach that results in the expression of a gene of choice in a target brain area. This approach takes advantage of the ability of viruses to package and transport genes into a host cell and use the host's cellular machinery to express these genes. There are a number of commonly used viral vectors that differ in terms of their specificity for cell populations, time-course and maintenance of gene expression, toxicity, and size limit of their gene-carrying capacity (reviewed in Barco & Marie, 2011; van den Pol *et al.*, 2009).

Herpes simplex viruses (HSV), adeno-associated viruses (AAV), and lentiviruses (LV) are the most common viral vectors used in behavioural studies. HSV has been used as a viral vector extensively in my lab because of its specificity for post-mitotic neurons and ability to transport relatively large amounts of DNA, such as the gene for GFP, which allows visualization of infected neurons (for reviews see Neve & Lim, 2001; Neve *et al.*, 2005). HSV vectors typically consist of an amplicon containing a gene of interest and HSV-1 viral particles needed for expression of the gene in the host, and low concentrations of the replication-defective HSV virus (helper virus) needed to infect cells (Neve *et al.*, 2005). An appropriate ratio of amplicon to helper virus ensures that the viral vector is non-toxic. Furthermore, the genes shuttled to the host cells remain episomal, circumventing problems associated with integration into the host genome (Carlezon *et al.*, 2000). A drawback of HSV vectors is that their expression is short lasting, peaking 24-72 hours after infection and essentially disappearing after 7 days (Han *et al.*, 2009). By contrast, replication-defective AAV vectors have been shown to induce gene expression for long periods, lasting up to a year (van den Pol *et al.*, 2004). AAV vectors also rely on a helper virus for expression, remain episomal, and are relatively non-toxic (reviewed in van den Pol *et al.*, 2009). A drawback to AAV vectors is the late onset of gene expression, and much smaller packaging capacity. Similarly, replication-defective LV vectors can induce gene expression lasting up to 8 weeks (Hioki *et al.*, 2007). These vectors infect both neurons and glia and have a much smaller carrying capacity than HSV (Adamantidis *et al.*, 2007). Many other viral vectors have important uses outside of behavioural testing. For instance, pseudorabies virus (PRV) can be used to trace neuronal circuits by virtue of its ability to propagate from one cell to the next in the anterograde or retrograde direction (Aston-Jones & Card, 2000; Card *et al.*, 1991).

However, each cell that it infects dies, eventually leading to death of the animal. Retroviruses are best suited for tracking developing cell lineages, since they integrate their genetic content into the host genome, ensuring that it is passed on when the host cell divides (reviewed in van den Pol *et al.*, 2009).

Viral-vector mediated gene transfer allows for precise temporal and regional control of genetic manipulations. This technique can be used to overexpress a gene of interest, leading to a gain of function, or to overexpress a dominant-negative transgene, leading to a loss of function. This approach allows for the easy visualization of infected cells through virus-mediated incorporation of a fluorescent protein, and generally is not influenced by compensatory genetic mechanisms. Overall, viral vectors are a useful complimentary technique to genetic mutations in mice. Significant drawbacks to this technique are that the duration of virus-mediated gene expression can be limited, and only a limited population of cells within a given brain region will be infected.

I have now reviewed the important signaling pathways regulated by CREB and BDNF, as well as some of the tools used to induce genetic manipulations in mice. I will now proceed to discuss how these tools have helped scientists elucidate the functional significance of the molecular signaling events modulated by CREB and BDNF.

2.3 Roles of CREB and BDNF in development

As proposed in the neurotrophic factor hypothesis (Thoenen & Barde, 1980), NTs secreted during development play a crucial role in modulating the connectivity of neuronal circuits. It has also become clear that during this period NTs exert modulatory influences on CREB-mediated gene expression. While my study was focused on animal behaviour in adulthood, I tested mice in which the function of a gene was disrupted from the time of conception, so it is important to understand the developmental abnormalities that could affect their behaviour.

2.3.1 Neuronal survival

Studies in which CREB or BDNF function is disrupted have revealed crucial roles for both proteins in the survival of developing neurons. CREB has been implicated in the survival of sensory neurons in the peripheral nervous system (PNS). Early evidence showed that NGF promotes the survival of sensory neurons by inducing CREB phosphorylation at Ser-133 (Riccio *et al.*, 1997). Sympathetic neurons infused with CREB inhibitors failed to survive in the presence of NGF for more than 5 days, and demonstrated a decrease in cytochrome c, a necessary event for the induction of apoptosis by caspases (Riccio *et al.*, 1999). Moreover, neurons transfected with VP16-CREB protein, a herpes simplex virus (HSV)-derived protein that binds to CREB target genes through the bZIP domain of CREB and constitutively activates CREB-mediated transcription, survived in the absence of NGF. NGF-dependent cell survival appears to be mediated by Ras-ERK signaling and activation of CREB (Bonni *et al.*, 1999), which induces expression of the anti-apoptotic factor B-cell lymphoma protein-2 (Bcl-2; Riccio *et al.*, 1999). It now appears that other CREB family members and interacting proteins are also important for neuronal survival during development. The CREB family member ATF-7 promotes cell survival by interfering with caspase-initiated apoptosis in mice (Persengiev & Green, 2003). The CREB transcriptional coactivator CBP has also been shown to protect neurons from neurotoxicity (Nucifora *et al.*, 2001), and reductions in CBP are associated with neurodegeneration in a model of Huntington's Disease (Jiang *et al.*, 2006).

A vast amount of insight into the roles of CREB in development has come from studies with transgenic mice that completely lack the CREB gene. $CREB^{-/-}$ mice die shortly after birth due to severe respiratory failure (Rudolph *et al.*, 1998). The same observation was made with mice overexpressing a dominant-negative CREB inhibitor (A-CREB) that prevents CREB binding to effector genes (Long *et al.*, 2001). These mice exhibited a defect in bone growth, resulting in atrophy of chondrocytes and reduced rib cage circumference, leading to respiratory distress. Lonze *et al.* (2002) observed an extensive depletion of sensory and sympathetic neurons in $CREB^{-/-}$ mice during embryonic development (E13.5-17.5). These mice had abnormal peripheral sensory projections to the fore- and hind-limbs, and abnormalities in the cranial and spinal nerves. Injection of anti-NGF antibodies into mice immediately after birth

also resulted in a marked decrease in CREB phosphorylation and apoptosis of sympathetic neurons 2 days later.

Perhaps more relevant to my study are the effects of CREB depletion on central nervous system (CNS) development. On E18.5, $CREB^{-/-}$ mice had substantial reductions of the corpus callosum, anterior commissure, and hippocampal commissure (Rudolph *et al.*, 1998). They showed normal embryonic development of the brain stem, spinal cord, and most other brain areas. A subsequent study showed that CREB is crucial for CNS development during embryogenesis (Montamadiotis *et al.*, 2002). $CREB^{NesCre}$ mice, which have a brain-wide deletion of CREB induced during early embryonic development, demonstrated severe neuronal atrophy in several brain areas by the time of birth. Neuron size and density was reduced in the pyramidal cell layer of the hippocampus, the mitral layer of the olfactory bulb, the rhinal and limbic cortical areas, and the cortical plate due to increased caspase-3 mediated apoptosis. CNS deficits were far less severe in $CREB^{CaMKCre4}$ mice, which exhibit a forebrain-specific deletion of CREB in post-mitotic neurons. However, these mice began to show neurological deficits at 6 months of age due to substantial neurodegeneration in the hippocampus and striatum. These findings indicate that depletion of CREB at earlier stages of life cause more severe developmental deficits.

Even though it is the primary neurotrophin in the CNS, BDNF has been implicated largely in the survival of PNS neurons. Sensory neurons from mice completely lacking BDNF ($BDNF^{-/-}$ mice) are undersized, show nuclear displacement, and undergo premature cell death (Ernfors *et al.*, 1994). These mice have a pervasive reduction in neuron number throughout the PNS, including the dorsal root ganglia (DRG), trigeminal ganglion, mesencephalic trigeminal nucleus, vestibular ganglion and nodose ganglia. Even though these mice did not survive beyond 2 weeks of age, they showed overt behavioural deficits shortly after birth, including defective motor coordination and balance, head tilting and bobbing, and hyperactive periods of spinning. By contrast, $BDNF^{+/-}$ mice develop normally but exhibit obesity and hyperactivity, which can be reversed by intracranial infusions of BDNF into the hypothalamus (Kernie *et al.*, 2000). Another study showed that infusion of antisense BDNF oligonucleotides (OGNs) into cultured neurons from the DRG of adult mice attenuated neuronal survival (Acheson *et al.*, 1995). These results implicate BDNF in an ongoing trophic function throughout the lifespan.

Genetic deletion of BDNF results in relatively mild developmental deficits in the CNS (Huang & Reichardt, 2001). In cultured neurons from the rat cerebellum, immature granule cells were dependent on BDNF for survival, whereas mature cells were dependent on NT-3 (Segal *et al.*, 1992). BDNF was also shown to exert neuroprotective effects by protecting cerebellar granule cells from glutamate-induced neurotoxicity (Lindholm *et al.*, 1993). However, $BDNF^{-/-}$ mice do not exhibit impairments in cerebellar or hippocampal development, suggesting that findings in culture do not necessarily reflect the function of BDNF *in vivo* (Ernfors *et al.*, 1994). Mice with a conditional deletion of BDNF in post-mitotic neurons of the CNS survived to at least 8 months of age and showed no overt physical abnormalities (Rauskolb *et al.*, 2010). These mice showed a reduction in striatal volume attributable to a decrease in medium spiny neuron (MSN) size and a substantial (~50%) reduction in dendritic branching. Pyramidal neurons in the hippocampus were morphologically similar to wild-type neurons, suggesting that only certain neuronal populations in the CNS require BDNF during development.

While the effects of CREB on neuronal survival are dependent on NGF signaling, it is unclear whether CREB and BDNF interact in a similar way. Evidence for the divergent and independent roles of BDNF and NGF during development suggests that they activate different survival-promoting mechanisms. In cultured hippocampal neurons, NGF is secreted in a constitutive manner, while BDNF is secreted in a regulated manner in response to electrical stimulation (Mowla *et al.,* 1999). The constitutive secretion of NGF would appear to be an ideal mechanism for its target-derived trophic effects, while the activity-dependent secretion of BDNF better fits the role of a synaptic modulator. Interestingly, a recent study showed that neurons from mouse embryonic stem cells underwent apoptosis *in vitro* and *in vivo* following inhibition of TrkA or TrkC, but not TrkB (Nikoletopoulo *et al.*, 2010). This suggests that BDNF-TrkB signaling does not play an important role in preventing apoptosis during development, in marked contrast to NGF-TrkA signaling (Riccio *et al.*, 1997, 1999). Instead, the pro-form of BDNF, which has a high affinity for the $p75^{NTR}$ receptor appears to be responsible for apoptosis in developing neurons (Lee *et al.*, 2001). This suggests that the regulation of proBDNF cleavage to BDNF has an important role in neuronal survival. BDNF protein levels in the hippocampus rise 12-fold over the first 12 weeks postnatally, while proBDNF levels decline into adulthood, likely contributing to the stabilization of neuronal circuits (Rauskolb *et al.*, 2010; Yang *et al.*, 2009). Although BDNF expression can be induced by NGF *in vivo* (Apfel *et al.*, 1996), other studies

suggest that the expression of BDNF during development is independent of other neurotrophins. Kolbeck *et al.* (1999) showed that $NGF^{-/-}$ and $NT3^{-/-}$ knock-out mice, which die shortly after birth, had similar levels of surviving neurons in the CNS compared to wild-type mice, and BDNF protein levels were equivalent among transgenic and wild-type mice in all brain areas examined. Although deletion of NGF or NT3 resulted in significant reductions in cell survival in the PNS, BDNF levels in the DRG were not reduced in transgenic mice when differences in total neuron number were accounted for. These findings suggest that BDNF and NGF have separate roles during development and that the link between NGF and CREB in neuronal survival does not imply an important role for a CREB-BDNF interaction during development.

2.3.2 Ocular dominance plasticity in the visual cortex

The formation of ocular dominance columns in the visual cortex is one of the most extensively studied developmental phenomena in the CNS. Ocular dominance plasticity is activity-dependent and has a critical period during early postnatal life (for reviews see Thoenen, 1995; Wiesel, 1982). Normally, visual input from the environment is converted to a sensory signal by the retina and relayed to the lateral geniculate nucleus (LGN) of the thalamus before being transmitted to the visual cortex. At the LGN, input from each eye is segregated, and afferents to the visual cortex project to distinct zones corresponding to each eye. When visualized autoradiographically, the visual cortex is dividing into distinct columnar bands, topographically arranged to correspond to different parts of the retinal receptive field, with adjacent ocular dominance columns corresponding to input from separate eyes. While ocular dominance columns in layer 4 (L4) of the visual cortex receive strictly monocular input from the LGN, the superficial and deep layers of the visual cortex also tend to be more responsive to input from the eye corresponding to the dominance column in which they reside. By suturing one eye shut to induce monocular deprivation (MD) during the first weeks of life, Hubel, Wiesel and colleagues showed that cats and monkeys are blind in the deprived eye when the suture is removed and that ocular dominance columns in L4 of the visual cortex respond exclusively to input in the non-deprived eye (Hubel & Wiesel, 1968; Hubel *et al.*, 1977; Wiesel & Hubel, 1963; reviewed in Wiesel, 1982). While the suturing procedure did not affect ocular dominance columns or visual responses in adult animals (Hubel & Wiesel, 1970), only a few days of MD were needed to induce substantial changes in eye preference in the visual cortex during development (LeVay *et*

al., 1980), a finding that has since been replicated in mice (Gordon & Stryker, 1996; Tagawa *et al.*, 2005).

In the mouse, a maximal shift in ocular dominance towards the non-deprived eye is achieved within 4 days of MD during the critical period, which lasts until approximately P34 (Gordon & Stryker, 1996). Pham *et al.* (1999) showed that LacZ-reporter mice, which carry a reporter construct that tags newly expressed genes containing a CRE consensus sequence, had an 8-fold increase in CREB-mediated gene expression after 24 hours of MD in the visual cortex contralateral to the open eye. This expression level was 4-fold higher than in the ipsilateral visual cortex, which receives input corresponding to the deprived eye. Moreover, LacZ expression was widely distributed in the contralateral hemisphere but confined primarily to a narrow binocular zone in the ipsilateral hemisphere. MD resulted in these changes in juvenile (P26-28) animals, but did not induce shifts in ocular dominance or LacZ expression in adult (P40-44) animals. CREB-mediated gene expression during MD was further shown to be dependent on PKA and ERK signaling (Cancedda *et al.*, 2003; Suzuki *et al.*, 2004). MD has since been shown to induce changes in ocular dominance plasticity in adult mice up to 1 year of age with techniques measuring *c-fos* expression after MD (Pham *et al.*, 2004) and single-unit recordings (Fischer *et al.*, 2007). However, the MD-induced shift in ocular dominance in adult mice is only transient, corresponding to a developmental decrease in CREB levels in the visual cortex (Pham *et al.*, 2004). Pham *et al.* showed that MD induced a persistent shift in ocular dominance in VP16-CREB mice that overexpressed CREB in the visual cortex during adulthood. Taken together, these findings show that CREB mediates the refinement of ocular dominance columns in the visual cortex in an experience-dependent manner.

Appropriate regulation of BDNF is also crucial for visual cortex development. Visual experience results in an upregulation of BDNF expression in the visual cortex, while visual deprivation results in a decrease in BDNF (Majdan & Shatz, 2006). Although $BDNF^{+/-}$ mice show normal development of the visual cortex and recovery after an episode of MD (Bartoletti *et al.*, 2002; Kaneko *et al.*, 2012), infusion of BDNF into the visual cortex of kittens during the critical period prevents the formation of ocular dominance columns (Cabelli *et al.*, 1995). Strikingly, this manipulation actually results in a shift in ocular dominance to the visual cortex corresponding to the deprived eye following an episode of MD (Galuske *et al.*, 2000). Both TrkB receptor blockade in the visual cortex of cats (Cabelli *et al.*, 1997) and TrkB deletion in

mice (Kaneko *et al.*, 2008b) block recovery after short periods of MD. It is likely that such increases or decreases in BDNF-mediated signaling disrupt ocular dominance plasticity due to interference with the normal activity-dependent neuronal competition for BDNF among afferent neurons from the LGN (Thoenen, 1995). Consistent with this explanation, a requirement for BDNF in the dendritic arborization and axon growth of retinal ganglion cells has been demonstrated extensively in the tadpole *Xenopus laevis* (Cohen-Cory & Fraser, 1995; Lom *et al.*, 2002; Hu *et al.*, 2005) and in rodents (Ma *et al.*, 2010; Meyer-Franke *et al.*, 1995). BDNF mediates the growth of connecting dendrites in the visual cortex *in vitro* (McAllister *et al.*, 1995) and transgenic mice with deficits in the transportation of BDNF to apical dendrites demonstrate an abnormal shift in ocular dominance during MD and impaired recovery of binocular vision (Kaneko *et al.*, 2012). Furthermore, mice overexpressing BDNF in the visual cortex showed accelerated development of cortical inhibitory circuits and visual acuity (Huang *et al.,* 1999). Like CREB, BDNF appears to modulate experience-dependent changes in the visual cortex during the critical period of development. Two intriguing questions that emerge from this discussion are whether or not these proteins interact, and if so, what is the role of this interaction in visual cortex plasticity.

2.3.3 Excitatory-inhibitory balance

The link between CREB and BDNF in visual cortex development appears to reside in the regulation of inhibitory responses. The main inhibitory synapses in the CNS contain receptors for gamma-aminobutyric acid (GABA). The development of GABAergic synapses is required for cortical plasticity (Fagiolini *et al.*, 2004; Hensch *et al.*, 1998; reviewed in Hensch, 2005), and there is now a wealth of evidence showing that endogenous BDNF is required for inhibitory synapse development (Bosman *et al.*, 2006; Huang *et al.*, 1999; Kohara *et al.*, 2007; reviewed in West, 2008). Recently, Hong *et al.* (2008) linked the CREB-mediated expression of *Bdnf* promoter IV to the development of inhibitory synapses in the mouse visual cortex.

As described earlier, Hong *et al.* (2008) generated a transgenic mouse with a knock-in mutation in the CaRE3/CRE-like binding sequence on BDNF promoter IV (CREmKI$^{-/-}$ mouse). These mice expressed normal basal levels of CREB and BDNF, but demonstrated a complete absence of CREB or CBP associated with *Bdnf* exon IV mRNA. In response to stimulation of NMDA receptors *in vitro*, CREmKI$^{-/-}$ mice had a dramatic (~90%) reduction in *Bdnf* promoter

IV mRNA expression and a 50% reduction in overall *Bdnf* mRNA expression. This deficit in activity-dependent *Bdnf* expression was replicated *in vivo* by exposing dark-reared mice to light for 90 minutes. CREmKI$^{-/-}$ mice showed a 75% reduction in visual cortex expression of *Bdnf* exon IV mRNA and a 50% overall reduction in *Bdnf* mRNA. There was evidence of compensatory effects in the mutant mice, as the IEGs *c-fos*, *Arc*, and neuronal pentraxin-2 (*Np-2*) were upregulated. Critically, Hong *et al.* demonstrated that cultured cortical neurons and L2/3 neurons from the visual cortex of 8-12 week old CREmKI$^{-/-}$ mice had reductions in inhibitory synapse number as shown by GAD65 immunostaining and $GABA_A$ receptor β2/3 and γ subunit inhibitory markers. Electrophysiological recordings from L2/3 of the visual cortex showed that miniature inhibitory post-synaptic currents (mIPSCs) in the CREmKI$^{-/-}$ brain were reduced in amplitude and frequency. However, the number of excitatory synapses in CREmKI$^{-/-}$ brains did not differ from wild-type brains, and the number of inhibitory neurons in CREmKI$^{-/-}$ brains was not reduced, pointing to a specific deficit in the development of inhibitory synapses.

Since the CREmKI mutation disrupts the CREB-mediated expression of *Bdnf* promoter IV throughout the entire brain, the relevance of the developmental deficit in these mice extends beyond the visual cortex. Shifts towards increased excitation in the nervous system have been linked to behavioural abnormalities and neuropsychiatric diseases (Dani *et al.*, 2005; Lisman *et al.*, 2008; Rubenstein & Merzenich, 2003). My remaining discussion will focus on the roles of CREB and BDNF in the behaviour of adult mice.

2.4 Roles of CREB and BDNF in memory

The capacity for memory does not simply allow us to maintain a detailed, accessible record of our past experiences. It also helps us to behave in appropriate, adaptive ways in familiar environments in the future. Increasingly sophisticated transgenic, viral, and other approaches have allowed us to gain insight into many of the basic physiological processes underlying memory and behavioural adaptations.

2.4.1 Memory processes and the brain

Three different types of physiological processes are relevant to my discussion of memory: acquisition, consolidation, and retrieval. *Acquisition processes* are those processes that allow information accessible to our sensory organs at the time of a learning event to be captured by the brain and stored as a memory. The acquisition of memories is a subjective process dependent on the particular individual's mental faculties and past experiences; therefore, any two individuals experiencing the same event are unlikely to form an identical representation of that event in memory. After the acquisition of a memory, it enters a labile state in which it is vulnerable to disruption (Müller & Pilzecker, 1900). During this time, *consolidation processes* reinforce the existing memory representation and stabilize the memory (Dudai, 2004). Consolidation occurs at the level of individual cells (*synaptic consolidation*) followed by a more prolonged process of systems reorganization of memory circuits (*systems consolidation*). Synaptic and systems consolidation have vastly different time-scales (seconds, minutes, hours for synaptic consolidation, and days, months, years for systems consolidation) and likely depend on independent processes in the brain (McGaugh, 2000). *Retrieval processes* depend on the ability of consolidated memory representations to influence behaviour.

Two brain areas critically involved in memory are the central focus of my discussion: the hippocampus and the amygdala. Both are limbic system structures situated in the medial temporal lobe. The hippocampus and amygdala are interconnected but subserve distinct memory functions. The hippocampus is best known for supporting spatial information based on the finding of place cells in rodents (O'Keefe & Dostrovsky, 1971; O'Keefe & Nadel, 1978), and amnesia for past events (i.e. *episodic memory*) in brain-damaged patients (Bayley & Squire, 2002; Kesner & Hunsaker, 2010; Squire & Zola, 1998; Tulving, 1969). It has since become clear that the hippocampus supports other types of relational memories (Eichenbaum, 1996). The hippocampus appears to be necessary for updating existing memory representations with new information (Eichenbaum *et al.*, 1989), and information initially stored in the hippocampus becomes reorganized to cortical circuits over time (Frankland *et al.*, 2004; Restivo *et al.*, 2009; Squire, 1992).

The hippocampal formation consists of the hippocampus, as well as parahippocampal areas, including the entorhinal, perirhinal and parahippocampal cortices, which receive unimodal

and multimodal sensory input through a complex network of afferents from many areas of the cortex (see Fig. 2.5; reviewed in Burwell *et al.*, 1995; Squire *et al.*, 2004). The entorhinal cortex (EC) serves as the gateway of sensory input into the hippocampus (reviewed in O'Keefe & Nadel, 1978). The hippocampus is divided into the dentate gyrus (DG), CA1, CA2, and CA3 (standing for *cornu ammonis*, Latin for the ram's horn) subfields (Lorente de No, 1934). The flow of information through the hippocampus is largely unidirectional, as each subfield consists of a tightly packed layer of cell bodies with excitatory axons projecting directly to the dendrites of the next subfield (Ishizuka *et al.*, 1990; Witter, 1993). Axons from the EC constitute the perforant pathway, which carries the main input into the hippocampus by projecting to granule cells in the DG (Amaral *et al.*, 2007). Mossy fiber axons from the DG granule cells project to the large pyramidal neurons of the CA3 subfield, which then project to the small pyramidal neurons of the CA1 subfield via the Schaffer collateral pathway. There does not appear to be any important anatomical distinction between the CA2 and CA3 subfields (Blackstad, 1956; Blackstad & Flood, 1963). The pyramidal neurons of the CA1 send out the main hippocampal output through excitatory projections to the subiculum (SB), lateral septum, or back to the EC (O'Keefe & Nadel, 1978). Hippocampal processing is also modulated in all subfields by orthogonally projecting inhibitory neurons (Klausberger & Somogyi, 2008). Functional distinctions have been proposed for all of the hippocampal subfields (see Kesner, 2007a,b; Kesner *et al.*, 2004, 2010; Moser & Moser, 1998), and there is a gradient of increasing place cell size from dorsally- to ventrally-located pyramidal neurons that may account for the differential involvement of these areas in spatial and contextual processing in rodents (Maren *et al.*, 1997; Moser *et al.,* 1993; Richmond *et al.*, 1999; Pittenger *et al.*, 2002).

The amygdala is well known for its role in the processing of emotional information. Initially it was thought to process information about aversive stimuli based on findings of impairments in responses to fearful stimuli in amygdala-lesioned rodents (LeDoux, 1995), and impaired recognition of fearful facial expressions in brain-damaged humans (Adolphs *et al.*, 1994). However, animals with amygdala lesions also demonstrate a reduction in approach behaviour towards appetitive stimuli such as food, implicating the amygdala in the processing of positive emotions as well (Parkinson *et al.*, 2000). The amygdala has been extensively implicated in Pavlovian conditioning in animals (reviewed in Balleine & Killcross, 2006; LeDoux, 2003; Robbins *et al.*, 2008), a process by which a previously neutral stimulus

(conditioned stimulus; CS) acquires motivational value through an association with an emotionally salient stimulus (unconditioned stimulus; US), such that the CS becomes capable of eliciting an emotional response (Pavlov, 1927; reviewed in LeDoux, 2000).

The amygdala is a heterogeneous structure consisting of many anatomically distinct cellular populations with different developmental origins (LeDoux, 2000; Swanson, 2003). Functionally, the nuclei of the amygdala can be divided into three broad areas, the basolateral complex of the amygdala (BLA), consisting of the lateral, basolateral, and basomedial nuclei, the accessory basal amygdala (AB), and central amygdala (CeA), which has medial and lateral nuclei (Fig. 2.6; Balleine & Killcross, 2006; Davis *et al.*, 2010; LeDoux, 2000). Nuclei of the BLA receive input from the geniculate bodies of the thalamus relaying information from sensory organs through direct projections, as well as through indirect projections from cortical sensory areas (LeDoux *et al.*, 1990; LeDoux, 2000). Meanwhile, the AB receives projections directly from the hippocampus (Canteras & Swanson, 1992). It is believed that the BLA is the point of CS-US convergence between sensory/perceptual cues and biologically salient information, while the AB is a point of convergence for context-US associations (LeDoux, 2000). The CeA is thought to integrate information from the other nuclei of the amygdala for higher order processing through its outputs to hypothalamic, cortical, and striatal regions of the brain (reviewed in Balleine & Killcross, 2006; LeDoux, 2000; Pitkänen *et al.*, 2003). The BLA and CeA may also have independent parallel functions, with the BLA attributing emotional value to discrete sensory or perceptual cues, and the CeA being required for general affective processing (Balleine & Killcross, 2006). Consistent with this interpretation, rats with BLA lesions demonstrated normal aversive responses to a painful footshock but did not show aversion to a CS previously associated with the shock, while rats with CeA lesions demonstrated the opposite pattern of behaviour (Killcross *et al.*, 1997). The medial and lateral nuclei of the CeA also appear to have dissociable roles in fear and anxiety (reviewed in Davis *et al.*, 2010).

Having given a brief overview of memory-related processes and important brain areas underlying memory, I will now discuss what is known about the effects of CREB and BDNF on synaptic mechanisms of memory storage and behavioural manifestations of memory.

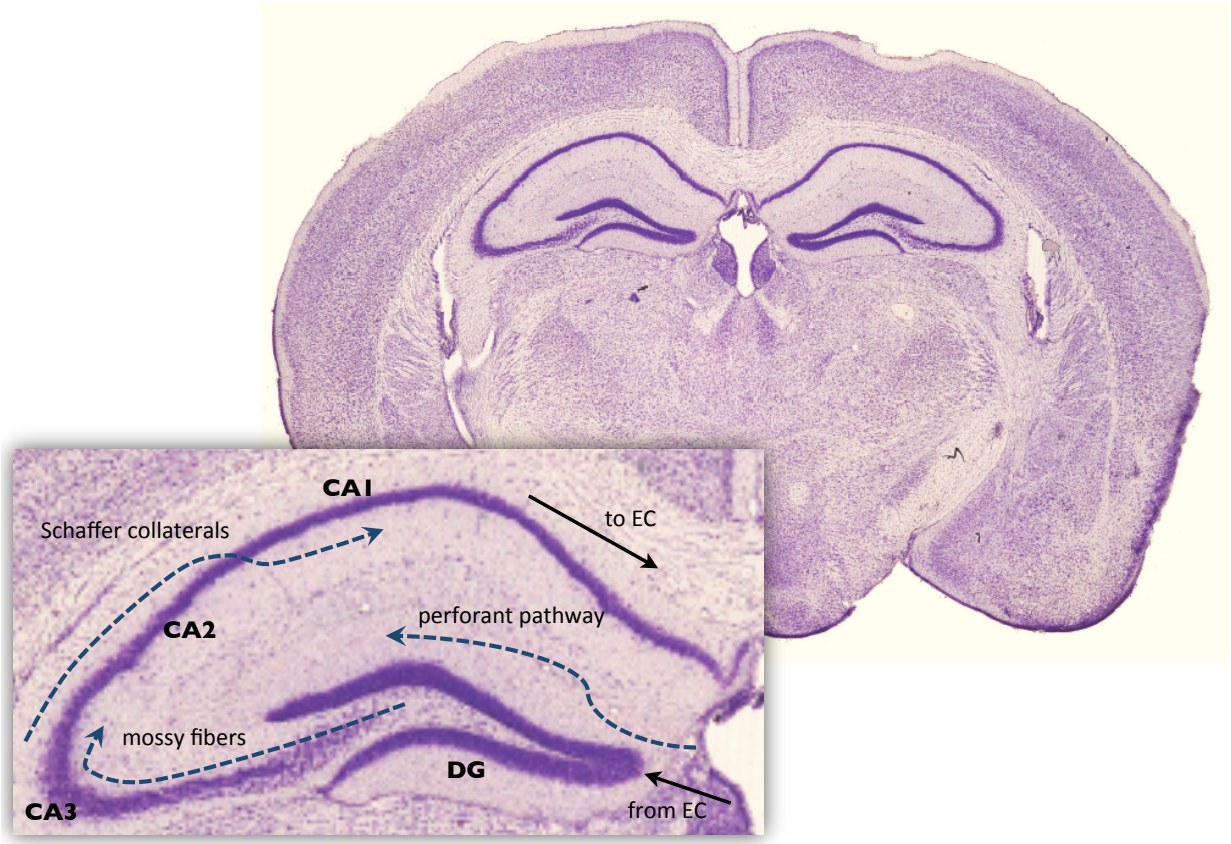

Figure 2.5: The hippocampus is the primary structure involved in the processing of spatial information in rodents
Information is propagated unidirectionally through the hippocampus by a series of excitatory pathways. Sensory input to the hippocampus converges in the entorhinal cortex (EC) and projects to the dentate gyrus (DG) via the perforant pathway. Mossy fibers from the DG project to the large pyramidal neurons of the CA3 subfield, which send information to the small pyramidal neurons of the CA1 subfield via the Schaffer collateral pathway. Pyramidal neurons of the CA1 send the main hippocampal output back to the EC, completing the loop. Inhibitory neurons also project orthogonally to the hippocampal subfields (not shown). Adapted from Paxinos and Franklin (2001).

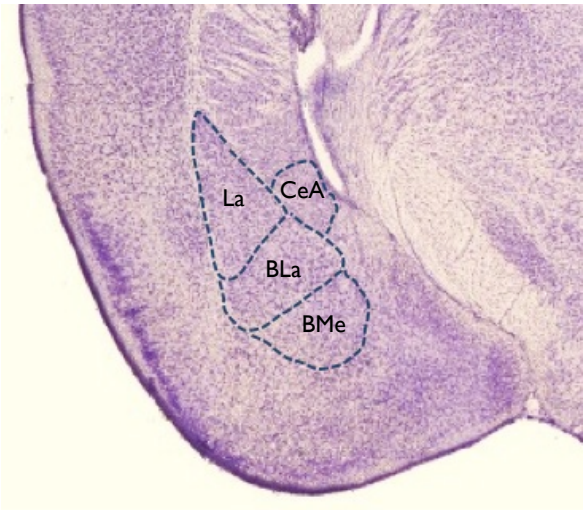

Figure 2.6: The amygdala is the primary structure involved in the processing of emotional information
The amygdala can be broadly divided into three functional regions: the basolateral complex of the amygdala (BLA), the accessory basal amygdala (AB) and the central amygdala (CeA). The BLA can be further subdivided into lateral (La), basolateral (BLa) and basomedial (BMe) nuclei. The AB is sometimes considered a separate region within the BMe nucleus (not shown). One prevalent view is that the BLA and AB are involved in sensory-perceptual and contextual processing, respectively, and that this information is integrated for higher processing in the CeA. Alternatively, regions of the amygdala may have independent, parallel functions. Adapted from Paxinos and Franklin (2001).

2.4.2 The CREB-BDNF interaction in synaptic plasticity

The ability of the brain to adapt through experience was, for the longest time, a perplexing phenomenon for scientists. By the early 1900s it was known that the number of neurons and their topographic organization in the brain were largely stable in adulthood due to highly organized developmental programs. This fact ruled out the formation or elimination of neural structures as a mechanism for the acquisition and forgetting of memories. By the mid-1900s, prominent scientists advocated for such mechanisms as changes in chemical gradients surrounding neurons or experience-dependent changes in the base sequence of DNA (reviewed in Kandel, 2001). However, it was an idea proposed by Santiago Ramon y Cajal half of a century earlier that proved to be the most instrumental in deriving how memories are formed.

2.4.2.1 Long-term potentiation

Cajal postulated that memory is mediated by structural changes in neurons that strengthen neuronal connections (Jones, 1994; reviewed in Bailey *et al.*, 2000). Nearly 60 years later, Kandel and his colleagues began to characterize the molecular biology of short- and long-term changes in synaptic strength in the sea snail, *Aplysia californica* (Kandel & Tauc, 1964). Neurons in *Aplysia*, like neurons in mammals, undergo experience-dependent changes in synaptic strength, a capacity referred to as *synaptic plasticity*. Plasticity at the synapse can result in changes in synaptic strength that are transient or prolonged, corresponding to two distinct phases of a physiological process called *long-term potentiation* (LTP; reviewed in Bailey *et al.*, 2000; Bliss & Collingridge, 1993; Goelet *et al.*, 1986; Kandel, 2001).

LTP is an enduring, experience-dependent increase in synaptic strength believed to underlie memory storage. The early phase of LTP (E-LTP) lasts 1-2 hours, involves post-translational modifications of existing proteins or changes in AMPA receptor internalization, and corresponds to short-term memory (STM; Lüscher & Frerking, 2001; Soderling & Derkach, 2000; Racaniello *et al.*, 2010). The late phase of LTP (L-LTP) typically lasts 6-24 hours or longer, requires synthesis of new proteins, and corresponds to long-term memory (LTM; Davis & Squire, 1984; Frey *et al.*, 1993; Goelet *et al.*, 1986; Huang & Kandel, 1994; Kandel, 2001; Silva *et al.*, 1998).

The most well understood form of LTP involves the strengthening of a homosynaptic connection between two neurons. This homosynaptic form of LTP was first observed in 1972 by Terje Lomo and Tim Bliss in neurons of the perforant pathway projecting from the EC to the DG of the hippocampus (reviewed in Bliss & Collingridge, 1993). By repeatedly stimulating the angular bundle of the EC to activate a perforant pathway fibre, Lomo and Bliss observed a persistent increase in the field evoked post-synaptic potential (fEPSP) in the stimulated population of neurons (Bliss & Lomo, 1973). Whereas a single bout of high frequency stimulation (called a *tetanus*) evoked a moderate increase in fEPSP amplitude that lasted approximately 30 minutes, four tetani administered a few minutes apart resulted in a more marked increase in EPSP amplitude that lasted seven hours or more. Although they did not propose a formal distinction of the two types of potentiation at the time, these are now known as E- and L-LTP, and appear to occur in all neurons that can undergo LTP (Bailey *et al.*, 2000).

LTP in the perforant pathway is caused by the coincident activation of both the pre- and post-synaptic neuronal populations, a property known as *associativity* (Bliss & Collingridge, 1993). Associative LTP results in a pre-synaptic facilitation of neurotransmitter release and a post-synaptic increase in excitability. However, as Kandel and colleagues first elucidated in experiments with *Aplysia*, LTP can also be non-associative, driven by an increase in efficiency of the pre-synaptic neuron (Kandel & Tauc, 1965a, 1965b). Kandel and Tauc showed that non-associative LTP occurs in a form of learning called long-term sensitization. Sensitization results when a stimulus causes a behavioural response to become increasingly pronounced or prolonged. *Aplysia* demonstrates sensitization of a reflex to withdraw its gill after a noxious stimulus is applied to its tail. In response to a single shock to the tail, *Aplysia* demonstrates behavioural sensitization that lasts a few minutes. However, five or more shocks to the tail cause a prolonged sensitization that last days to weeks. Kandel and Tauc discovered that synaptic sensitization could be induced by applying puffs of serotonin (5-hydroxytryptamine; 5-HT) to a sensory neuron. Applying one puff of 5-HT caused a short-lived potentiation that lasted only minutes, while applying five puffs induced LTP lasting days, as well as protein synthesis, paralleling the time-course of behavioural sensitization. While LTP in *Aplysia* depends on repeated depolarization induced by the 5-HT neurotransmitter signal, glutamate is the main neurotransmitter responsible for LTP at excitatory synapses in the mammalian brain (Malenka *et al.*, 1989).

Both non-associative and associative forms of LTP are prevalent in the mammalian brain. Associative forms of LTP, such as those occurring in the perforant and Schaffer collateral pathways of the hippocampus, differ critically from non-associative LTP in that they are dependent on the activation of NMDA receptors on the post-synaptic cell (Malenka *et al.*, 1989; Morris *et al.*, 1990; Salin *et al.*, 1996b). LTP in thalamic inputs to the lateral amygdala (LA; Lange *et al.*, 2012; Rogan & LeDoux, 1995) and cortical neurons (Vickery *et al.*, 1997) is also associative and blocked by NMDA receptor antagonists. In the non-associative mossy fibre pathway, LTP is not affected by blocking NMDA receptors but demonstrates enhanced AMPA receptor-mediated currents at the post-synaptic cell, suggesting that LTP is mediated by enhanced pre-synaptic release of glutamate (Kauer *et al.*, 1988). Similar observations have been made in purkinje cells of the cerebellum, which undergo non-associative LTP (Salin *et al.*, 1996a).

A great deal is now known about the pre- and post-synaptic signaling pathways involved in LTP. Non-associative LTP is driven by a depolarization-induced influx of Ca^{2+} into the pre-synaptic neuron through VSCCs, resulting in the activation of the second messenger cAMP (Brunelli *et al.*, 1976; Schacher *et al.*, 1988). In associative LTP, where glutamate is the synaptic transmitter, the post-synaptic cell must be sufficiently depolarized to expel the Mg^{2+} ions that block the calcium-permeable channels of NMDA receptors (Huang & Kandel, 1994; Malenka *et al.*, 1989; Fig 2.7). This allows the influx of Ca^{2+} ions into the post-synaptic cell, which binds to calmodulin and recruits the alpha isoform of CaMKII (Lee *et al.*, 2009). Binding to Ca^{2+}/calmodulin induces a conformational change in αCaMKII that allows it to undergo autophosphorylation at threonine 286 (Thr-286). Although Ca^{2+}/calmodulin rapidly dissociate from αCaMKII, once phosphorylated, αCaMKII remains active and carries out several important functions (reviewed in Lisman *et al.*, 2002). Firstly, αCaMKII can traffic and anchor AMPA receptors to the post-synaptic membrane. Secondly, αCaMKII can associate directly with membrane-bound NMDA and AMPA receptors to potentiate the flow of ions through these channels. Finally, some αCaMKII may translocate directly into the nucleus and activate CREB (Hardingham *et al.*, 2001).

While αCaMKII signaling is necessary for the induction of LTP, activation of the cAMP pathway leads to the nuclear signaling events that result in protein synthesis and the late phase of LTP. The importance of cAMP in LTP was first demonstrated in cultured neurons, where

injecting cAMP directly into the pre-synaptic neuron resulted in E-LTP following one injection, and L-LTP following multiple injections (Brunelli *et al.*, 1976; Schacher *et al.*, 1988). The activation of metabotropic G-protein coupled receptors (GPCRs) and their coupling with adenylyl cyclase triggers the synthesis of cAMP, which activates protein kinases such as PKA and PKC (Huang & Kandel, 1994). PKA can translocate directly into the nucleus or activate MAPK through the extracellular regulated kinase (ERK) pathway (Adams & Sweatt, 2002; Ahmad & Frey, 2005; Racaniello *et al.*, 2010). As discussed earlier, nuclear kinase activity via PKA, PKC, MAPK, and CaMKs contributes to the phosphorylation of CREB at Ser-133, initiating gene transcription necessary for L-LTP. Cyclic AMP formation can also be triggered by intracellular Ca^{2+} signaling or activated heterosynaptically by binding of modulatory neurotransmitters, such as dopamine, serotonin or norepinephrine, to their receptors (Bernabeu *et al.*, 1997; Hersi *et al.*, 1995; McGaugh, 2000). Ca^{2+} also interacts with calcineurin to activate phosphatases that negatively regulate the activity of CREB.

Among the CREB effector proteins activated at synapses is BDNF (Aid *et al.*, 2007; Patterson *et al.*, 1992). As I will discuss below, both CREB and BDNF are extensively implicated in L-LTP, but they do not appear to be involved in LTP under all circumstances. Their effects on LTP are dependent on the stimulation protocol used to induce LTP, and possibly on other experimental conditions. Furthermore, neither protein has a role restricted to the late phase of LTP *per se*. This observation reflects the fact that the regulation of both molecules is complex and dynamic. BDNF also shows high basal levels of activity and participates in a variety of other cellular functions throughout the brain.

Early experiments suggested that both CREB and BDNF were necessary for the induction of LTP. In a now classic study, Dash *et al.* (1990) injected CRE DNA oligonucleotides into cultured sensory neurons from *Aplysia*. The oligonucleotides outcompeted CREB for effector binding sites and prevented the induction of L-LTP, but not E-LTP, by multiple pulses of 5-HT. Bourtchuladze *et al.* (1994) carried out the first investigation of synaptic plasticity and memory in $CREB^{\alpha\delta-/-}$ mice by stimulating the Schaffer collateral pathway and recording from the stratum radiatum in CA1 of hippocampal slice preparations. $CREB^{\alpha\delta-/-}$ mice were not impaired in two

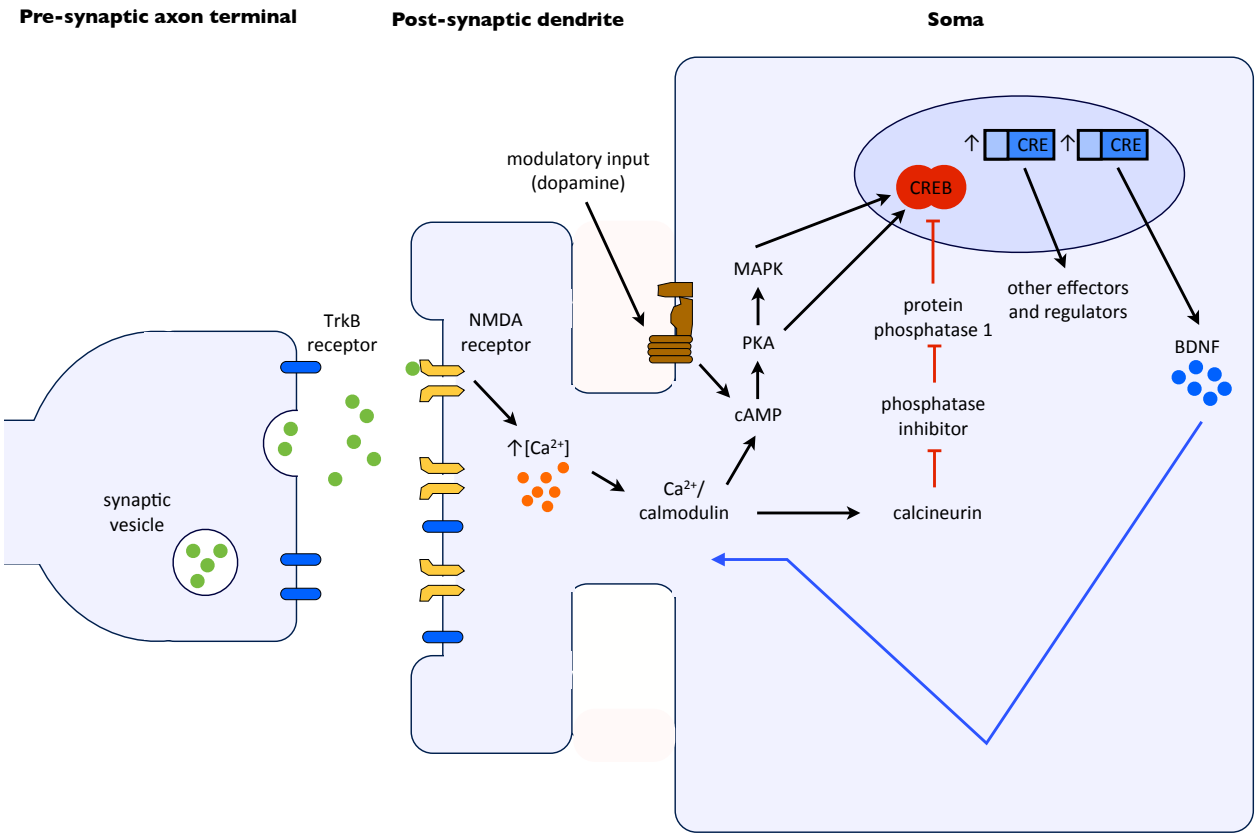

Figure 2.7: CREB-mediated expression of BDNF underlies associative L-LTP in the Schaeffer collateral pathway
Associative LTP in the Schaffer collateral pathway requires depolarization of the post-synaptic neuron coupled with glutamate binding to NMDA receptors. These events trigger Ca^{2+} influx into the post-synaptic neuron inducing the association of Ca^{2+}/calmodulin complexes with αCaMKII. Prolonged neuronal activation leads to the synthesis of cAMP and modulation CREB activity in the nucleus by protein kinases and phosphatases. These signals can be further potentiated heterosynaptically by modulatory neurotransmitters. *De novo* gene expression driven by activated CREB induces L-LTP. BDNF is an important CREB effector that contributes to the maintenance of L-LTP. Adapted from Kandel (2001).

tests of short-term plasticity, paired-pulse facilitation (PPF) or post-tetanic potentiation (PTP)[3], but demonstrated weak and rapidly decaying LTP compared to wild-type mice. It is important to note that the stimulation protocol used to induce LTP in this study, a single 2-second tetanus of

[3] PPF is a pre-synaptic form of short-term plasticity induced by a pair of stimuli spaced closely apart (usually ~50-200 ms). It is thought to occur because of an accumulation of Ca^{2+} in the pre-synaptic terminal (Katz & Miledi, 1968; Patterson *et al.*, 1996). PTP is a measure of the characteristic population spike in fEPSP amplitude evoked immediately after tetanization.

100 pulses with a frequency of 100 Hz, is usually only sufficient for the induction of E-LTP (Frey & Morris, 1997; Kandel, 2001), and was previously shown to be cAMP independent (Frey *et al.*, 1993). Since fEPSPs were only recorded for 2 hours following stimulation, generally not considered long enough to demonstrate L-LTP, it is possible that the impairment observed in $CREB^{\alpha\delta-/-}$ mice was actually related to a deficit in the induction of E-LTP.

Shortly after studies began implicating CREB in LTP, similar experiments were conducted with BDNF by several independent groups of researchers. Korte *et al.* (1995) investigated LTP in the Schaffer collateral pathway of $BDNF^{-/-}$ and $BDNF^{+/-}$ mice. Three tetani of 30 pulses (100 Hz) with an inter-stimulus interval (ISI) of 5 seconds between trains induced LTP in 87% of wild-type hippocampal slices, but only 27.7% of $BDNF^{+/-}$ brains, and none of the $BDNF^{-/-}$ brains. These effects were somewhat age-dependent, as the probability of inducing LTP in $BDNF^{-/-}$ brains rose to 32.6% in slightly older (>P16) animals. In two subsequent studies, adenovirus-mediated overexpression of BDNF under the control of cytomegalovirus (CMV) promoter (Ad-CMV-BDNF vector) in the hippocampus rescued the deficit in LTP in $BDNF^{+/-}$ and $BDNF^{-/-}$ mice using the same stimulation protocol, but LTP in mutant mice never reached wild-type levels (Korte *et al.*, 1996a, 1996b). In these studies, mutant mice did not show deficits in measures of short-term plasticity. Contrastingly, Patterson *et al.* (1996) showed that $BDNF^{-/-}$ mice had a considerable (63%) reduction in basal synaptic transmission compared to wild-type mice, while $BDNF^{+/-}$ mice had a 35% reduction in basal transmission. Both mutants with the knockout mutation were impaired in PPF and PTP, and had deficits in LTP following a stimulation protocol of 2 tetani of 60 pulses (100 Hz, 20-s ISI). These deficits could be rescued by incubating hippocampal slices in a bath containing BDNF for 5-8 hours. However, both the deficit in short-term plasticity and the rescue of LTP in this study have been difficult to replicate (Patterson *et al.*, 2001). Notably, none of the above studies measured LTP beyond 1.5 hours after stimulation, suggesting a role for BDNF in the induction of E-LTP, but not necessarily in L-LTP. Figurov *et al.* (1996) showed that BDNF applied in bath for 2.5-4 hours in young (P12-13) hippocampal neurons that normally only undergo short-term potentiation (STP) could induce LTP using a variety of stimulation protocols, which could be prevented by co-incubation of cells with a BDNF scavenger. A strong protocol of 4 trains of 100 pulses (100 Hz, 20-s ISI) induced LTP for at least 3 hours, suggesting that BDNF contributes to the induction of L-LTP. Interestingly, an earlier study showed that incubation of hippocampal slices with BDNF can

itself induce LTP (referred to as BDNF-LTP) lasting 2-3 hours, and that these neurons can be further potentiated by subsequent high-frequency stimulation (Kang & Schuman, 1995). This raised the interesting possibility that mechanisms of BDNF-LTP and LTP induced by high frequency stimulation (HFS-LTP) are distinct. It also implies that the observed rescue of deficits in LTP through the application of exogenous BDNF could be due to the induction of BDNF-LTP.

More recent studies have helped clarify some of the questions surrounding the roles of CREB and BDNF in LTP. Reducing levels of CREB using transgenic approaches has by-and-large been unsuccessful in generating deficits in LTP, suggesting that LTP can be induced under conditions in which the expression of CREB is reduced. Both $CREB^{\alpha\delta-/-}$ and $CREB^{comp}$ mice demonstrated normal LTP for up to 6 hours using a strong stimulation protocol (3 trains of 100 pulses, 100 Hz, 10-min ISI; Balschun *et al.*, 2003; Gass *et al.*, 1998). They also demonstrated normal long-term depression (LTD), a habituation-like effect that results in a decrease in synaptic strength following low-frequency stimulation (3 trains of 2000 pulses, 2 Hz, 10-min ISI). Balschun *et al.* (2003) replicated these findings in $CREB^{NesCre}$ mice, in which floxed CREB is excised in early development throughout the entire brain, and $CREB^{CaMKCre7}$ mice, in which the αCaMKIIa promoter induces a more restricted knockout of CREB in the forebrain and hippocampus. Mice expressing the dominant-negative S133A mutation, which contains a serine to alanine mutation, preventing phosphorylation of CREB at the critical serine residue ($CREB^{S133A}$ mice; Gonzalez *et al.*, 1989), under the control of the αCaMKIIa promoter also showed normal LTP (5 trains of 60 pulses, 100 Hz, 20-s ISI) and LTD (1 Hz for 15-min) in fibers connecting the lateral and basolateral nuclei of the amygdala (Rammes *et al.*, 2000). By contrast, at least one model of CREB overexpression suggests that CREB is involved in LTP, particularly in the late-phase. Barco *et al.* (2002) generated mice expressing VP16-CREB under the control of the tTa-TetOp inducible system. VP16-CREB is 25-fold more active than endogenous CREB and induces transcription of many of the same effectors, including CREB itself (Barco *et al.*, 2005). Using stimulation that only induced E-LTP for less than 2 hours in wild-type mice (1 train of 100 pulses, 100 Hz), L-LTP was induced for 6 or more hours in VP16-CREB mice. In another study, overexpression of a constitutively active form of CREB in the hippocampus enhanced the magnitude and maintenance of E-LTP (Marie *et al.*, 2005). The overexpression of CREB may more accurately capture the importance of CREB in L-LTP since

the expression of CREB cannot be completely abolished in transgenic mice, and these mice show compensatory upregulation of other CREB isoforms as well as CREM (Balschun *et al.*, 2003; Blendy *et al.*, 1996; Hummler *et al.*, 1994; Rudolph *et al.*, 1998).

As in the case of CREB, a number of caveats have emerged to the role of BDNF in LTP. Certain stimulation protocols seem to be more dependent on BDNF than others. Generally, high-frequency patterned stimulation occurring in a short time-period or 'burst,' referred to as theta-burst stimulation (TBS), is dependent on BDNF, while spaced tetanic stimulation is not. Patterson *et al.* (2001) showed that tetanic stimulation (4 trains of 100 pulses, 100 Hz, 5-min ISI) induced long-lasting LTP in the Schaffer collateral pathway of $BDNF^{-/-}$ mice, but 12 TBS (4 pulses of 100 Hz, 200-ms ISI) only induced E-LTP. Incubation of brain slices with a TrkB scavenger, which blocks extracellular BDNF, inhibited L-LTP induced by TBS and by forskolin in wild-type mice, suggesting that BDNF-dependent LTP involves cAMP signaling. Indeed, both TBS and forskolin increased the distribution of phosphorylated MAPK in the soma of neurons within 30 minutes of stimulation, demonstrating a mechanism by which extracellular BDNF could induce CREB-mediated gene expression in response to electrical activity. In a recent study, Lu *et al.* (2011) generated mice that have a knock-in mutation in which phenylalanine 616 is mutated to alanine in the ATP binding pocket of endogenous TrkB ($TrkB^{F616A}$ mutation). Expression of this mutation can be induced by a single i.p. injection of a small membrane-permeable molecule, 1NMPP1 (Chen *et al.*, 2005). $TrkB^{F616A}$ mice injected with 1NMPP1 demonstrated an impairment in L-LTP, but not E-LTP, following TBS (12x 4 pulses, 100 Hz, 200-ms ISI) in the Schaffer collateral pathway (Lu *et al.*, 2011). Inducible BDNF knockout mice under the control of the tTa-TetOp system that showed a 70% reduction of BDNF protein levels in hippocampal neurons had an impairment in the induction of LTP, implicating BDNF in E-LTP as well (Monteggia *et al.*, 2004). However, mice with a truncated mutant isoform of TrkB (TrkB.T1 mice) underwent LTP following TBS, revealing that not all manipulations that affect BDNF signaling necessarily interfere with LTP (Saarelainen *et al.*, 2000).

Studies have also shed light on the mechanism of BDNF-LTP. It appears that BDNF-LTP and HFS-LTP have different mechanisms of induction but produce similar long-term synaptic changes. In the rat DG, BDNF-LTP lasting more than 7 hours can be induced by infusing BDNF into the perforant pathway of an anesthetized animal (Ying *et al.*, 2002). Like

HFS-LTP, this form of BDNF-LTP was dependent on post-synaptic Ca^{2+} influx and NMDA receptor-mediated signaling (Kovalchuk *et al.*, 2002). Like HFS-LTP, BDNF-LTP was characterized by a potentiation of fEPSP amplitude, an increase in excitability of the post-synaptic neuron for a given stimulus input (E-S coupling), and dependence on RNA synthesis for L-LTP (Messaoudi *et al.*, 2002). Infusion of BDNF into the DG 1-hr after the induction of saturating HFS-LTP that lasts 1-5 days (4 trains of 8 pulses, 400 Hz, 10-s ISI) caused further potentiation; however, this potentiation was completely occluded when the BDNF infusion took place 260 min after the induction of HFS-LTP, suggesting that the late phases of both forms of LTP rely on the same cellular machinery. This is further suggested by the facts that MEK inhibitors prevent the induction of BDNF-LTP, and that BDNF-LTP increases ERK2 and CREB phosphorylation, as well as the activation of the immediate early gene *Arc* (activity-regulated cytoskeleton-associated gene; Arg3.1) for up to 3 hours (Ying *et al.*, 2002). ERK2 is part of a kinase pathway that leads to the activation of CREB, and *Arc* is important in synaptic consolidation processes (Guzowski *et al.*, 2000).

While the evidence presented thus far points to the involvement of CREB and BDNF in regulating the induction of LTP, there is also a substantial body of evidence showing that the activity of both proteins is altered when neurons enter the potentiated state. Using the CRE-reporter mouse, in which the binding of CREB to its target genes can be easily detected with histological analyses, it was shown that CRE-dependent gene expression is increased by stimulation that induces L-LTP (3 tetani of 100 Hz, 5 min ISI) but not by stimulation that induces E-LTP (1 tetanus of 100 Hz; Impey *et al.*, 1996). Interestingly, both E- and L-LTP were associated with increased phosphorylation of CREB at Ser-133, consistent with other studies (Ahmed & Frey, 2005; Racaniello *et al.*, 2010). Ahmed and Frey (2005) observed that following strong tetanization that induces L-LTP, the phosphorylation of CREB peaks after 45-min and then again after 6-hr. The 45-min peak is unaffected by pre-treatment with the protein synthesis inhibitor anisomycin, while phosphorylation 2-6 hours after LTP induction is completely abolished. Consistent with the data from electrophysiological experiments in transgenic mice, these results implicate CREB in both early and late stages of LTP.

Both tetanic stimulation and TBS that induces LTP have been shown to increase BDNF expression that is dependent on Ca^{2+} signaling (Balkowiec & Katz, 2000, 2002; Hong *et al.*, 2008). In experiments with rats, Lee *et al.* (2005) showed that LTP-inducing TBS (20 bursts, 20

pulses, 100 Hz) resulted in a 2.9-fold increase in *Bdnf* mRNA expression in the hippocampus, while LTD-inducing low frequency stimulation decreased the expression of *Bdnf* 1.7-fold. Interestingly, blockade of glutamatergic transmission while stimulating the pre- and post-synaptic neurons induced a 7.9-fold decrease in *Bdnf* expression, suggesting the BDNF not only plays a large role in LTP, but depends on associative, coordinated firing between the pre- and post-synaptic neurons. However, it is difficult to infer the generalizability of these findings since the investigation was limited to *Bdnf* promoter I.

All of the parallel findings between the roles of CREB and BDNF in LTP beckons the question of what role the interaction between these proteins plays. Some clues come from the observation that increased phosphorylation of TrkB 30 minutes after the induction of LTP by TBS is congruent with the observed up-regulation of CREB during E-LTP (Lu *et al.*, 2011). Furthermore, the transcription of *Bdnf* exon IV was recently shown to be upregulated following CREB phosphorylation during E-LTP (Racaniello *et al.*, 2010), consistent with an earlier observation that *Bdnf* exon IV behaves like an IEG (Castrén *et al*., 1998). One possibility is that early activation of CREB and BDNF contributes to the progression from the early to the late phase of LTP, while the sustained activation of CREB-mediated BDNF expression at later time points contributes to the maintenance of L-LTP.

2.4.2.2 Spinogenesis and the formation of new synapses

Experimentally elucidating the mechanisms responsible for the maintenance of LTP presents some unique challenges. While the induction of E- and L-LTP can be studied within a time-scale of a few hours (electrophysiological recordings of LTP are rarely carried out for more than 12 hours), memories persist for months, years, or even a lifetime. LTP can indeed be induced on a similar time-scale, lasting months to years, in the rodent hippocampus (Abraham, 2003; Abraham *et al.*, 2002). However, all of the functional and regulatory molecules in the nervous system experience a much more rapid turnover (Crick, 1984; Kasai *et al.*, 2010). The autophosphorylation state of CaMKII, considered a potential mechanism for the maintenance of LTP through the continuous cycling of AMPA receptors, lasts for only minutes during E-LTP (Lee *et al.*, 2009). Even post-synaptic density protein-95 (PSD-95), one of the most stable synaptic molecules, is re-distributed in cortical neurons within 3 hours (Gray *et al.*, 2006). Changes in gene expression regulated by transcription factors during the induction of LTP can be

more persistent, but are typically returned to baseline after several hours (reviewed in Greer & Greenberg, 2008; Lin *et al.*, 2008). These observations beg the question of how LTP can be maintained for periods long enough to support memories. A prevalent hypothesis is that experience-dependent neuronal activity induces structural changes at existing synapses or the formation of new synapses, a phenomenon called *structural plasticity* (reviewed in Holtmaat & Svoboda, 2009; Kasai *et al.*, 2010).

Activity-dependent changes in synapse structure occur at dendritic spines. Spines are tiny protrusions extending ~1 μm from the dendritic shaft (reviewed in Segal, 2005). They typically have a distinct neck and head area, but there are several well-characterized forms (Peters & Kaiserman-Abramof, 1970). The size of spines is directly related to the size of the post-synaptic density (PSD; Harris *et al.*, 1992), and the quantity of AMPA receptors in the post-synaptic membrane (Benke *et al.*, 1998; Nusser *et al.*, 1998). Spines are the most actin-rich structures in the brain and their structural integrity is modulated by a balance of F-actin and G-actin (Okamoto *et al.*, 2004). Several features of dendritic spines make them likely sites for functional synaptic changes. Most of the excitatory synapses in the mammalian brain occur at dendritic spines (Farb *et al.*, 1992), and spines are effectively tiny compartments, which makes them sensitive to slight (μM scale) fluctuations in Ca^{2+} needed to trigger intracellular signaling cascades (Lee *et al.*, 2009). Spines also contain NMDA and AMPA receptors needed for the induction of plastic changes as discussed above (Matsuzaki *et al.*, 2001). Finally, plasticity at the level of spines would explain how networks of synapses form memories and how one neuron can be involved in multiple memories activated by different synapses (Ziv & Ahissar, 2009).

There is now an abundance of experimental evidence confirming that structural synaptic changes during learning have functional significance. Activity-dependent structural changes at individual spines can be observed using high-resolution 2-photon microscopy (Lee *et al.*, 2009; Matsuzaki *et al.*, 2001, 2004; Tanaka *et al.*, 2008; Yang *et al.*, 2009). By shining a high intensity beam of light of a particular wavelength on *ex vivo* or cultured neurons in a solution containing caged glutamate and Mg^{2+}, conditions of synaptic stimulation can be imitated within a ~1 μm radius of the beam. The beam of light causes glutamate uncaging in the focal area: uncaging is a process in which glutamate undergoes photorelease from a biologically inert precursor. If

glutamate uncaging is combined with depolarization of the spine, similar conditions are generated to those needed for the induction of LTP[4] (Dan & Poo, 2006; Tanaka *et al.*, 2008). Matsuzaki *et al.* (2004) demonstrated that uncaging of caged glutamate increased the size of individual spines by over 50% within 1-5 minutes, a time-course comparable to the induction of LTP. Interestingly, the enlargement in spine head diameter was more likely to be persistent (lasting 100 minutes or more) in spines that were initially small compared to spines that were large. Since the size of a spine head is dependent on AMPA receptors (Benke *et al.*, 1998; Nusser *et al.*, 1998), this result suggested that spines with a higher proportion of NMDA receptors, called *silent synapses*, were more viable sites for the induction of structural changes. Persistent increases in spine diameter were believed to correspond to LTP, as the NMDA antagonist AP5 blocked all changes in spine head diameter, while a CaMKII inhibitor blocked persistent, but not transient changes in spine size, consistent with the roles of NMDA receptors and CaMKII in the induction and maintenance of LTP, respectively (Matsuzaki *et al.*, 2004; Tanaka *et al.*, 2008).

Changes in spine head diameter occur rapidly following neuronal activity, while the formation of new spines takes at least an hour, perhaps corresponding to a later phase of LTP (reviewed in Segal, 2005). Consistent with this, most studies of dendritic spines investigating the induction of LTP demonstrate changes in spine size (Okamoto *et al.*, 2004; Otmakhov *et al.*, 2004; Zhou *et al.*, 2004), while those investigating memory processes over a longer time-scale typically note changes in spine density (Leuner *et al.*, 2003; Restivo *et al.* Xu *et al.*, 2009; Yang *et al.*, 2009). Evidently, spine dynamics can be altered in an activity-dependent manner. They are also regulated by developmental programs resulting in an excess of new spines in early development that are subsequently pruned, and defects in these developmental programs have been linked to a number of neurological disorders (An *et al.*, 2008; Kaneko *et al.*, 2012; Penzes *et al.*, 2011). Importantly, spine dynamics are also responsive to intrinsic fluctuations (reviewed in Kasai *et al.*, 2010). Throughout development and adulthood, existing spines dynamically shrink and grow; some existing spines are pruned and disappear, and new spines spontaneously

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[4] A more conventional procedure is to uncage glutamate in Mg^{2+}-free solution, which does not require depolarization to unblock NMDA receptors on the target spine (Matsuzaki *et al.*, 2001). However, this does not accurately simulate physiological conditions and appears to induce LTP that is not as robust (Dan & Poo, 2006).

form. Although these intrinsic fluctuations are somewhat random, large spines are much less likely to be pruned than small spines, and on average, the size of a given spine does not change over a period of time. This suggests that large spines involved in LTP or memory, though susceptible to intrinsic fluctuations, are unlikely to be eliminated over time, providing a structural substrate capable of maintaining LTM. Another implication is that the spontaneous formation of new spines, which are likely to be small, silent synapses susceptible to LTP, on particular neuronal populations can increase the likelihood that these neuronal populations will capture and store new information during a learning event.

The presence of high levels of an endogenous substance may increase the spontaneous generation of new spines in a particular neuronal population, effectively priming these neurons to capture a memory. Viral transfection of CA1 hippocampal neurons with a constitutively active form of CREB (caCREB) resulted in an increase in spine density, and corresponding neurons demonstrated an increase in LTP magnitude and duration (Marie *et al.*, 2005). A recent study suggested that CREB and microRNA-132 (miR132) mediate changes in spine density through the Rac-PAK signaling pathway (Impey *et al.*, 2010), while another study suggested that CREB is involved in activity-dependent adjustments of spine head size and density in the rat visual cortex (Suzuki *et al.*, 2007). In our lab, we have observed that HSV viral vector-mediated gene transfer of CREB fused with GFP (HSV-CREB-GFP vector) into neurons in the mouse LA increased the spine density of these neurons compared to control neurons transfected with HSV-GFP (G. Higgs, M. Florczynski, V. Mercaldo, S. Josselyn, unpublished observations). Infusions of HSV-mCREB-GFP into the LA, which overexpresses dominant negative $CREB^{S133A}$, resulted in reduced spine density.

Since we conducted our spine analysis at the same time-point (3 days) after surgery at which we had previously trained mice in an auditory fear conditioning task, we can directly relate the structural changes induced by CREB to behavioural outcomes. Mice that received infusions of HSV-CREB-GFP into the LA demonstrated enhanced freezing in response to an auditory cue previously paired with a foot shock (Han *et al.*, 2007, 2009). Moreover, neurons transfected with CREB were more likely than control neurons to express the IEG *Arc* after testing, an indicator of recent neuronal activity (Guzowski *et al.*, 1999), suggesting that CREB-overexpressing neurons were preferentially incorporated into the newly formed fear memory trace. Subsequent targeted ablation of neurons transfected with CREB appeared to erase

memory for the tone-shock association (Han *et al.,* 2009). These results suggest that neurons in the LA that expressed higher levels of CREB prior to auditory fear conditioning were more likely to be recruited to the memory trace and thus had a competitive advantage over other neurons. Increased spine density in these neurons at the time of learning, perhaps in cohesion with CREB-mediated increases in neuronal excitability (see Lopez de Armentia *et al.*, 2007; Silva *et al.*, 2009; Zhou *et al.*, 2009), may provide a mechanistic explanation for this competitive advantage.

BDNF has been extensively implicated in the regulation of spine structural dynamics, both in development and adulthood. As discussed in Section 2.3, during development BDNF is needed for normal formation of ocular dominance columns in the visual cortex (Galuske *et al.*, 2000; Thoenen, 1995). During episodes of MD, BDNF is down-regulated in the visual cortex, while re-opening the closed eye to restore binocular vision is correlated with a return of normal BDNF levels (Majdan & Shatz, 2006). Acute administration of BDNF-TrkB inhibitors following MD prevents the recovery of normal ocular dominance (Kaneko *et al.*, 2008). An *et al.* (2008) developed a transgenic mouse in which post-transcriptional polyadenylation of long 3'UTR transcripts was disrupted ($BDNF^{klox/klox}$ mouse). *Bdnf* transcripts with long (2.85-kb) 3' UTRs are transported to dendrites for synthesis, while transcripts with short (0.35-kb) 3' UTRs are translated in the soma. The long transcripts account for approximately 60%, 35% and 70% of *Bdnf* mRNA in the hippocampus, cortex, and cerebellum, respectively. $BDNF^{klox/klox}$ mice synthesized a truncated form of the long *Bdnf* transcript and showed a complete absence of *Bdnf* mRNA outside of the soma in CA1 pyramidal neurons, as well as essentially no BDNF protein expression in distal dendrites more than 20 μm from the soma. $BDNF^{klox/klox}$ mice showed a 20% reduction in spine head diameter and a 54% increase in spine density. HFS and TBS induced LTP if stimulation was applied directly to the soma of these mice, but not if it was applied to dendrites, and young mutant mice showed normal spine properties at P21, a time point before the developmental onset of spine pruning. $BDNF^{klox/klox}$ mice showed a 15% increase in spine density in the visual cortex and had a higher proportion of immature (small and thin) spines (Kaneko *et al.*, 2012). Young (P25-26) mutant mice were also unable to recover responsiveness in the previously closed eye following a 4-day episode of MD, despite demonstrating normal basal visual responses. Adult (P70-85) $BDNF^{klox/klox}$ mice showed normal recovery from MD, but had depressed closed-eye responses during MD in both the monocular and binocular zones of

the visual cortex, suggesting that they had a deficit in normal inhibitory responses. Taken together, these findings suggest that dendritic BDNF expression during development is needed for the appropriate pruning and maturation of dendritic spines.

BDNF also appears to have a more general role in dendritic spine dynamics throughout life. Bath application of BDNF increases dendritic spine density in culture (Chapleau *et al.*, 2008). Bath application of BDNF paired with glutamate uncaging caused a marked enlargement of individual spines in rat CA1 pyramidal neurons that was protein-synthesis dependent (Tanaka *et al.*, 2008). In the same study, the long-term, but not transient, enlargement of spines by glutamate uncaging paired with depolarization was blocked by the Trk receptor antagonist K252a, as well as by the specific blockade of TrkB receptors with antibodies and or a BDNF scavenger. Blockade of BDNF-induced Ca^{2+} transients to dendritic spines has also been shown to block LTP in the mouse DG (Kovalchuk *et al.*, 2002). These studies suggest that BDNF has a direct role in mediating structural changes corresponding to LTP.

Insights into the mechanism of BDNF-induced spine formation link the role of BDNF to the cAMP pathway. BDNF-induced increases in spine density depend on the recruitment of microtubules to the PSD, as stabilization and inhibition of microtubules with pharmacological agents enhanced and impaired BDNF-mediated spine formation in cultured hippocampal neurons (Gu *et al.*, 2008). A recent study showed that BDNF induced microtubule mobilization to dendritic spines within 20 minutes of stimulation, resulting in an increase in PSD-95 in the spine head (Hu *et al.*, 2011). Interestingly, Ji *et al.* (2005) showed that the effects of BDNF on spine density are dependent on cAMP. BDNF-induced TrkB phosphorylation was blocked by inhibitors of cAMP signaling, was potentiated by cAMP analogs, and was not induced by cAMP alone, consistent with earlier findings that cAMP is necessary, but not sufficient, for BDNF-LTP (Boulanger & Poo, 1999). Moreover, enhancement of cAMP signaling, either through direct application or stimulation of dopaminergic or noradrenergic receptors on the spine, enhanced translocation of phosphorylated TrkB to the spine (Ji *et al.*, 2005). cAMP also modulated the BDNF-mediated increase in spine density. Since elevated levels of cAMP contribute to the activation of CREB, these findings bring to light a possible avenue of interplay between CREB and BDNF in spine structural dynamics.

2.4.2.3 Synaptic tagging and capture

I have now reviewed evidence that both CREB and BDNF are implicated in synaptic and structural forms of plasticity. Since both molecules are expressed at high endogenous levels throughout the brain, an important question concerns how these proteins carry out synapse-specific functions. Studies investigating synaptic tagging and capture have indeed elucidated synapse-specific roles for CREB and BDNF consistent with their roles in LTP and spine dynamics (Frey & Morris, 1997; reviewed in Barco *et al.*, 2008; Frey & Frey, 2008; Reymann & Frey, 2007). Frey & Morris (1997) showed that a synapse (synapse 2; S2) in the Schaffer collateral pathway stimulated by a weak tetanus (TBS of 80 pulses at 100 Hz), which normally only induces E-LTP, undergoes L-LTP if a nearby, but independent synapse (synapse 1; S1) has recently undergone L-LTP following a strong stimulus (3 trains of 100 pulses, 100 Hz, 10-min ISI; referred to as the *strong-then-weak* protocol). L-LTP is even induced in S2 if it is stimulated while a protein synthesis inhibitor is applied during stimulation of S2. However, if stimulation of S1 takes place in the presence of the protein synthesis inhibitor, neither synapse undergoes L-LTP. Frey and Morris proposed that there are two requirements for long-term changes in synaptic plasticity. The first is the local setting of *synaptic tags* at stimulated synapses, likely to occur during E-LTP. The second requirement is the expression of plasticity-related proteins (PRPs) during L-LTP. Synaptic tags are localized to the stimulated synapse, while PRPs travel non-specifically throughout the stimulated cell. Therefore, any synapse that expresses a synaptic tag can recruit, or *capture*, PRPs and undergo L-LTP, and the induction of the synaptic tag and expression of PRPs need not occur at the same time. The ability of distant synapses to capture PRPs explains why a synapse (S2) that only underwent E-LTP, presumably inducing synaptic tag expression, could undergo L-LTP in the presence of a protein synthesis inhibitor.

It also appears that the expression of the synaptic tag is transient (< 3 hours), consistent with the time-course of E-LTP (Frey & Morris, 1997). It was recently shown that stimulating a synapse with a strong tetanus did not induce L-LTP in the presence of a pharmacological inhibitor of CaMKII but following a brief washout period, L-LTP was induced at a distinct, convergent synapse with a weak tetanus (Redondo *et al.*, 2010). The finding suggested that PRPs available from the first synapse were available to the second synapse, but that the first synapse did not express a synaptic tag, implicating CaMKII as a potential synaptic tag.

Interestingly, pharmacological inhibition of CaMKIV, which phosphorylates CREB in the nucleus, blocked L-LTP, but not E-LTP, in two synapses that had undergone the same tetanization protocol as above. This implicated the CaMKIV-CREB pathway in the expression of PRPs. While CaMKII is likely implicated in synaptic tagging as suggested by this study, it is unlikely that CaMKII is the synaptic tag itself. A recent study used uncaging of caged glutamate to stimulate individual CA1 spines, and phosphorylated αCaMKII was tagged with a fluorescence-resonance based energy transfer (FRET)-based sensor, green-Camuiα (Lee *et al.*, 2009). Individual αCaMKII molecules were activated for only 1-2 minutes immediately after stimulation of the spine and diffused into the dendrite within 20 minutes. This is inconsistent with the experimental observation that the synaptic tag must be activated in a synapse-specific manner for ~3 hours (Frey & Morris, 1997). Furthermore, Redondo *et al.* (2010) did not identify PRPs needed for synaptic capture.

Several studies now point to TrkB and BDNF as putative synaptic tags and PRPs, respectively. This possibility was first suggested by Barco and colleagues in studies using VP16-CREB mice that express a constitutively active form of CREB (Barco *et al.*, 2002, 2005). In VP16-CREB mice, one tetanus of 100 Hz induced L-LTP even though this type of stimulation only induced E-LTP in wild-type mice, and experiments with pharmacological inhibitors showed that L-LTP in VP16-CREB mice was dependent on protein synthesis but not on mRNA synthesis (Barco *et al.*, 2002). This initial study suggested that VP16-CREB mice constitutively express the mRNA needed for synthesis of PRPs used in synaptic capture, so induction of the synaptic tag, which can be accomplished with weak stimulation, is enough to induce L-LTP. In a subsequent study, Barco *et al.* (2005) revealed that the enhanced L-LTP phenotype (i.e. the induction of L-LTP by stimulation that induces E-LTP in wild-type mice) in VP16-CREB mice was blocked by a BDNF scavenger and in VP16-CREB/BDNF$^{+/-}$ double transgenic mice, which express reduced levels of BDNF. Importantly, similar manipulations with other important CREB target genes (e.g. prodynorphin, CD3δ) did not affect the enhancement of LTP in VP16-CREB mice. Barco *et al.* then used the strong-then-weak protocol (4 trains of 100 Hz at S1, followed 40-min later by 1 train of 100 Hz at S2) to determine whether the enhanced LTP phenotype would be impaired in double transgenic VP16-CREB/BDNF$^{-/-}$ mice with the BDNF knockout mutation throughout the forebrain (including CA3 and CA1) or in a second line of VP16-CREB/BDNF$^{-/-}$ mice with the BDNF knockout mutation restricted to CA1. Both transgenic lines

had an impairment in synaptic capture, as L-LTP could not be induced at S2, suggesting that BDNF is an important PRP. Interestingly, the CA1 mutant line demonstrated relatively stable LTP at S2 that slowly decayed after 2 hours, and was distinct from L-LTP. Meanwhile, the CA3-CA1 line, in which both pre- and post-synaptic BDNF synthesis were presumably inhibited, showed a much more rapid decay in LTP at S2, suggesting that both pre- and post-synaptic BDNF expression contributes in important ways to L-LTP.

Recently, Lu *et al.* (2011) further delineated the potential role of TrkB-BDNF signaling in synaptic tagging and capture. The phosphorylation of TrkB occurred to a similar extent following strong (12 TBS, 4 pulses, 100 Hz, 200-ms ISI) and weak (4 TBS, 4 pulses, 100 Hz, 200-ms ISI) stimulation, peaking after 30-min and receding to baseline after 2-hrs. Using a platform containing fixed fluorescent beads that secrete BDNF, they further showed that phosphorylation of TrkB resulted in local secretion of BDNF within 5-10 μm of the stimulation site. In $TrkB^{F616A}$ inducible transgenic mice, but not wild-type mice, treatment with 1NMPP1 prior to and up to 40-min after strong TBS prevented the phosphorylation of TrkB and induction of L-LTP, revealing a time window during which phosphorylation of TrkB is needed for synaptic potentiation. The strong-then-weak protocol (same as protocol in Barco *et al.*, 2005) also failed to induce L-LTP at S2 when $TrkB^{F616A}$ mice were treated with 1NMPP1 40-80 min after strong TBS at S1. Treatment of mutant mice with 1NMPP1 prior to stimulation at S1 failed to induce L-LTP at S1, but resulted in L-LTP at S2 following weak TBS, strongly suggesting that the treatment impaired synaptic tagging, but not PRP production in $TrkB^{F616A}$ mice. This study and those discussed above strongly implicate CREB-mediated BDNF synthesis in the production of PRPs and suggest that phosphorylated TrkB could be a putative synaptic tag. Since TrkB receptors are membrane-bound and immobile, phosphorylated TrkB fits the important criterion of a synapse-specific synaptic tag. Of course, it is possible that some as of yet unidentified ligand and its receptor would be equally suitable candidates for ligands and PRPs, and there are still many questions to be answered about the roles of CREB-BDNF-TrkB signaling in synaptic and structural plasticity.

2.4.3 The CREB-BDNF interaction in memory

Changes in synaptic strength and structure in response to experience provide the physiological underpinnings for learning and memory. CREB and BDNF are not only involved in experience-

dependent synaptic changes, but in behaviours mediated by these changes. The association between CREB and LTM has a rich experimental history that began with investigations of olfactory learning in the fruit fly, *Drosophila melanogaster* (Yin *et al.*, 1994, 1995). Yin and colleagues used a paradigm in which flies received multiple training sessions in which two odours were presented, one paired with an electric shock, and another without, over multiple training trials, and LTM was assessed 7 days later as a function of aversion to the odour paired with the shock. Massed training (10 training trials with no interval between trials) did not induce LTM in wild-type flies, but just one training trial induced LTM in transgenic flies that overexpressed the inducible CREB *activator* dCREB2-a under the control of a heat shock protein (Yin *et al.*, 1995). Spaced training (10 training trials with a 15-min interval between trials) induced robust LTM in wild-type flies, but did not induce LTM retention in transgenic flies overexpressing the CREB *repressor* dCREB2-b (Yin *et al.*, 1994). These studies indicated that overexpressing CREB enhances memory, while repressing CREB impairs memory.

Interest in the role of BDNF in memory was ignited by a study carried out by Falkenberg *et al.* (1992) in which rats housed in either enriched or impoverished environments for 34 days were trained in the Morris water maze, a task that requires animals to use spatial cues to navigate a circular pool of water to find a platform submerged below its surface. Rats that had been housed in an enriched environment demonstrated an enhancement in ability to find the platform coincident with a 48% increase in *Bdnf* mRNA expression in the CA1 compared to rats in the impoverished housing condition. Even though memory performance was assessed based on the time needed to find the hidden platform and probably did not assess LTM *per se*, this study suggested that BDNF plays a role in a task that is heavily dependent on the structural integrity of the hippocampus (see Section 2.4.2.1 below). Subsequent studies have supported these initial observations, implicating both CREB and BDNF in memory across a wide range of modalities.

2.4.3.1 Spatial memory

The vast body of evidence suggesting that CREB and BDNF play important roles in hippocampal LTP and spinogenesis strongly implicates these proteins in hippocampal-dependent LTM. Perhaps the one form of memory thought to be the exclusive domain of the hippocampus in rodents is spatial memory. O'Keefe and Dostrovsky (1971) first showed that the hippocampus has 'place cells' that fire differentially depending on a rat's location in its environment, and

single-unit electrophysiological recordings show that hippocampal place-cell representations for different environments diverge with experience (Lever *et al.*, 2002). Hippocampal lesions (McNaughton *et al.*, 1986; Morris *et al.*, 1982) and NMDA receptor blockade (Bannerman *et al.*, 1995; Morris *et al.*, 1986) prevent rats from learning to navigate a complex environment, suggesting that the encoding of spatial information in memory is dependent on the hippocampus. Furthermore, neuroimaging studies in humans have repeatedly demonstrated that activation of the medial temporal lobe (MTL), which contains the hippocampus, amygdala, and entorrhinal and perirrhinal cortices, is correlated with spatial memory (Kumaran & Maguire, 2005; Maguire *et al.*, 1997) or memory for analogous forms of relational information (Henke *et al.*, 1999; Ryan *et al.*, 2000). Consistent with their physiological roles in the hippocampus, both CREB and BDNF appear to be necessary for spatial memory.

The quintessential spatial memory task in rodents is the Morris water maze (Maei *et al.,* 2009; Morris *et al.*, 1982; Teixeira *et al.,* 2006). In the hidden platform version of this task, rodents use visual cues in the surrounding room to find a hidden platform submerged below the surface of a pool of water. In the visible platform version of the task, which does not depend on the encoding and retrieval of spatial information, the location of the platform is in plain sight, usually cued by a flag placed on top of it. Bourtchuladze *et al.* (1994) first showed that $CREB^{\alpha\delta-/-}$ mice displayed a substantially slower improvement than wild-type mice in time needed to find the hidden platform during training (1 trial/day for 15 days) and did not spend more time in the target quadrant compared to the other quadrants of the pool when the platform was removed for a LTM test (commonly called a *probe test*) on day 15. Pittenger *et al.* (2002) showed that mice overexpresseing a potent dominant negative inhibitor of CREB, CREM and ATF-1 (KCREB; Jean *et al.*, 1998; Walton *et al.*, 1992) under the control of the inducible tTa-TetOp system (Mayford *et al.*, 1996) and with hippocampal expression restricted to the dorsal CA1 by the αCaMKII promoter (dCA1-KCREB mice) had substantial impairments in the hidden platform version of the water maze, but performed normally in the visible platform version of the task.

As in the case of LTP, studies investigating LTM in mice with genetically engineered deficits in CREB expression have generated conflicting results about whether or not CREB is necessary for spatial memory. Gass *et al.* (1998) showed that $CREB^{\alpha\delta-/-}$ mice performed comparably to wild-type mice using two training protocols (strong: 6 trials/day, 1-hr inter-trial interval or ITI, 9 days; weak: 2 trails/day, 1-min ITI, 9 days). $CREB^{comp}$ mice, which are

produced by crossing the $CREB^{\alpha\delta-/-}$ mutation with the $CREB^{-/-}$ mutation and thus have a slightly higher level of CREB knockdown, improved at a slower rate during training and showed a weaker target quadrant preference than wild-type and $CREB^{\alpha\delta-/-}$ mice in the weak protocol. However, even the deficit in $CREB^{comp}$ mice could not be attributed to spatial memory *per se*, as the mice demonstrated a persistent tendency to swim in circles around the periphery of the pool throughout training, a behaviour known as thigmotaxis that can be reflective of reluctance to explore the pool. Balschun *et al.* (2003) generated similar findings in three lines of CREB transgenic mice, including $CREB^{\alpha\delta-/-}$, $CREB^{comp}$, and conditional $CREB^{NesCre}$ mice, which completely lacked CREB but showed an upregulation of CREM throughout the brain. All three lines had a reduced preference for the target quadrant during a probe test that appeared to be attributable to an increase in thigmotaxis. A fourth line of mice with a CREB mutation restricted to the forebrain ($CREB^{CaMCre7}$ mice), resulting in a 70-80% reduction in CREB expression in the CA1, demonstrated a milder increase in thigmotaxis and performed at wild-type levels during both training and probes. Recently, Sekeres *et al.* (2010) found that $CREB^{\alpha\delta-/-}$ mice were impaired during training (6 trials per day for 3 days) and in a subsequent probe test, but also demonstrated increased levels of thigmotaxis. While the failure to replicate the initial findings of Bourtchuladze *et al.* (1994) with $CREB^{\alpha\delta-/-}$ mice casts some doubt on the role of CREB in spatial memory, the interpretation of negative findings is confounded by the fact that CREB-mediated deficits in water maze learning appear to be influenced by experimental conditions such as number of training trials per day (Bourtchuladze *et al.*, 1994) and ITI (Kogan *et al.*, 1997). Kogan *et al.* (1997) showed that the magnitude of the spatial memory deficit during a probe trial 24-hrs after a 10-day training protocol (2 trials/day) was greatest in $CREB^{\alpha\delta-/-}$ mutants that received an ITI of 1-min, and was reduced when the ITI was raised to 10-min. $CREB^{\alpha\delta-/-}$ mice showed no deficit in training or probe trials when the ITI was 60-min.

Other methods of reducing CREB expression in mice have also produced conflicting findings. Intracranial infusion of CREB antisense oligonucleotides (As-OGNs) but not sense oligonucleotides (S-OGNs) into the hippocampus prior to water maze training (two series of 7 trials, 40-min apart) resulted in an impairment in a spatial memory test 48 hours later (Guzowski & McGaugh, 1997). Performance on a memory test 4 hours after training was not affected, implicating CREB in LTM but not STM. Contrary to these findings, a more recent study showed that infusion of CREB As-OGNs into the dorsal CA3 did not impair performance in a probe test

24 hours after training using a similar, but weaker, massed training protocol (4 consecutive trials; Florian *et al.*, 2006).

Studies in which CREB is genetically overexpressed point more convincingly to a role for CREB in spatial memory. Suzuki *et al.* (2011) generated two lines of mice overexpressing a dominant active form of CREB with a high affinity for PKA ($CREB^{Y134F}$), in which the mutation was specific to the forebrain. Mice underwent water maze training (2 trials per day for 10 days) and received probe tests 24-hrs after the last training trial on days 5 and 10. The Y134F-C mutant line showed an increased preference for the target quadrant during the first probe test compared to the Y134F-A mutant line and wild-type mice. Coincidently, the Y134F-C mutant line showed a higher level of CREB overexpression than the Y134F-A line, suggesting that the benefit of CREB to LTM is dependent on the magnitude of overexpression. Sekeres *et al.* (2010) infused a replication-defective HSV virus containing CREB (CREB vector), a mutant ($CREB^{S133A}$) form of CREB (mCREB vector), or GFP and LacZ (control vector) into the dorsal hippocampus prior to weak (3 trials per day for 3 days) or strong (6 trials per day for 3 days) training on the spatial version of the water maze. While mice infused with control or mCREB vectors did not demonstrate evidence of spatial memory in a probe test following weak training, there was a robust enhancement in memory in mice transfected with CREB vector. Following strong training, which induced spatial LTM in mice infused with control vector, CREB vector infusion further potentiated probe test performance and rescued a memory deficit in $CREB^{\alpha\delta-/-}$ mice. These results suggested that CREB is sufficient for the induction of LTM under conditions that do not normally promote LTM formation.

Consistent with its well-defined role in LTP, BDNF is strongly implicated in hippocampal-dependent spatial memory. Linnarson *et al.* (1997) showed that $BDNF^{+/-}$ mice took twice as long as wild-type mice to reach the same level of performance in the water maze over 10 days of training (4 trials/day). The deficit was more potent in aged (10-month-old) $BDNF^{+/-}$ mice, which showed a further reduction in hippocampal *Bdnf* mRNA compared to young $BDNF^{+/-}$ mice, did not show an improvement over training, and spent most of their time swimming in the incorrect quadrants during the probe test. Intracerebroventricular infusion of antibodies specific for BDNF was also shown to increase latency to find the platform during training (2 blocks of 8 trials per day for 4 days) and impair probe test performance in rats (Mu *et al.*, 1999). In another study, mice expressing a mutant truncated isoform of TrkB (TrkB.T1

mice) showed improvements in latency to reach the platform during training (4 trials per day for 9 days), but never reached the same level of performance as wild-type littermates (Saarelainen *et al.*, 2000). TrkB.T1 mice also took longer to reach the platform compared to wild-type mice when tested 60 days after training, indicating a remote memory impairment. These results show that both BDNF and its receptor, TrkB, are needed for the learning and retention of spatial information.

Studies investigating brain-region specific and inducible mutations have also supported the role of BDNF and its receptor in spatial memory. BDNF-floxed mice that underwent lentivirus-mediated intracranial infusions of Cre-recombinase (LV-Cre virus) into the dorsal hippocampus had deficits during water maze training (4 trials/day for 5 days, 60-s ITI) and a probe test 48-hr later (Heldt *et al.*, 2007). Similar deficits were observed in bitransgenic mice expressing floxed TrkB and Cre-recombinase under control of the αCaMKII promoter (TrkB-Cre mice; Minichiello *et al.*, 1999). These mice showed no improvement after extensive training of one trial per day for 36 days. However, they also performed poorly in the visible platform version of the task and showed persistent thigmotaxis, suggesting that the observed deficits may have been anxiety-related. Furthermore, $TrkB^{+/-}$ mice performed similarly to wild-type mice in both water maze tasks. Interestingly, TrkB-Cre mice also made more working memory errors and failed to improve over days in the 8-arm radial maze compared to $TrkB^{+/-}$ and wild-type mice. While the water maze requires animals to escape from a stressful situation, the radial arm maze depends on the animal's willingness to search for a food reward, so impairments in the latter task may have been more reflective of a spatial memory deficit. Indeed, rats that received infusions of BDNF antisense OGNs into the hippocampus before training showed increased working and reference memory errors in the radial arm maze (Mizuno *et al.*, 2000). Taken together, studies involving the genetic knockdown and overexpression of BDNF or TrkB support a role for BDNF-mediated signaling in spatial memory.

Studies investigating the molecular basis of spatial memory reveal yet another function subserved by both CREB and BDNF. Yet the question remains as to whether these proteins contribute to spatial memory through a single pathway or multiple parallel pathways. There are several reasons for believing that CREB and BDNF mediate spatial memory via a common mechanism. Firstly, forms of LTP that are dependent on BDNF require cAMP and CREB activation (Patterson *et al.*, 2001; Ying *et al.*, 2002). Although a particular form of LTP cannot

be easily related to behavioural observations of LTM, there is no reason to believe that forms of LTP that involve CREB and BDNF are not physiologically relevant. Secondly, both CREB phosphorylation and increased *Bdnf* mRNA and protein levels are induced by learning spatial tasks (Bernebeu *et al.*, 1997; Kesslak *et al.*, 1998). Thirdly, the repression of these proteins before, not after training, impairs LTM, but not STM (Guzowski & McGaugh, 1997; Heldt *et al.*, 2007). These observations suggest that CREB and BDNF are needed for the consolidation, but not necessarily retrieval, of spatial memories. This is an appealing hypothesis because it explains why overexpression or constitutive activation of either protein almost invariably confers benefits to memory, inducing L-LTP or LTM under conditions that do not normally support these processes (Barco *et al.*, 2005; Sekeres *et al.*, 2010). Interestingly, Viosca *et al.* (2009b) recently showed that mice with the inducible VP16-CREB mutation, which show enhancements in LTP (Barco *et al.*, 2002) and neuronal excitability (Lopez de Armentia *et al.*, 2007) contingent on elevated BDNF expression (Barco *et al.*, 2005), showed impaired acquisition and long-term memory in the water maze. While this finding is in obvious discord with the usual memory enhancements mediated by increased levels of CREB and BDNF, it does not refute the importance of these proteins in LTM. Indeed, it suggests that appropriate regulation of CREB and BDNF, perhaps influenced by their ability to regulate each other, is important for the formation of stable spatial memories.

2.4.3.2 Fear memory

Another behavioural paradigm in which both CREB and BDNF have been extensively studied is fear conditioning. Fear conditioning is a Pavlovian conditioning paradigm that requires animals to form an association between a previously neutral stimulus, such as an auditory or visual cue (CS), and an aversive or fearful stimulus, such as a painful foot-shock (US). Memory for the CS-US association is demonstrated by conditioned freezing in mice, characterized by the cessation of all movements other than respiration, in response to future presentations of the CS, reflecting anticipation of the impending US (reviewed in Balleine & Killcross, 2006). Two forms of fear memory are most commonly studied in mice. Cued fear memory, in which an animal forms an association between a neutral sensory cue (CS), such as a tone or flashing light, and an aversive footshock (US) is subserved by the amygdala and largely independent of the hippocampus (Kim & Fanselow, 1992; Phillips & LeDoux, 1992; reviewed in LeDoux, 2003).

Contextual fear memory, in which the CS associated with the shock is the context in which the animal received the shock, is dependent on the amygdala and the hippocampus, but is not considered to be a spatial memory task (Frankland *et al.*, 1998; Phillips & LeDoux, 1992). Dissociable regions of the hippocampus are better suited for supporting memory in contextual and spatial tasks in rodents (Maren *et al.*, 1997; Moser *et al.,* 1993; Richmond *et al.*, 1999; Pittenger *et al.*, 2002). The larger place fields of the ventral hippocampus may have a greater role in encoding contextual information and the smaller place fields of the dorsal hippocampus may be better suited for encoding detailed spatial information. Both CREB and BDNF appear to have roles in the consolidation of fear memory.

Consistent with water maze experiments, studies using mice in which the expression of CREB is knocked down have produced mixed results about the role of CREB in conditioned fear memory. Bourtchuladze *et al.* (1994) found the $CREB^{\alpha\delta-/-}$ mice demonstrated considerable deficits (~50% decrease in freezing compared to wild-type mice) in contextual and cued fear memory. These deficits were specific to LTM, as $CREB^{\alpha\delta-/-}$ mice showed normal freezing 0.5 hours or 1 hour after fear conditioning, but were impaired in 2- or 24-hour memory tests. Using the same conditioning protocol (single 0.75 mA footshock paired with tone), two other studies failed to replicate the contextual fear memory deficit in $CREB^{\alpha\delta-/-}$ mice (Balschun *et al.*, 2003; Gass *et al.*, 1998). Gass *et al.* (1998) showed that $CREB^{comp}$ mice had LTM deficits in cued and contextual fear, whereas $CREB^{\alpha\delta-/-}$ mice did not. Kogan *et al.* (1997) also observed that increasing the ITI between consecutive CS-US pairings during contextual fear conditioning helped rescue the LTM deficit in $CREB^{\alpha\delta-/-}$ mice, highlighting the importance of training protocol in memory experiments. Nevertheless, discrepancies between studies do not seem to be easily explained in terms of differences in the extent of CREB knockdown or task protocol. $CREB^{S133A}$ mice showed a deficit in cued, but not contextual fear memory (Rammes *et al.*, 2000). Contrastingly, inducible CREB repressor ($CREB^{IR}$) mice in which $CREB^{S133A}$ overexpression was induced with tamoxifen prior to conditioning showed deficits in LTM, but not STM, in both contextual and tone fear (Kida *et al.*, 2002). Mice overexpressing KCREB throughout the dorsal CA1 did not show deficits in contextual or cued fear memory, although this mutation did not appear to affect the amygdala (Pittenger *et al.*, 2002).

As was the case for spatial memory, increasing CREB expression invariably enhances fear memory. Fear-potentiated startle (FPS), a paradigm in which cued fear memory is reflected

by a more intense reaction of a mouse to an unexpected, startling stimulus such as a sudden blaring noise (*startle stimulus*) when it is paired with the familiar CS, was enhanced in mice that had received pre-training infusions of CREB into the LA (Josselyn *et al.*, 2001). Han *et al.* (2007, 2009) convincingly demonstrated an enhancement in freezing after auditory fear conditioning in mice that received HSV-mediated viral vector infusions of CREB into the LA prior to training. Infusions of CREB into the LA also rescued auditory fear memory deficits in $CREB^{\alpha\delta-/-}$ mice and induced LTM following a weak training protocol (single 0.3 mA foot shock) that did not induce LTM in wild-type mice. Furthermore, targeted ablation of neurons transfected with CREB restored freezing to baseline in a subsequent memory test, suggesting that CREB was needed to encode fear memories. Zhou *et al.* (2009) similarly showed that the targeted inactivation of neurons transfected with CREB prior to an auditory fear memory test reversed a CREB-induced enhancement in memory.

BDNF has also been implicated in contextual and cued fear memory, although it appears to have distinct brain-region specific roles in these tasks. *Bdnf* mRNA is upregulated in the hippocampus, but not LA, following contextual fear learning (Hall *et al.*, 2000) and in the amygdala, but not hippocampus, following cued fear learning (Ou *et al*., 2010; Rattiner *et al.*, 2004). $BDNF^{+/-}$ mice demonstrated drastically impaired performance (~15% of wild-type freezing level) in a contextual memory test 24-hrs after fear conditioning, but no impairment in a cued memory test in a new context (Liu *et al.*, 2004). Adult BDNF-floxed mice in which forebrain-specific deletion of BDNF was mediated by the tTa-TetOp inducible system had deficits in contextual, but not cued fear memory, measured 24-hrs and 7-days after training (Monteggia *et al.*, 2004). However, juvenile mice with this mutation showed a deficit in cued fear memory, albeit their deficit in contextual fear memory was more dramatic. Contrastingly, mice with a conditional forebrain deletion of TrkB showed normal levels of freezing in contextual memory tests 24-hrs and 72-hrs after conditioning, but had deficits in cued fear memory at the same time points (Minichiello *et al.*, 1999). Therefore, the deficits in contextual and cued fear memory in mice with impaired BDNF-TrkB signaling are sensitive to specific experimental conditions, such as the genetic manipulation and the age of mice.

Ressler and colleagues have convincingly linked BDNF-TrkB signaling in the amygdala to cued fear memory in two studies using the FPS paradigm (Ou *et al.*, 2010; Rattiner *et al.*, 2004). Rattiner *et al.* (2004) demonstrated that pre-training infusion of the non-specific Trk

receptor antagonist K252a or LV-mediated infusion of a dominant negative TrkB mutation (TrkB.T1) bilaterally into the BLA impaired memory 48-hrs after training. Post-training infusion of the TrkB.T1 vector had no effect, suggesting that the inhibition of BDNF-TrkB signaling affected memory consolidation but not retrieval. Ou *et al.* (2010) replicated these findings by infusing K252a or TrkB-specific IgGs into the BLA prior to training, but also showed that post-training infusions impaired memory 7 days after training. These findings suggested that BDNF-TrkB signaling is necessary for the long-term maintenance that supports the retrieval of amygdala-dependent fear memories. Interestingly, two other studies from the same lab failed to demonstrate a role for BDNF in memory consolidation, but showed that the disruption of BDNF-TrkB signaling prevented normal extinction of fear memories. Chhatwal *et al.* (2006) showed that *Bdnf* mRNA levels were increased in the amygdala following extinction of FPS, and that mice that received TrkB.T1 infusions into the amygdala showed normal acquisition, but prolonged extinction, of this response. Heldt *et al.* (2007) showed that BDNF-floxed mice infused with Cre-recombinase into the dorsal hippocampus prior to conditioning showed normal conditioned freezing and FPS on subsequent memory tests that assessed responses to the tone in the training context. However, they showed impaired extinction of both responses, suggesting that BDNF signaling in the hippocampus, so long as the task has a contextual component, is also involved in the long-term maintenance of memories. Choi *et al.* (2010) recently showed that mice expressing an inducible deletion of BDNF in the prelimbic cortex had impaired auditory fear memory but normal extinction. Overall, these results point to a role of BDNF in the amygdala, and perhaps hippocampus, in the extinction of fear memory, whereas BDNF in the hippocampus and prelimbic cortex plays a role in consolidation of fear memories.

Another large body of insight into the role of BDNF in cued fear conditioning has come from mouse models of a common human genetic polymorphism in the BDNF gene. The substitution of valine for methionine on codon 66 of the *Bdnf* gene prodomain (Val66Met mutation) has an allele frequency of 20-30% in Caucasian populations and has been linked to cognitive function and anxiety-related disorders (Egan *et al.*, 2003; Shimizu *et al.*, 2004; reviewed in Mahan & Ressler, 2011). Neurons from mice heterozygous ($BDNF^{+/Met}$) or homozygous ($BDNF^{Met/Met}$) for the polymorphism demonstrated reductions in activity-dependent BDNF secretion in culture, with $BDNF^{Met/Met}$ neurons demonstrating the more substantial

reduction (~30%), of comparable magnitude to neurons from $BDNF^{+/-}$ mice (Chen *et al.*, 2006a). Functional MRI studies with humans have shown that carriers of Val66Met polymorphism have impaired episodic memory and hippocampal activation (Egan *et al.*, 2003), and reduced fear potentiated startle responses to stimuli previously associated with an ankle shock (Hajcak *et al.*, 2009; Lonsdorf *et al.*, 2010), suggesting that they have deficits in hippocampal- and amygdala-dependent memory. However, other studies have failed to replicate deficits in hippocampal dependent memory in tasks that required participants to remember face-scene pairs (Dennis *et al.*, 2010), or demonstrate a deficit in amygdala-dependent memory for emotionally salient stimuli (van Wingen *et al.*, 2010). An important limitation in human studies is the low number of participants homozygous for the Val66Met polymorphism, who appear to have greater deficits than heterozygous carriers. Studies in mice point more directly to a role for the Val66Met mutation in extinction, but not acquisition of cued fear memory. Chen *et al.* (2006a) showed that $BDNF^{+/Met}$, $BDNF^{Met/Met}$, and $BDNF^{+/-}$ mutants had impairments in contextual fear memory, and the deficits were greatest in $BDNF^{Met/Met}$ and $BDNF^{+/-}$ mice. All of the mutants demonstrated a similar level of freezing to wild-type mice on a cued fear memory test. Soliman *et al.* (2010) recently found that both human Met allele carriers and $BDNF^{Met/Met}$ mice showed normal acquisition of cued fear, but impaired extinction. Levels of fMRI activation and *c-fos* expression in human carriers and mutant mice, respectively, were reduced in the vmPFC and increased in the amygdala. Therefore, findings in human and mouse carriers of the Val66Met polymorphism also point to a role for BDNF in the amygdala-dependent extinction of cued fear memories.

As in the case of spatial memory, both CREB and BDNF appear to play a role in the consolidation of fear memories. If a CREB-BDNF interaction underlies fear memories, it would be interesting to determine to what extent the regulation of BDNF differs in the hippocampus and amygdala. These regions have different activity-dependent expression profiles of *Bdnf* exons (Aid *et al.*, 2007), and it has been suggested that CREB activates *Bdnf* to a greater extent in the hippocampus than in other brain regions (Lonze & Ginty, 2002). If differential patterns of CREB-mediated *Bdnf* regulation exist in the hippocampus and amygdala, it is tempting to speculate that they could account for the apparent brain region-specific roles of BDNF in the encoding, consolidation and long-term maintenance of memories.

2.4.3.3 Conditioned taste aversion

Conditioned taste aversion (CTA) is another form of learning in which an animal learns to associate a previously neutral or appetitive CS, typically a food or drink, with an aversive experience, such as intestinal malaise induced by injection with a noxious substance (US), such as nausea-inducing lithium chloride (LiCl). Memory for the CS-US association is easily observed, as the animal will avoid consumption of the CS. CTA learning is sensitive to lesions of the amygdala (Josselyn *et al.*, 2004) and is also dependent on the insular cortex (Bahar *et al.*, 2004; Shema *et al.*, 2007), but does not implicate the hippocampus to an important extent.

Behavioural evidence suggests that CREB is involved in the consolidation of CTA, while BDNF has been implicated in consolidation and extinction. LTM in the CTA task was attenuated in $CREB^{NesCre}$ mice (Balschun *et al.*, 2003) as well as $CREB^{\alpha\delta-/-}$ mice and $CREB^{IR}$ mice injected with tamoxifen prior to conditioning (Josselyn *et al.*, 2004). Bilateral infusion of CREB antisense OGNs into the amygdala also impaired LTM but did not affect STM in rats tested several hours after training (Lamprecht *et al.*, 1997). BDNF mRNA and protein levels, as well as levels of phosphorylated TrkB, were found to rise in the CeA and insular cortex, but not in the BLA, hippocampus or vmPFC, between 1 and 8 hours after training in the CTA task (Ma *et al.*, 2011). Rats infused with BDNF antibodies 1 hour after conditioning, but not prior to conditioning, failed to show these increases and had impaired LTM, but not STM. This deficit was rescued by a subsequent infusion of BDNF into the CeA, demonstrating the importance of BDNF in the consolidation of CTA in the amygdala. In another study, rats that received infusions of BDNF into the insular cortex 1 hour before training showed enhanced CTA in a LTM test 3 days later, and this enhancement was blocked by inhibiting Akt/PI-3K signaling or the ERK/MAPK pathway (Castillo & Escobar, 2011). Contrasting these findings, both $BDNF^{+/-}$ and $BDNF^{Met/Met}$ mice showed normal levels of CTA 3, 7 and 30 days after conditioning but showed delayed extinction of the aversion response (Yu *et al.*, 2009). These results parallel the observed role for BDNF-TrkB signaling in the extinction of amygdala-dependent fear memories.

2.4.3.4 Inhibitory avoidance

In the step-through inhibitory avoidance (IA) task, animals are conditioned to avoid a chamber in which they previously received a foot-shock. Animals are able to choose whether to spend time

in a light chamber that has not been paired with a shock (unpaired chamber) or an adjacent dark chamber in which they received the shock (paired chamber). Importantly, rodents normally prefer the safety of the dark chamber, and will avoid the open light chamber under control conditions. Accordingly, memory in the IA task can be measured as a function of the latency to enter the paired (dark) chamber during a test. The IA task has a contextual component and requires the formation of a CS-US association, thus recruiting both the hippocampus and the amygdala (Cahill & McGaugh, 1990; Taubenfeld *et al.*, 1999).

Studies investigating IA in rodents have implicated both CREB and BDNF in this form of learning. Rats with lesions of the fornix, an important relay region for information from the hippocampus, showed normal avoidance responses when tested immediately or 6 hours after training, reflective of intact STM, but showed a greatly reduced latency to enter the dark chamber compared to sham lesioned rats when tested 24 or 48 hours after training, indicating a LTM deficit (Taubenfeld *et al.*, 1999). Control, but not fornix-lesioned rats, demonstrated increases in levels of CREB phosphorylation in the hippocampus 3-6 hours after training, implicating CREB in the LTM deficit. Bernabeu *et al.* (1997) demonstrated that levels of phosphorylated CREB in the hippocampus peak twice after IA conditioning, with the first peak immediately after training, and the second peak 3-6 hours later. Rats that received bilateral infusions of cAMP or forskolin into the CA1 demonstrated memory enhancements 3 and 6 hours, but not 9 hours after training, while rats that received infusions of a PKA inhibitor or D1/D5 receptor antagonist had memory impairments at these time points. Bernabeu *et al.* concluded that agents that increase or decrease CREB expression lead to enhanced or impaired memory, respectively, at these time points. The involvement of CREB in IA was supported by a recent study in which $CREB^{Y134F\text{-}C}$ mice, which have an enhancement in CREB expression, showed higher crossover latency, indicative of stronger IA memory, than wild-type mice to the paired chamber in tests 2 and 24 hours, but not 30 minutes, after conditioning (Suzuki *et al.*, 2011).

BDNF appears to have roles in both STM and LTM in the IA task. Alonso *et al.* (2002) gave rats one trial of IA training and tested STM or LTM at time points 1.5 or 24 hours later, respectively. They demonstrated that infusions of anti-BDNF antibodies into the dorsal hippocampus impaired STM, while infusions of BDNF enhanced STM. The modulation of STM appeared to be contingent on BDNF-mediated ERK1/2 activation, as an ERK1/2 inhibitor

impaired STM, but not LTM. Infusions of anti-BDNF antibodies impaired LTM when administered 15 minutes before, or 1 or 4 hours after training, but not when infusions were administered immediately or 6 hours after training, revealing a time window during which BDNF expression is needed for LTM. Comparable time windows have been observed for CREB phosphorylation following IA training (Bernabeu *et al.*, 1997) and electrical stimulation of CA1 pyramidal neurons (Ahmed & Frey, 2005).

The role of TrkB in IA is less clear, as one study showed that mice with a conditional deletion of TrkB in the forebrain had normal memory performance in the IA task (Minichiello *et al.*, 1999). These mice actually performed better than control mice in an active avoidance task in which they had to escape from a chamber when a familiar cue signaled an impending footshock, reflecting heightened anxiety-related locomotor activity. Perhaps the most definitive illustration of the role of BDNF-TrkB signaling in IA was given by Lu *et al.* (2011) using mice with the $TrkB^{F616A}$ knock-in mutation, in which forebrain TrkB signaling can be inhibited by injecting mice with 1NMPP1. Lu *et al.* first placed mice in an open field with 4 novel objects for 15 minutes, and then trained them in a single-trial IA task 1 hour later. Exposure to the open field was intended to induce the expression of PRPs prior to the induction of another memory by IA training, creating a paradigm analogous to the strong-then-weak electrophysiological protocol (see Section 2.4.1.3: Synaptic tagging and capture). When trained in IA alone, mutant mice injected with 1NMPP1 demonstrated intact STM in a test 60 minutes after training but a lack of LTM 24 hours after training. Interestingly, mice that had been exposed to the open field 1 hour before IA training showed an enhancement in LTM, suggesting that the induction of PRPs prior to IA training facilitated the consolidation of STM to LTM. Mutant mice treated with 1NMPP1 did not demonstrate this behavioural tagging and capture, implicating TrkB in the role of a synaptic tag.

Overall, findings in the IA task are consistent with a role for CREB and BDNF in the consolidation of memories in the hippocampus. Both the time-lines of CREB activation and BDNF expression following a learning event and the observed LTM deficits suggest that these proteins are needed to facilitate the conversion of STM to LTM.

2.4.3.5 Recognition memory

CREB and BDNF have also been implicated in several tasks in which animals have to discriminate between novel and familiar features in the environment. Upon re-exposure to a familiar object, animals may spend less time investigating that object compared to a novel one, providing a measure of memory for the familiar object. Memory for familiar objects based on past experience is thought to be a form of episodic memory, which has a well-known association with the hippocampus based on studies in humans with brain damage (Bayley & Squire, 2002; Squire & Zola, 1998; Tulving, 1969), and more recently, neuroimaging studies (Greicius *et al.*, 2003). There is also some debate about the extent to which successful discrimination depends on familiar versus novel information, as in humans the hippocampus is believed to be especially responsive to novel information (Eichenbaum, 1999; Kirchhoff *et al.*, 2000).

In the object recognition task, an animal is first exposed to an object in an empty chamber and is later placed in a chamber with the familiar object and a novel object. Animals usually spend more time examining the novel object in this memory test. Mice that conditionally expressed the KCREB mutation in the dorsal CA1 under the control of dox demonstrated a normal preference for the novel object when tested 1 hour after the familiarization trial, but spent the same amount of time examining the novel and familiar objects 24 hours later, indicating a LTM deficit (Pittenger *et al.*, 2002). Similarly, BDNF-floxed mice that received bilateral infusions of Cre-recombinase into the dorsal hippocampus had a deficit in a memory test 24 hours after the familiarization trial (Heldt *et al.*, 2007).

Suzuki *et al.* (2011) recently conducted a study that demonstrated interactive effects of CREB and BDNF in a social recognition task that does not involve discrimination. In this task, mice were exposed to the same juvenile mouse twice, separated by 5-min, 30-min, 2-hr, 24-hr or 48-hr time intervals. Memory was assessed by computing a recognition index based on the amount of time that mice spent investigating the juvenile mouse during the second exposure compared to the first exposure. The duration of the first exposure was also varied from 45-s to 180-s in order to modulate the strength of the memory. Using several lines of mice overexpressing CREB, Suzuki *et al.* showed that the magnitude of memory enhancement in CREB transgenic mice was correlated with the magnitude of CREB overexpression, and this memory enhancement was further modulated by BDNF. While none of the mice showed

enhanced STM compared to wild-type mice when the interval between the first and second exposure was 5 minutes, $CREB^{DIEDML}$ mice, which express a constitutively active form of CREB, showed memory enhancements when the interval was 30 minutes or longer, $CREB^{Y134F-C}$ mice showed enhancements starting at the 2-hr time-point, and $CREB^{Y134F-A}$ mice only had enhanced LTM compared to wild-type mice after 24 hours. Bilateral infusions of BDNF into the hippocampus 3 hours before the social recognition task further enhanced STM at several time points for all lines of mice and enhanced LTM in both wild-type and $CREB^{Y134F-A}$ mice 48 hours after a short first exposure (45-s) to the juvenile mouse. These findings are consistent with the interpretation that both CREB and BDNF facilitate the consolidation of STM to LTM. Furthermore, the magnitude of LTM enhancement following BDNF infusions in $CREB^{Y134F-A}$ mice was greater than in wild-type mice, suggesting that CREB and BDNF act in synergy to accelerate the consolidation process.

2.4.4 Summary

Taken together, results from experiments involving electrophysiological manipulations, spatial memory tasks, contextual and cued fear conditioning, conditioned taste aversion, inhibitory avoidance, object and social recognition, and other tasks show that CREB and BDNF have important roles in memory processes. Although there are marked inconsistencies across studies, particularly when genetic manipulations are used to knock down the expression of a protein, it is important to note the consistency of findings within large studies evaluating multiple behaviours. For example, in the initial characterization of behaviour in CREB transgenic mice, Bourtchuladze *et al.* (1994) showed that $CREB^{\alpha\delta-/-}$ mice had deficits in LTP, spatial memory in the water maze, and both contextual and auditory fear memory. Heldt *et al.* (2007) showed that mice in which BDNF was deleted from the dorsal hippocampus had deficits in the water maze, object recognition task, and extinction of conditioned freezing responses. Given that genetic knockdown in mice is incomplete and other mechanisms can compensate for the lack of a particular protein, it is not surprising that transgenic mice exhibit deficits only under certain experimental conditions. Furthermore, the much more replicable enhancement of function observed in LTP and memory tests by overexpressing either CREB or BDNF strengthens the argument that they play essential roles in memory. It is still difficult to narrow down the roles of CREB and BDNF to a specific memory process, as these proteins have been found to affect both

STM and LTM, as well as the initial consolidation and long-term maintenance of memories, with brain-region specific effects. However, the most consistent finding across electrophysiological, neurostructural and behavioural experiments is that both CREB and BDNF are necessary for the consolidation of memories to a stable, long-term form.

2.5 Roles of CREB and BDNF in motor learning

In addition to learning about relational and emotional components of past experiences, humans and animals are able to learn motor skills with practice. Like the other forms of learning discussed above, motor learning depends on the synthesis of new proteins (Luft *et al.*, 2004). By contrast, motor learning is relatively independent of the amygdala and hippocampus, and is influenced by the sensorimotor cortex, cerebellum and striatum (reviewed in Hikosaka *et al.*, 2002). The sensorimotor cortex is directly responsible for motor output and receives independent input from the cerebellum, regarding the timing, balance and coordination of movements, and from the basal ganglia, regarding motivational and goal-directed components of the behaviour. The striatum is thought to be crucial for the procedural memory formed during motor learning (van der Meer *et al.*, 2010; reviewed in Robbins *et al.*, 2008). The initial performance of a motor skill is thought to be mediated by dopaminergic innervations from the ventral tegmental area (VTA) to the nucleus accumbens and ventral regions of the striatum, while performance of motor skills after extensive practice appears to be dependent on dopaminergic projections from the substantia nigra (SN) to dorsal regions of the striatum (Belin & Everitt, 2008; Everitt et al., 2008). Consistent with these observations, monkeys failed to learn a new sequence of hand movements following deactivation of the anterior striatum but not posterior striatum, while inactivation of the medial-posterior striatum impaired performance on the task once monkeys were extensively trained (Miyachi *et al.*, 1997). In another study, devaluation of lever pressing for food by inducing intestinal malaise in rats after extensive training rapidly extinguished lever pressing in rats with lesions of the dorsolateral striatum, but not in sham-lesioned rats (Yin *et al.*, 2004). The involvement of CREB and BDNF in other protein synthesis-dependent memory processes raises the question of whether they are also important for motor learning.

The role of CREB in motor learning has been investigated using the rotarod task. In this task, mice are placed on a rotating beam that gradually accelerates. The latency of animals to fall from the beam is recorded across trials within a day and across days. Improvements in this task are associated with dopaminergic neurotransmission in the striatum (Yin *et al.*, 2009) and the formation of dendritic spines in pyramidal neurons of the motor cortex (Xu *et al.*, 2009; Yang *et al.*, 2009). Both $CREB^{\alpha\delta-/-}$ mice and mice homozygous or heterozygous for a mutation in the KIX domain of CBP, which binds CREB ($CBP^{kix/kix}$ and $CBP^{kix/+}$ mice), fell from the rotarod consistently faster than wild-type mice using a 3-day training protocol (3 trials/day, 1-hr ITI) in which the rotarod accelerated from 4-40 rotations per minute (RPM) over 5 minutes (Oliveira *et al.*, 2006). PC1 mice, which overexpress αCREB in the cerebellum, leading to repression of CREB-mediated gene expression, were hyperactive and severely impaired in a one-trial version of the rotarod task (acceleration from 2-4 RPM over 3 minutes) that was successfully completed by wild-type mice (Brodie *et al.*, 2004). This finding likely reflects a role of cerebellar CREB in coordination and balance, but not motor learning *per se*. However, other studies in which CREB expression is attenuated (Kobayashi *et al.*, 2005) or increased (Viosca *et al.*, 2009a) have failed to demonstrate effects on motor learning. Interestingly, LTM in a cross-maze task was associated with CREB phosphorylation in the hippocampus when rats were trained to perform the task by learning the spatial layout of the maze, while CREB phosphorylation in the striatum was associated with LTM in rats that learned to perform a specific sequence of actions to navigate the maze (Colombo *et al.*, 2003). Therefore, CREB may act in different regions of the brain depending on whether task demands require spatial or procedural learning.

BDNF has also been implicated in some forms of motor learning. BDNF is expressed at high levels in the cerebellum (Aid *et al.*, 2007; Rauskolb *et al.*, 2010) and is involved in the development of cerebellar granule cells *in vitro* (Segal *et al.*, 1992). BDNF, as well as glial cell-line derived neurotrophic factor (GDNF), also contributes to the development and maintenance of dopaminergic neurons in the SN (Do *et al.*, 2007; Strand *et al.*, 2007). Although BDNF synthesis does not occur in striatal MSNs, BDNF synthesized in the SN or cortical pyramidal neurons is transported anterogradely to the striatum (Altar & DiStefano, 1998; Altar *et al.*, 1997). In $BDNF^{+/-}$ mice, striatal levels of BDNF are reduced compared to wild-type mice and decline at a more rapid rate with age (Boger *et al.*, 2011). $BDNF^{+/-}$ mice demonstrated normal levels of locomotor activity at 3, 12 and 21 months of age. Contrastingly, $BDNF^{+/-}$ mice were consistently

impaired in a 3-day version of the rotarod task when the beam was set to rotate at different speeds ranging from 4 to 40 RPM, reflecting an impairment in motor learning that was most substantial in aged mice. Saylor *et al.* (2006) also found that $BDNF^{+/-}$ mice demonstrated a reduction in locomotor activity at 3 and 24 months of age. Contrasting these findings, other studies found that $BDNF^{+/-}$ mice (Baker *et al.*, 2005) and mice overexpressing BDNF (Papaleo *et al.*, 2011) showed normal locomotor activity and performance on the rotarod. While this mix of behavioural findings is not particularly conclusive, the knockdown of BDNF expression in mice has substantial effects on dopaminergic neurons projecting from the SN to the dorsal striatum, strongly suggesting that BDNF is needed to maintain the integrity of this circuitry. Dopamine neurons in $BDNF^{-/-}$ mice are reduced in size and complexity at P14-18 (Baker *et al.*, 2005). Aged 12 and 21 month old $BDNF^{+/-}$ mice exhibit deficits in dopamine uptake by the dopamine transporter (DAT) and vesicular monoamine transporter-2 (VMAT2), as well as impaired potassium-induced dopamine release (Boger *et al.*, 2011; Dluzen *et al.*, 2004). To my knowledge, no study has implicated a mechanism involving both CREB and BDNF in motor learning. However, it would be interesting to determine whether the apparent roles of CREB and BDNF in consolidating hippocampal- and amygdala-dependent memories also apply to procedural memories.

2.6 Roles of CREB and BDNF in anxiety-related behaviour

So far, this review has focused on how memories of past experiences direct future behaviour. However, memory and cognitive reasoning abilities are not the sole determinants of how an individual will behave in a given situation. The emotional and motivational states of an individual are very much in constant interplay with their abilities to make rational decisions based on experience. Whether consciously or subconsciously, mood affects the choices that we make and can even impair our ability to behave in ways that are adaptive. Anxiety is of course a normal emotional state, and a certain level of anxiety helps animals to pick up on signals in the environment and avoid dangerous or harmful situations. On the other hand, heightened levels of anxiety can interfere with an animal's ability to appropriately distinguish harmless from potentially dangerous situations, leading to maladaptive avoidance responses. The genetic determinants of anxiety-related behaviour in mice have drawn considerable interest because of

their potential link to stress-induced behaviour and anxiety-related neuropsychiatric disorders in humans.

Given what we know about the roles of CREB and BDNF in neuronal development and synaptic and structural plasticity, some obvious links can be drawn between these proteins and anxiety-related responses. In humans and animals, several brain regions in which CREB and BDNF mediate learning and memory experience changes in volume or activity in response to stress (Davidson & McEwen, 2012). High levels of anxiety and aggression are associated with amygdala hyperactivity, whereas chronic stress and low self-esteem are associated with decreased hippocampal volume in humans (McEwen & Gianaros, 2011). Epigenetic changes in hippocampal neurons in response to early maternal care shape lifelong responses to stress in mice (Weaver *et al.*, 2004), and are correlated with a history of early childhood abuse in humans (McGowan *et al.*, 2009). In mice, exposure to increased levels of stress-related glucocorticoid hormones leads to abnormal dendritic spine development and turnover in developing and adult neurons (Liston & Gan, 2011). Furthermore, stress has been linked to the regulation of inhibitory neurons during development (Davidson & McEwen, 2012). The activity-dependent regulation of BDNF by CREB directly modulates inhibitory neuron development in the mice used in my experiments (Hong *et al.*, 2008), pointing to a potential role of the CREB-BDNF interaction in the development of anxiety-related responses. Below I discuss some experiments that have explicitly investigated the roles of CREB and BDNF in anxiety-related behaviours.

2.6.1 Anxiety-related behaviour in response to acute stress

One stereotypical manifestation of anxiety-related behaviour in mice is a fear of open spaces. This simple response can be investigated without actively inducing stress in animals and thus serves as a good baseline measure of anxiety. One type of paradigm used to investigate fear of open spaces in mice is the open field (OF) test, where an animal is placed in a large open chamber. In this paradigm, anxious mice are reluctant to explore the open areas of the chamber and spend more time close to the walls. A more complex test is the elevated-plus maze (EPM), an apparatus that consists of two straight, intersecting hallways, making four arms in the shape of a ‘plus’ sign. Time spent in the two *open* arms, which have no walls and are uncovered, compared to two *closed* arms, which have walls, can be used as an index of anxiety. Stress has

been shown to reduce exploratory behaviour in the OF and time spent in the open arms of the EPM (Ma *et al.*, 2011), while anxiolytic agents have the opposite effects (Li *et al.*, 2010).

Studies investigating the roles of CREB and BDNF in the OF and EPM have produced mixed results. Because there are few studies that have expressly investigated the role of CREB in anxiety-related behaviour, there is a lack of evidence for CREB-mediated impairments in the OF or EPM. Neither mice expressing a potent dominant-negative inhibitor of CREB (KCREB mice; Pittenger *et al.*, 2002), nor mice that constitutively overexpress CREB (VP16-CREB mice; Viosca *et al.*, 2009a) behaved differently from wild-type mice in the OF or EPM. Notably, Balschun *et al.* (2003) found that four different transgenic lines of CREB mice ($CREB^{\alpha\delta-/-}$, $CREB^{comp}$, $CREB^{NesCre}$, $CREB^{CaMKCre7}$) had persistently increased thigmotaxis during water maze training, a tendency to swim close to the periphery of the pool that may reflect anxiety.

The role of BDNF in these tasks has been more thoroughly investigated. $BDNF^{Met/Met}$ mice, and to a greater extent, $BDNF^{+/-}$ mice, spend less time in the center of the OF and in the open arms of the EPM than wild-type mice (Chen *et al.*, 2006; Li *et al.*, 2010). However, TrkB transgenic mice with developmental (e.g. $TrkB^{+/-}$ or TrkB deletion in the forebrain) deficits in TrkB expression (Minichiello *et al.*, 1999), and $TrkB^{F616A}$ mice, in which forebrain-specific deletion of TrkB is induced by 1NMPP1 (Lu *et al.*, 2011), did not show heightened anxiety-related responses in the OF or EPM. Although these findings could suggest that the effects of BDNF on anxiety are modulated by a mechanism that is relatively independent of TrkB receptor signaling, this is probably an oversimplification. While Minichiello *et al*. (1999) found that mice with the forebrain deletion of TrkB performed normally in the OF and EPM, their behavioural experiments revealed several indices that the transgenic mice had elevated levels of anxiety. These mice showed persistently increased thigmotactic behaviour in the water maze, as well as hyperactivity and facilitated performance in an active avoidance task in which they had to anticipate a foot-shock based on a familiar cue. The authors concluded that these mice developed inappropriate coping mechanisms for stressful situations. It also appears that heightened anxiety-related responses result from developmental deficits due to a reduction in BDNF expression, as BDNF-floxed mice in which BDNF deletion was induced by a LV vector containing Cre-recombinase did not differ from control mice in anxiety-related measures in the OF or EPM (Choi *et al.*, 2010).

Acute stress induced by traumatic events also changes CREB and BDNF expression in the brain. Following a painful foot-shock in fear conditioning tasks, mice demonstrate an upregulation of CREB (Taubenfeld *et al.*, 1999) and BDNF (Hall *et al.*, 2000). Social isolation following contextual fear conditioning induced a decrease in BDNF in the hippocampus and impaired LTM (Barrientos *et al.*, 2003). Acute restraint stress induced by physically immobilizing a mouse for 2 hours reduced *Bdnf* mRNA levels in the hippocampus of 3-4 month old rats, and produced a greater reduction in 24 month old rats (Smith & Cizza, 1996). Acute social defeat stress induced by subjecting an experimental mouse to multiple attacks from a larger, aggressive mouse induced dramatic decreases in *Bdnf* mRNA in the hippocampus, BLA, and piriform cortex 24 hours later (Pizarro *et al.*, 2004). Social defeat stress was associated with a dramatic increase in corticosterone, a well-known physiological response to stress. Acute stress manipulations such as shocks, social isolation, restraint, and social defeat would be expected to induce higher levels of anxiety than tasks such as the OF and EPM, so it is not surprising that they are more reliably associated with changes in BDNF-mediated signaling.

2.6.2 Effects of chronic stress on anxiety

A limitation of the tests described above is that they can only be used to investigate anxiety under conditions of acute stress. Conclusions derived from the results of such tests grossly oversimplify the phenomenon of anxiety. Pathological levels of anxiety, such as those shown by humans with neuropsychiatric disorders, are better reflected in animals by experimental conditions that induce chronic stress.

Both CREB and BDNF are responsive to manipulations that induce stress over a prolonged period of time. Hu *et al.* (2009) induced chronic mild stress (CMS) in mice, using a protocol that involved 4 weeks of varied and unpredictable exposure to stressors including paired caging, cage tilting and shaking, food and water deprivation, wet cage bedding, and a continuous light cycle, and measured intake of a sucrose solution for 1 hour each day. CMS resulted in a progressive decrease in sucrose intake, corresponding to a reduction in phosphorylated CREB and BDNF protein levels in the hippocampus. Chronic (7-day) restraint stress for 2 hours per day caused a decrease in hippocampal *Bdnf* mRNA in rats, and a similar effect was achieved with intraperitoneal (i.p.) administration of corticosterone (Smith *et al.*, 1995). Finally, mice that underwent repeated bouts of social defeat with various aggressor mice over a 10-day period

demonstrated marked social avoidance of a new non-aggressive mouse 1 day and 4 weeks later that was associated with an increase in BDNF in the nucleus accumbens (Berton *et al.*, 2006). Manipulations that increase CREB and BDNF in the nucleus accumbens are correlated with decreases in other brain areas, such as the hippocampus and amygdala (see Dong *et al.*, 2006, for a demonstration of this effect with CREB). Infusions of an AAV vector containing Cre-recombinase into the VTA 20 days prior to the social defeat protocol prevented the increase in BDNF in the nucleus accumbens and reversed the effects of chronic social defeat stress.

Findings from studies measuring anxiety-related behaviour in response to acute and chronic stress point to roles for CREB and BDNF that are independent of their roles in memory-related processes. Since many of the same brain areas are recruited by memory processes and responses to stress, including the hippocampus and amygdala, it is likely that the two types of processes are more heavily influenced by different signaling cascades. CREB-mediated expression of dynorphins and induction of Akt/PI-3K pathway signaling by BDNF are likely to play prominent roles in responses to stress (Berton *et al.*, 2006; reviewed in Berton & Nestler, 2006). Interestingly, structural changes in dendritic spine density in the nucleus accumbens are also associated with stress, demonstrating another common process by which CREB and BDNF might modulate anxiety-related responses (reviewed in Nestler *et al.*, 2002). The ability for CREB and BDNF to modulate both memory- and anxiety-related processes suggests that these proteins have dynamic effects on behaviour that are also influenced by emotional states.

2.7 Human cognition and psychiatric disorders

Scientific enquiry has an end point that involves the translation of basic knowledge about physiological and behavioural processes to an understanding that can be applied for the betterment of human conditions. Throughout this review, I have discussed many attributes of CREB and BDNF that, with continued study, can help us understand how to ameliorate a number of debilitating clinical disorders.

I began my review of the literature by discussing the molecular structures and interactions of CREB and BDNF. Here I elucidated several molecular players implicated in human disorders. Mutations in CBP, a critical mediator of CREB activation, are implicated in

Rubinstein-Taybi syndrome (Josselyn, 2005) and Huntington's disease (Jiang *et al.*, 2006), MeCP2 is implicated in Rett syndrome (Amir *et al.*, 1999), and the upregulation of MEF2 is implicated in autism spectrum disorders (Flavell *et al.*, 2008). Next, I discussed the effects of CREB and BDNF on the development of the nervous system, concluding that the CREB-mediated expression of BDNF modulates the balance of excitatory and inhibitory synapses in the nervous system. Shifts in this developmentally regulated balance are increasingly being implicated in neuropsychiatric disorders (West, 2008). Increased cortical excitation is associated with Rett syndrome and autism spectrum disorders (Dani *et al.*, 2005; Rubenstein & Merzenich, 2003) and reductions in inhibitory synapse number have been linked to schizophrenia (Lisman *et al.*, 2008). I then proceeded to discuss the roles of CREB and BDNF in physiological processes underlying learning and memory, focusing on the dynamic regulation of synaptic and structural plasticity throughout the life-span. Here it should be pointed out that developmental deficits in dendritic spine pruning have been linked to autism, while increased spine loss in adolescence and old age is associated with the pathologies of schizophrenia and Alzheimer's disease, respectively (reviewed in Penzes *et al.*, 2011).

About midway through my review, I began discussing the roles of CREB and BDNF in behavioural correlates of learning and memory in animals. I suggested that CREB and BDNF are involved in the consolidation of long-term spatial, contextual, and fear-related memories. I also pointed to a function of BDNF in the amygdala in fear extinction. This inability to extinguish fearful memories has been linked to post-traumatic stress disorder (PTSD), which is characterized by the inability to forget past traumatic events (reviewed in Mahan & Ressler, 2011). Interestingly, PTSD or history of trauma in humans are correlated with reductions in serum BDNF levels (Dell'osso *et al.*, 2009; Kauer-Sant'Anna *et al.*, 2007) and a recent clinical trial showed that changes in BDNF levels during treatment were associated with recovery from PTSD (Berger *et al.*, 2010; however, there are numerous conflicting findings; see Hauck *et al.*, 2010; Zhang *et al.*, 2006). Next, I discussed the roles of CREB and BDNF in motor learning, speculating that they are involved in the consolidation of procedural memories as well. I mentioned that both CREB and BDNF are needed for the development of dopaminergic neurons from the SN to the striatum. Neurodegeneration in this pathway underlies the debilitating motor symptoms of Parkinson's disease (Baker *et al.*, 2005).

Finally, I discussed the involvement of CREB and BDNF in anxiety-related responses to chronic and acute stress. Over the past 20 years, a substantial amount of evidence has emerged implicating the CREB-mediated expression of BDNF in depression (for reviews see Berton & Nessler, 2006; Duman *et al.*, 1997; Duman & Monteggia, 2006). Elevated levels of BDNF in the hippocampus, which result from the upregulation of CREB, are induced by chronic treatment with anti-depressant medications or electroconvulsive seizures (Nibuya *et al.*, 1995, 1996). Both CREB and BDNF have also been found to induce antidepressant-like effects in animal models of depression (Chen *et al.*, 2001; Gourley *et al.*, 2008; Monteggia *et al.*, 2004; reviewed in Duman & Monteggia, 2006), and are potential targets for the development of new therapeutic agents (reviewed in Berton & Nestler, 2006).

2.8 The current study

Given the broad roles of CREB and BDNF in physiology and behaviour in healthy and diseased states, it is surprising that so little is known about the behavioural effects of the interaction between these two key molecular players. In the current study, I took advantage of a transgenic mouse with a mutation at the CaRE-3/CRE binding site of *Bdnf* promoter IV (CREmKI$^{-/-}$ mouse). As described above, mutations at this site prevent the activation of *Bdnf* promoter IV transcription by CREB that is normally induced by neuronal activity (Hong *et al.,* 2008; Tao *et al.,* 1998). CREmKI$^{-/-}$ mice have normal basal levels of *Bdnf* mRNA but show markedly reduced transcription in response to physiologically relevant stimulation, resulting in a disruption of inhibitory synapse development throughout the brain. Using these mice allowed me to investigate the main neuronal activity-dependent component of BDNF function regulated by CREB. No previous study has examined the role of this CREB-BDNF interaction in memory- and anxiety-related behaviours.

Findings from the experiments discussed above, across several behavioural modalities (i.e. hippocampal-dependent memory, hippocampal-independent memory, motor learning, and anxiety-related behaviour), are summarized in Table 2.1. I selected a task from each of these modalities to create a behavioural battery. Although a more detailed behavioural characterization with a larger number of tasks might have been desirable, the nature of my

experiment necessitated that I limit the number of tasks administered. Since I used a repeated-measures design, using any more than a few tasks would likely cause mice to become overly habituated to the experimental setting, affecting performance on behavioural measures sensitive to stress, such as fear and anxiety. To evaluate a breadth of behaviours while minimizing the effect of experience on performance in subsequent tasks, I administered only one task for each behavioural modality, generally choosing the task in which both CREB and BDNF have been most extensively implicated in the literature. The tasks chosen have also been employed extensively by myself and others in my lab. This resulted in the choice of the Morris water maze to measure hippocampal-dependent memory, auditory fear conditioning to measure hippocampal-independent memory, and the rotarod to assess motor learning. While CREB-deficient mice do not usually show abnormal behaviour in the OF, they demonstrate elevated levels of thigmotaxis in the water maze (Balschun *et al.*, 2003), a tendency analogous to the reduction in exploratory behaviour in the OF shown by BDNF-deficient mice (Chen *et al.*, 2006; Li *et al.*, 2010), justifying the selection of this task.

Previous studies in my lab have shown that CREB levels in LA neurons directly modulate auditory fear learning (Han *et al*., 2007, 2009), and I was particularly interested to determine if these findings could be attributable to CREB-mediated expression of BDNF. Therefore, all naïve mice underwent auditory fear conditioning. The subsequent order of tasks was intended to subject mice to less invasive manipulations first and more invasive tasks at the end, so mice underwent the rotarod and OF tasks prior to the water maze. The experimental design, procedures and hypotheses are further summarized below.

2.8.1 Auditory fear conditioning

Eight-week-old mice first underwent an auditory fear conditioning task in which an association is formed between an auditory cue (CS) and an aversive electric foot-shock (US). During training, mice were placed in the fear conditioning chamber and allowed to explore for 2 minutes. The tone was then presented for 30 seconds and co-terminated with a 0.5 mA foot shock. Twenty-four hours later, mice were tested in a different context. Long-term memory was assessed as a function of conditioned freezing when the familiar tone was presented.

Table 2.1: Summary of experimental findings in mice with altered CREB or BDNF function

Behaviour Type	Task	Experimental findings
Hippocampus-dependent memory	Morris water maze	Impaired in CREB$^{\alpha\delta-/-}$, CREB^{IR}, KCREB, BDNF$^{+/-}$, floxed BDNF, and TrKB.T1 mice Enhanced by hippocampal infusions of HSV-CREB and in CREB^{Y134F} mice
	Radial arm maze	Impaired by hippocampal infusions of BDNF As-OGNs and in TrkB-Cre mice
	Contextual fear conditioning	Impaired in CREB$^{\alpha\delta-/-}$, CREB^{comp}, CREB^{IR}, BDNF$^{+/-}$, BDNF$^{Met/Met}$ and floxed BDNF mice Enhanced in CREB^{Y134F} and VP16-CREB mice, and by hippocampal BDNF infusions
	Inhibitory avoidance	Impaired in BDNF^{F616A} mice and by hippocampal BDNF antibody infusions
	Object recognition	Impaired in KCREB and floxed BDNF mice
	Social recognition	Synergistic enhancement in CREB^{Y134F} mice that received hippocampal BDNF infusions
Hippocampus-independent memory	Auditory fear conditioning	Impaired in CREB$^{\alpha\delta-/-}$, CREB^{comp}, CREB^{S133A}, CREB^{IR}, floxed BDNF, and TrKB-Cre mice Enhanced by amygdalar infusions of HSV-CREB and in VP16-CREB mice
	Fear-potentiated startle	Impaired by TrkB antibody infusions into the BLA Enhanced by amygdalar infusions of HSV-CREB
	Conditioned taste aversion	Impaired in CREB$^{\alpha\delta-/-}$, CREB^{NesCre} and CREB^{IR} mice and by amygdalar infusions of BDNF antibodies Enhanced by BDNF infusions to the amygdala or insular cortex
Motor learning	Rotarod	Impaired learning in CREB$^{\alpha\delta-/-}$, CREB$^{KIX/KIX}$ and BDNF$^{+/-}$ mice
	Cross-maze	Non-spatial strategy associated with CREB phosphorylation in the striatum
Anxiety-related behaviour (acute)	Open field	Reduced exploratory behaviour in BDNF$^{+/-}$ and BDNF$^{Met/Met}$ mice
	Elevated-plus maze	Reduced exploratory behaviour in BDNF$^{+/-}$ and BDNF$^{Met/Met}$ mice
	Thigmotaxis	Increased in CREB$^{\alpha\delta-/-}$, CREB^{comp}, CREB^{NesCre} and CREB^{CaMCre7} mice

See text for references and further explanation of all experiments. Contradictory findings are not shown in table.

Since mice carrying the CREmKI mutation have an impairment in the activity-dependent expression of BDNF, an important CREB target gene in the amygdala, I hypothesized that mutant mice would have impaired 24-hr LTM in this task. I expected that the impairment would be proportional to gene dosage, so CREmKI$^{-/-}$ mice would be more impaired than CREmKI$^{+/-}$ mice.

2.8.2 Rotarod

One week after fear conditioning, I assessed motor learning on the rotarod. In each trial, mice were placed on a rotating beam that accelerated from 4 to 40 RPM over the course of 5 minutes.

Each trial ended when the mouse fell from the rotarod. To assess learning-related improvements across sessions within a day and across days, mice underwent 10 trials per day (10-min ITI) for 8 straight days.

Like episodic memory in animals, procedural memory is dependent on the synthesis of new proteins (Luft *et al.*, 2004). I suspected that the CREB-BDNF interaction plays a role in the consolidation of procedural memory, and thus mice carrying the CREmKI mutation should demonstrate a slower learning curve in the rotarod task. I expected CREmKI$^{-/-}$ mice to be impaired to a greater extent than CREmKI$^{+/-}$ mice.

2.8.3 Open field

Two days after the last day of rotarod testing, I investigated anxiety-related behaviour in a single, 15-minute session in the OF. Mice were allowed to freely explore the chamber and anxiety was measured as a function of the relative amounts of time spent in three concentric zones of the chamber. I also measured overall locomotor activity during the trial. I hypothesized that CREmKI$^{-/-}$ mice, and to a lesser extent CREmKI$^{+/-}$ mice, would show increased levels of anxiety and spend less time in the central area of the chamber. Since BDNF$^{+/-}$ mice are hyperactive (Kernie *et al.*, 2000), I suspected that the mutant mice would also demonstrate heightened activity levels.

2.8.4 Water maze

Two days after the OF test, I trained mice in the hidden platform version of the Morris water maze, a task in which mice learn to navigate a circular pool of water by using visual cues in the environment in order to escape to an invisible platform below the pool's surface. Mice were trained for 6 trials per day for 6 days (*strong training*) or 3 trials per day for 3 days (*weak training*). The platform was removed, and mice were tracked as they swam in the pool for 60 s prior to the first day of training (*probe 1*), 24 hours after the third day of training (*probe 2*), 24 hours after the sixth day of training (*probe 3*) and 6 weeks after strong training (*remote probe*). During training, memory was evaluated based on the latency of mice to reach the hidden platform. In probe tests, memory was assessed based on the amount of time that mice spent searching in the zone of the pool in which the hidden platform was normally located.

Previous studies have shown that CREB-deficient mice have spatial memory impairments in the water maze (Bourtchuladze *et al.*, 1994), and CREB overexpression induces the late phase of LTP in pyramidal neurons (Barco *et al.*, 2002), as well as long-term spatial memory under conditions that do not normally support these processes (Sekeres *et al.*, 2010). Similarly, BDNF has been extensively implicated in hippocampal LTP (Korte *et al.*, 1995, 1996) and spatial memory in the water maze (Heldt *et al.*, 2007; Kesslak *et al.*, 1998). Since both CREB and BDNF, on their own, are crucial for the performance of this task, I hypothesized that CREmKI mutant mice would show a reduced improvement in latency to reach the platform over the course of training, and spend less time in the zone corresponding to the location of the platform during the 24-hour and 6-week probe tests. I expected CREmKI$^{-/-}$ mice to be impaired to a greater extent than CREmKI$^{+/-}$ mice.

Chapter 3
Materials and Methods

3.1 Subjects

3.1.1 Mice

In all of the experiments, I used male and female CREmKI mice with a mixed C57B/6 ×129s6/SvEvTac background. The original breeders, which were used to start our own colony in the lab, were obtained from Michael Greenberg's lab (Harvard University, Cambridge, MA). The generation of these mice has been described in a previous study (see Hong *et al.*, 2008). Briefly, a targeting vector was constructed from a 129s6/SvEvTac mouse genomic fragment that was amplified and cloned. A neomycin-zeomycin (NEO-ZEO) positive selection cassette flanked by loxP (5'-AGGGCGGTGAGCCACAGGCTGTGAGTTTG-3') was incorporated into this fragment, 2.4 kb downstream of *Bdnf* promoter IV, into a large intron located between promoters VII and VIII. The NEO-ZEO selection cassette ensured that the target construct was introduced into mouse embryonic stem cells by homologous recombination. The subsequent transfection of these cells with a plasmid containing Cre-recombinase led to excision of the NEO-ZEO cassette and half of each flanking loxP sequence. As a result, the remaining outer halves of each loxP sequence were ligated and incorporated into the embryonic stem cells. These cells were used to generate the loxP control mice.

To generate the CREmKI mutant mice, Hong *et al.* (2008) selected target constructs containing the loxP-flanked NEO-ZEO selection cassette for site-directed mutagenesis. The critical regulatory region that resembles the CRE consensus sequence (wild-type: 5'-TCACGTCA-3') and is a binding site for CREB was replaced with a mutated sequence (mutant: 5'-CAGCTGCA-3') in a region between 29 and 36 bp 5' of the initiation site of *Bdnf* promoter IV transcription. Following homologous recombination into embryonic stem cells and Cre-recombinase-mediated excision of the NEO-ZEO selection cassette, the CREmKI and loxP control stem cells were injected into C57B/6 blastocysts and implanted into female mice to generate independent mutant and control mouse lines. The chimeric offspring were then mated with C57B/6 mice and the resulting hybrid offspring were backcrossed into the C57B/6 background for four to six generations. Therefore, the independent lines of mutant mice generated by Hong *et al.* were either homozygous for both the mutant CaRE3/CRE sequence and the loxP sequence ($CREmKI^{-/-}$ mice) or homozygous for the loxP sequence only (loxP control

mice). Subsequently, mice heterozygous for the mutant CaRE3/CRE sequence and homozygous for the loxP sequence (CREmKI$^{+/-}$ mice) were generated by crossing these two lines.

The original breeders that we received from Dr. Greenberg's lab were heterozygous for the mutant CaRE3/CRE sequence and homozygous for the loxP sequence. We subsequently started our CREmKI mouse colony by mating the breeders to produce a mix of F1 offspring that were wild-type (+/+), heterozygous (+/-), or homozygous (-/-) for the mutant CaRE3/CRE sequence and homozygous for the loxP sequence (mouse cohort 1). LoxP control mice did not show phenotypic or developmental differences from wild-type C57B/6 ×129sv mice in the previous study (Hong *et al.*, 2008), so I did not use a pure wild-type control group in my experiments. Because the original parents did not produce a large number of homozygous mutant offspring (*N*s = 22, 20, and 10 for the loxP control, heterozygous, and homozygous mutant groups, respectively), I repeated my experiments using F2 mice from heterozygous and pure F1 crosses (cohort 2; *Ns* = 31, 9, and 29 for the loxP control, heterozygous, and homozygous mutant groups, respectively).

All mice were maintained in a colony at the Hospital for Sick Children (Toronto, ON) and group housed (3-5 mice per cage). Home cages were kept in an environmentally-controlled room on a 12-hr light/12-hr dark cycle with lights on from 7:00 a.m. to 7:00 p.m. Food and water were available *ad libitum*. New litters were kept in their home cages with their parents until 3-4 weeks of age when they were weaned. Although weaning typically occurs when mice are 3 weeks of age, the lab technician weaned some mice at 4 weeks because the mutants were consistently smaller in size. All mice were genotyped at 5-7 weeks of age. Briefly, mice were removed from their home cages and anaesthetized with isofluorane in an insulated glass jar. The mice were ear punched and a razor blade was used to clip a 0.5 cm tail sample before returning the mice to their home cages. The tail samples were genotyped using the polymerase chain reaction (PCR; see Section 4.1.2). Behavioural experiments were conducted during the light phase of the cycle. Mice were 8 weeks of age at the start of testing. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the Animals for Research Act, and were approved by the Hospital for Sick Children Animal Care and Use Committee.

3.1.2 Genotyping

Tail samples were incubated in 100 µL of working solution consisting of 2% proteinase K in one-step lysis buffer (50 mM KCl, 10 mM Tris-HCL, 0.1% Triton X-100) at 56°C for 24 hours. The samples were then genotyped using PCR (Gibbs, 1990; Mullis *et al.*, 1992; Saiki *et al.*, 1985). Oligonucleotide primers specific for the amplification of DNA fragments containing the loxP sequence (sense: 5'-AGGGCGGTGAGCCACAGGCTGTGAGTTTG-3' and antisense: 5'-ATCCCCAAGTCCCCATCCCCAGTTTCC-3') and CaRE3/CRE sequence (sense: 5'-GTTGCTGCCTAGATAATGACAGGC-3' and antisense: 5'-ATATGTACTCCTGTTCTGCAGC-3') were added to the PCR reaction mix (2 µL 10X buffer, 1 µL 50 mM $MgCl_2$, 0.4 µL, 10 mM dNTP mix, 1 µL tail DNA, and 0.1 µL Taq polymerase in 14.3 µL distilled water per reaction) and denatured at 94°C for 15 minutes. The samples then underwent 35 cycles of denaturation, annealing and elongation steps (94°C, 30 s; 62°C, 30 s; and 72°C, 60 s). There was a final elongation step at 72°C for 3 minutes. CaRE3/CRE samples (10 µL of PCR product, 7 µL distilled water, and 2 µL 10X restriction enzyme buffer) were then incubated with 1 µL of PvuII restriction enzyme for at least 2 hours at 37°C. PvuII selectively digests the mutant CaRE3/CRE fragment (Hong et al., 2008). The LoxP PCR products and digested CaRE3/CRE products were loaded onto 2% agarose gel and subjected to electrophoresis (130 V) for 30-35 minutes.

3.2 Histological analysis of brains

3.2.1 Perfusions

Following the completion of all behavioural experiments, mice from cohort 1 (*n*s = 3 for the loxP control and homozygous mutant groups, respectively) were deeply anaesthetized with an intraperitoneal (i.p.) injection of chloral hydrate at a dose of 400 mg/kg. The mice were perfused transcardially with a 0.1 M solution of phosphate-buffered saline (PBS; pH 7.4) and then with 4% paraformaldehyde (PFA). Brains were immediately removed and fixed overnight at 4°C. Twenty-four hours later the brains were weighed before being transferred to a 30% sucrose solution in 0.1 M PBS for 72 hours. The brains were then sliced into 50 µm coronal sections using a cryostat (Leica CM1850) and stained (see below).

3.2.2 Brain anatomical measurements

I selected every sixth section for histological analysis, resulting in an interval of 300 μm between sections. The bilateral sections were placed in well plates containing 1 μL of 4,6-diamidino-2-phenylindole (DAPI) diluted in 1 mL of 0.1 M PBS (6-8 sections per well) for 45 minutes to apply the fluorescent DNA stain. They were then rinsed with PBS and mounted on gel-coated slides. Slides were coverslipped with fluorescent mounting medium and allowed 24-72 hours to air-dry at room temperature. The slides were examined at 2 to 10x magnification with a Nikon Eclipse 80i light microscope and images of each section were captured with a Nikon DXM 1200F digital camera and recorded by computer software (Nikon Act-1). Using NIH ImageJ, I traced the whole hippocampus and LA in each section to obtain measures of area in mm^2. I then multiplied the average of the area measurements across sections (typically 6-9 sections per brain) by the interval between sections to obtain the volume in mm^3 (Mozhui *et al.*, 2007). In each section, I measured the cortical thickness through the primary motor cortex/mediomedial part 2 of the visual cortex and took the average across sections to obtain a global measure for each brain. I selected this area of the cortex because it was well preserved in all of the sections.

3.3 Behavioural Testing

Given the limited availability of transgenic mice and the exploratory nature of my study, I used a repeated measures design. Mice in cohorts 1 and 2 (*N*s = 52 and 69) first underwent auditory fear conditioning at 8 weeks of age. This was always the first task administered because the effect of CREB on fear memory is of particular interest to my lab (Han *et al.*, 2007, 2009; for review see Josselyn, 2010). Twenty-nine mice in cohort 1 and 8 mice in cohort 2 underwent the full set of behavioural tasks: after fear conditioning they were handled for 4-5 days (2-min/day) before starting rotarod training, followed two days later by the open field test, and two days later by water maze training. Alternatively, after fear conditioning mice were handled for 4-5 days before beginning water maze training (*n*s = 10 and 30 for cohorts 1 and 2, respectively). Subsets of mice from cohort 2 underwent a shortened version of the water maze protocol (*n* = 13) or fear conditioning only (*n* = 19). Mice were always weighed prior to behavioural testing. The time-

line of behavioural experiments is illustrated in Figure 3.1. A detailed breakdown of the experimental design is provided in Table 3.1.

3.3.1 Auditory Fear Conditioning

Using a paradigm that has been employed extensively in my lab, I tested mice in a Pavlovian conditioning task in which an association is formed between an auditory cue (CS) and an aversive electric foot-shock (US).

3.3.1.1 Apparatus

Training and testing was conducted in a dimly lit, windowless room, with a fan to generate white noise. The fear conditioning chamber (31 x 24 x 21 cm high; MED Associates, St. Albans, VT) consisted of two stainless steel walls, two clear acrylic walls, and had a stainless steel shock-grid floor (bars 2 mm diameter, spaced 7.9 mm apart). A stainless steel drop-pan was placed below the grid floor, and was lightly wiped with ethanol prior to conditioning. Two distinct contexts were used during training and testing. Context-A (CXT-A) was the chamber, paired with the fan and ethanol scent. To create context-B (CXT-B), an opaque white acrylic triangular wall insert was placed inside the chamber and an opaque white smooth acrylic floor panel was used to cover the floor bars. The door of the chamber was also covered with an opaque sheet of horizontal black and white stripes, and the fan and ethanol were not used.

3.3.1.2 Procedure

Mice were trained in CXT-A and memory for the CS-US association was tested in CXT-B 24 hours later. During the training trial, mice were placed in CXT-A and allowed to explore for 2 minutes. The tone (2800 Hz, 85 dB) was then presented for 30 seconds and co-terminated with a brief (2-second) 0.5 mA foot shock. Mice remained in the chamber for an additional 30 seconds before returning to their home cages. During the test, mice were placed in CXT-B for 2 minutes with no tone, and then the tone was sounded for 1 minute but no foot shock was delivered.

An overhead camera recorded the movement of mice during all trials, which was analyzed frame-by-frame using an automated tracking system (MedPC and Freezeview, Actimetrics, Wilmette, IL). Freezing (cessation of all movement other than respiration) was measured using Freezeframe (Actimetrics, Wilmette, IL). Long-term memory was assessed as a

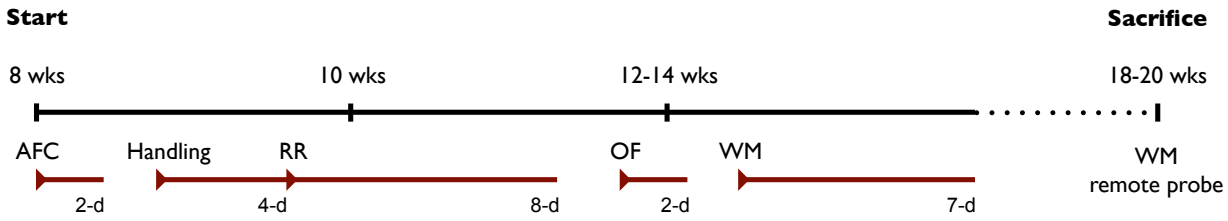

Figure 3.1: Time-line of the within-subjects design

Mice began behavioural experiments at 8 weeks of age and were sacrificed after 18-20 weeks so that their brains could be harvested. Timespans above the time-line correspond to the approximate age of animals, while timespans below correspond to the length of each behavioural experiment. Not all animals underwent all behavioural experiments (see Table 3.1 below). AFC = auditory fear conditioning; RR = rotarod; OF = open field; WM = water maze.

Table 3.1: Experimental design broken down by task, genotype and cohort

	Number of Animals					
	Cohort 1			Cohort 2		
	+/+	+/-	-/-	+/+	+/-	-/-
AFC	12	15	7	31	0	29
RR	12	12	5	3	3	2
OF	16	12	7	7	8	5
WM strong	18	13	8	17	4	9
WM weak	-	-	-	4	5	4
WM remote	18	13	8	3	4	2
Total [†]	22	20	10	31	9	29

[†] Note that the total number of animals does not reflect the genotype ratio of offspring born in each cohort, as heterozygotes were excluded from the fear conditioning experiments as the study progressed.
WM strong: 6 trials/day for 6 days with probe tests on days 1, 4 and 7
WM weak: 3 trials/day for 3 days with probe test on days 1 and 4
WM remote: probe test held on day 5 (6 weeks after completion of strong training protocol)
AFC = auditory fear conditioning; RR = rotarod; OF = open field; WM = water maze

function of conditioned freezing, the typical anticipatory response resulting from the expectation of an aversive event (Balleine & Killcross, 2006). Time spent freezing before the tone was presented (pre-CS freezing) compared to time spent freezing during the tone (CS freezing) was used as an index of long-term memory. The shock reactivity index was computed to assess sensitivity to the foot-shock during training by comparing the distance travelled in the 2 seconds prior to the onset of shock (pre-US) to the distance travelled in the 2 seconds during the shock (US). Reactivity index = (US – pre-US) / max (US + pre-US). As an additional control measure, I assessed post-shock freezing over 30 seconds following the foot-shock during training.

3.3.2 Rotarod

I assessed motor learning using a rotarod protocol previously shown to generate the typical logarithmic learning curve associated with learning a skill (Yin *et al.,* 2009). Mice were individually placed on a rotating beam in the rotarod apparatus that accelerated from 4 to 40 RPM over the course of each 5-minute trial. There were 10 trials per day, with 10-minute ITIs, for 8 straight days. In my lab, this protocol has previously demonstrated robust skill learning, typified by a rapid initial improvement followed by a plateau in performance, and is difficult enough to avoid a performance ceiling. Performance on each trial was measured by latency to fall from the rotarod. The rotarod was manually stopped if a mouse did not fall within the 5-minute duration of a trial, but this was uncommon.

3.3.3 Open Field

I used the OF test as a general measure of anxiety and exploratory behaviour in a novel environment (Chen *et al.*, 2006; Li *et al.*, 2010). Mice were placed in the center of a square chamber (45 x 45 x 19 cm high) in a dimly lit room with a fan to mask external noise. The chamber had white acrylic walls and a white acrylic floor that was lightly wiped with ethanol prior to testing. Mice were allowed to freely explore for 15 minutes while a camera connected to tracking software recorded their movement (Limelight 2.0). Anxiety was measured by comparing the amount of time spent in three concentric zones (15 cm across) of the chamber. I also measured overall locomotor activity during the trial.

3.3.4 Water Maze

I assessed spatial memory using the hidden platform version of the Morris water maze (Morris *et al.*, 1982). In this task, mice learn to navigate a pool of water by using visual cues in the environment in order to escape to an invisible platform below the pool's surface.

3.3.4.1 Apparatus

The circular water maze tank (120 cm diameter, 50 cm deep) was located in a dimly lit room and surrounded by white curtains displaying distinct visual cues (1 m from pool perimeter). The tank was filled with a pool of water 30 cm deep made opaque by nontoxic white acrylic paint. A circular escape platform (10-cm diameter) was submerged 0.5 cm below the water surface and kept in the same location throughout training. Water temperature was maintained at 28±1°C.

3.3.4.2 Training

There were two training protocols. The *strong protocol* consisted of 6 trials per day for 6 days, and the *weak protocol* consisted of 3 trials per day for 3 days. In each trial, the mouse was released from the north, south, east, or west end of the pool, varied pseudo-randomly across trials. The platform was always located in the northwest quadrant of the pool. Prior to each trial, the mouse was placed on the platform for 15 seconds. The trial was complete once the mouse reached the platform. If 60 seconds elapsed and the mouse was unable to locate the platform, the trial was stopped and the mouse was guided to the platform by the experimenter.

3.3.4.3 Probe Tests

During probe trials, the platform was removed from the water maze but visual cues around the room were left in place. Mice were not placed on the platform prior to probe trials, and instead were immediately released from the south end of the pool. For the strong protocol, individual probe trials were administered on day 1 (after 0 trials; *probe 1*), day 4 (after 18 trials; *probe 2*), day 7 (after 36 trials; *probe 3*), and day 50 (6 weeks after the last trial; *remote probe*). For the weak protocol, individual probe trials were administered on day 1 (after 0 trials; *probe 1*) and day 4 (after 9 trials; *probe 2*). Notably, probes 2 and 3 were administered 24 hours after the most recent training trial.

3.3.4.4 Analysis

The movement of mice in each trial was acquired with an overheard camera connected to an automated tracking system (Actimetrics, Wilmette, IL). For training trials, I analyzed latency to find the platform, swim speed, and thigmotaxis (time spent within 5 cm of the walls of the maze). Memory for the spatial location of the platform was assessed during probe trials, based on the amount of time that mice spent within a 15 cm radius of the platform location (target zone) located in the northwest quadrant of the pool. This was compared to the time that mice spent swimming in equally sized zones in the other three quadrants (Moser *et al.*, 1993; Moser & Moser, 1998).

3.4 Data Analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences 19.0 (IBM, Chicago, IL). An α-value of 0.05 was the criterion for statistical significance. For anatomical measures, paired-samples *t*-tests were used to make quantitative comparisons between the loxP control and homozygous mutant groups. For behavioural measures, overall tests were mixed model analyses of variance (ANOVAs) with either one or two within-subjects factors and *Cohort* and *Genotype* as the between-subjects factors unless specified otherwise. I followed up *Cohort* × *Genotype* interactions by conducting mixed model ANOVAs for each cohort using the within-subjects factors from the overall test and *Genotype* as the only between-subjects factor. Follow-up tests for significant within-subjects main effects were Tukey HSD tests collapsed across levels of the between-subjects factor. Significant mixed interactions were evaluated with repeated measures ANOVAs for each level of the between-subjects factor and univariate ANOVAs for each level of the within-subjects factor, followed by Tukey HSD *post-hoc* tests. The Greenhouse-Geisser correction for degrees of freedom and its corresponding *F*-statistic are reported for all comparisons that violated Mauchly's test of sphericity ($p < .05$). Violation of Levene's test of equality of variances was uncommon and parametric tests were used for all between-subjects comparisons.

Chapter 4
Results

4.1 Verification of the genotype

Polymerase chain reaction amplification of tail sample DNA from 5-7 week old mice confirmed that all of the mice were homozygous for the loxP sequence, represented by a 150 bp band on the electrophoretic gel. As expected, CREmKI$^{+/+}$ mice had a single 400 bp band corresponding to the wild-type CaRE3/CRE site, while CREmKI$^{+/-}$ mice had a 400 bp corresponding to the wild-type sequence and a 200 bp band corresponding to the mutant allele. CREmKI$^{-/-}$ mice had two 200 bp bands, showing that they did not contain the wild-type allele. Representative images of the gels are shown in Figure 4.1A.

Interestingly, while breeding these mice for several generations, I noticed that all of the F1, F2 and F3 offspring had an agouti coat colour characteristic of the 129s6 background. This suggested that the 129s6 parental strain made a larger genetic contribution to the background of our mice. Although the exact genetic contribution from the pure C57B/6 and 129s6 strains in the background of the breeders obtained by our lab was unknown, this observation was unexpected because the original mutations had been backcrossed many generations into the C57B/6 background (see Materials and Methods section 3.1.1). Furthermore, this observation implies that mice in cohort 2, despite carrying the same genetic mutations as mice in cohort 1, were likely to show a higher contribution of the 129s6 strain to their genetic background.

4.2 Variations in mouse weight were dependent on sex and genotype

A previous study demonstrated that CREmKI$^{-/-}$ mice were viable, fertile and indistinguishable from control littermates (Hong *et al.*, 2008). My study supported these findings, as CREmKI$^{+/-}$ and CREmKI$^{-/-}$ mutant mice survived to adulthood and did not show any overt physical or phenotypic abnormalities compared to wild-type littermates. Since other studies have reported that BDNF$^{+/-}$ mice are obese (Boger *et al.*, 2011; Kernie *et al.*, 2000), I compared the weights of all mice at 3 months of age (Fig. 4.1C). I decided to compare the weights of mice at this set time-point since I did not observe any notable fluctuations in weight during experiments.

The effects of the CREmKI mutation were sex-dependent, as revealed by a 3-way factorial ANOVA with between-subjects factors of *Cohort* (cohort 1, cohort 2)*, Genotype* (CREmKI$^{+/+}$, CREmKI$^{+/-}$, CREmKI$^{-/-}$) and *Sex* (male, female). Overall, male mice were larger than female mice (main effect of *Sex*, $F_{(1,83)} = 143.024$, $p < .001$) and CREmKI$^{+/-}$ mice were larger than their wild-type and homozygous littermates (main effect of *Genotype,* $F_{(2,83)} = 5.819$, $p = .004$). The effect of *Cohort* was not significant, $F_{(1,83)} = 0.894$, $p = .347$. There was also a significant *Genotype* × *Sex* interaction, $F_{(2,83)} = 3.211$, $p = .045$ (ps > .094 for other interactions). Tukey's HSD *post-hoc* tests revealed that among males, loxP control mice were smaller than heterozygous mice ($p = .021$), and among females, homozygous mice were smaller than loxP control mice ($p = .004$) and heterozygous littermates ($p = .001$).

4.3 Brain anatomy of CREmKI$^{-/-}$ mice did not differ from control littermates

Hong *et al.* (2008) previously observed that the brains of CREmKI$^{-/-}$ mice were not noticeably different from brains of wild-type mice. To probe several brain anatomical measures related to my investigation, I perfused the brains of loxP control and homozygous mutant mice from cohort 1 (ns = 3) following the completion of behavioural experiments. Using independent samples *t*-tests, I found no significant differences between genotypes in average hippocampal volume, $t_{(4)} = 0.097$, $p = .928$, average LA volume, $t_{(4)} = 1.461$, $p = .218$, average cortical thickness, $t_{(4)} = 1.220$, $p = .290$, or brain mass, $t_{(4)} = 0.010$, $p = .992$. These results supported previous findings that the neural structure of the CREmKI$^{-/-}$ mouse brain was not altered on a gross anatomical level (Fig. 4.1B,D-F).

4.4 CREmKI$^{-/-}$ mice in cohort 1, but not cohort 2, have a deficit in auditory fear memory

Lateral amygdala levels of both BDNF (Chhatwal *et al.,* 2006; Ou *et al.,* 2010; Rattiner *et al.,* 2004) and the transcription factor CREB (Bourtchuladze *et al.,* 1994; Han *et al.,* 2007, 2009) are necessary for the consolidation of cued fear memories, raising the possibility that the CREB-mediated activity-dependent component of *Bdnf* expression in the LA is important for memory.

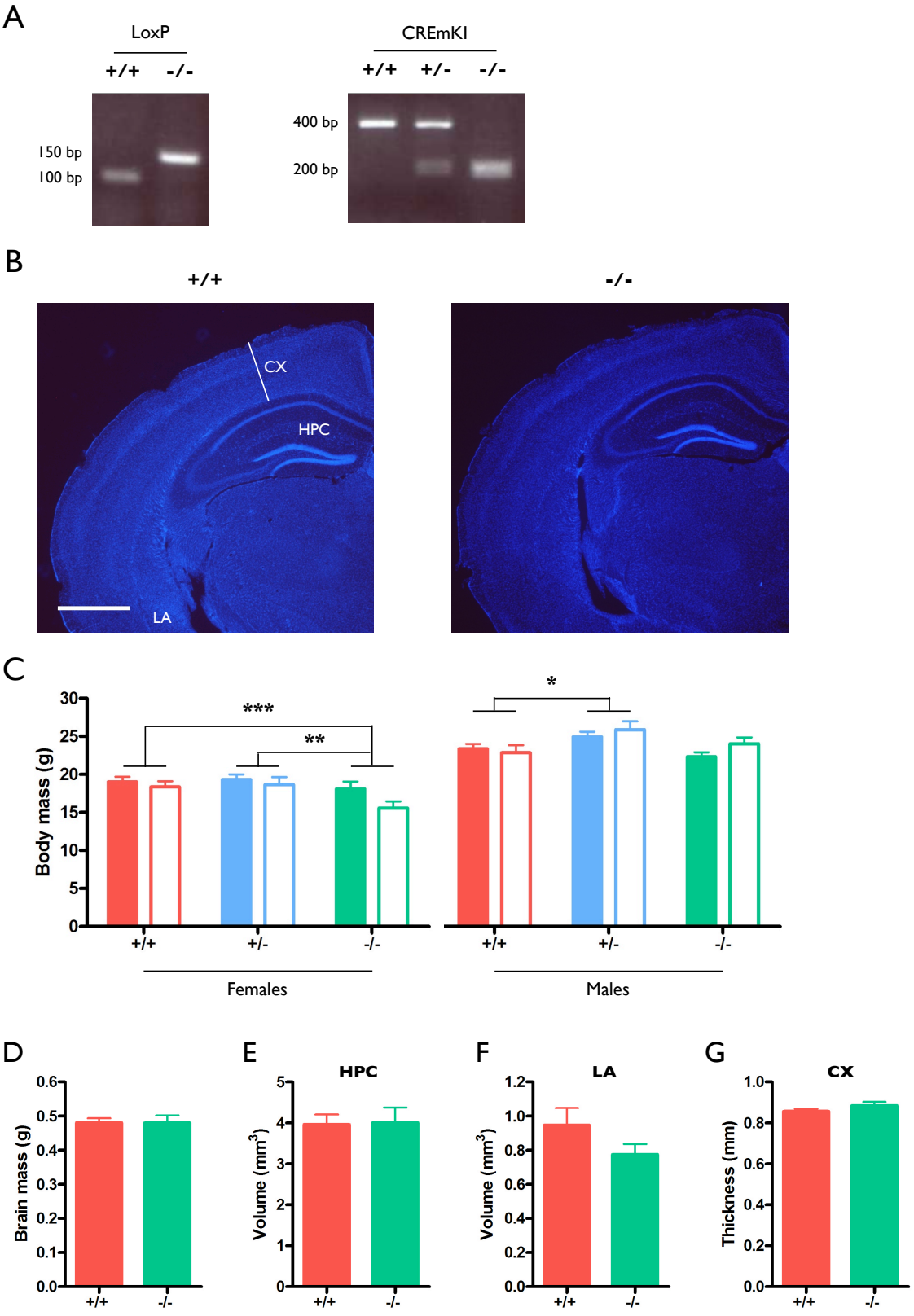
A
LoxP
+/+ -/-
150 bp
100 bp
CREmKI
+/+ +/- -/-
400 bp
200 bp
B
+/+
-/-
CX
HPC
LA
C
Body mass (g)
***
**
*
+/+ +/- -/-
Females
Males
D
Brain mass (g)
E
HPC
Volume (mm3)
F
LA
Volume (mm3)
G
CX
Thickness (mm)

Figure 4.1: Genotypic and phenotypic variations in control and mutant mice
(A) Representative gel electrophoresis blots of LoxP and CREmKI genotypes resulting from PCR in experimental mice. The blots show genomic DNA digested with the PvuII restriction enzyme. LoxP: wild-type, 200 bp; mutant, 150 bp; CREmKI: wild-type, 400 bp; mutant, 200 bp.
(B) Representative fluorescent DAPI images contained right dorsal hippocampus and cortex of mice from cohort 1.
(C) Body weight of mice at 3 months of age. Filled bars represent cohort 1 and white bars represent cohort 2.
(D-G) Gross brain anatomical measurements of mice from cohort 1. From left to right: mass of whole brain, volume of hippocampus (HPC), volume of lateral amygdala (LA), thickness of cortex (CX).
*$p < .05$, **$p < .01$, ***$p < .001$.

I investigated this possibility using an auditory fear memory protocol in which training and testing trials took place in distinct contexts 24 hours apart. Memory for the tone-shock association was assessed by comparing conditioned freezing in the presence and absence of the tone during the test trial. CREmKI$^{-/-}$ mice in the F1 generation (cohort 1) showed a lower level of freezing in response to the tone than CREmKI$^{+/+}$ littermates, with CREmKI$^{+/-}$ mice demonstrating an intermediate level of freezing. However, both groups from the F2 generation (cohort 2) showed similar freezing to the tone (Fig. 4.2A,B).

4.4.1 Overall findings

Thirty-four mice from cohort 1 and 60 mice from cohort 2 underwent fear conditioning at the start of behavioural testing. CREmKI$^{-/-}$ mice showed a modest (~25%) reduction in overall freezing that did not appear to be dependent on presentation of the tone, and thus may not have been representative of a memory deficit *per se*. A mixed model ANOVA with the within-subjects factor of *Time* (pre-CS, CS) and between-subjects factors of *Cohort* and *Genotype* revealed that, overall, mice showed increased freezing during the CS (main effect of *Time*, $F_{(1,89)} = 256.491$, $p < .001$), and that CREmKI mutants froze less than loxP control littermates (main effect of *Genotype*, $F_{(2,89)} = 4.639$, $p = .012$). There was a significant *Time* × *Cohort* interaction, $F_{(1,89)} = 4.656$, $p = .034$, indicating that levels of freezing before and during the tone differed between cohorts. There were no other significant main effects or 2- or 3-way interactions (ps > .100).

I also conducted 2-way ANOVAs to assess sensitivity to the shock (reactivity index) and post-shock freezing (Fig. 4.2C,D). Only 23 mice from cohort 2 were included in the analysis of shock sensitivity due to a software failure that prevented me from retrieving the data

corresponding to the remaining mice in this cohort. There was no significant effect of *Cohort,* $F_{(1,52)} < 0.001$, $p = 1.000$, *Genotype*, $F_{(2,52)} = 0.249$, $p = .781$, or *Cohort* × *Genotype* interaction, $F_{(1,52)} = 3.081$, $p = .085$, on shock sensitivity. There was a significant effect of *Cohort* on post-shock freezing, $F_{(1,79)} = 16.672$, $p < .001$, but no effect of *Genotype*, $F_{(2,79)} = 0.209$, $p = .812$, or *Cohort* × *Genotype* interaction, $F_{(1,79)} = 0.004$, $p = .949$, suggesting that mice in cohort 1 (M = 35.751%, SE = 2.524%) froze more than mice in cohort 2 (M = 24.112%, SE = 1.979%) irrespective of genotype.

4.4.2 Cohort 1

To further probe the results of the overall test, I carried out separate follow-up analyses for each cohort. In cohort 1, CREmKI$^{-/-}$ mice showed a pronounced (~50%) reduction in freezing compared to loxP control littermates during presentation of the tone 24 hours after training (Fig. 4.2B), as revealed by a mixed model ANOVA with the within-subjects factor of *Time* and between-subjects factor of *Genotype*. There were significant effects of *Time*, $F_{(1,31)} = 70.893$, $p < .001$, and *Genotype*, $F_{(2,31)} = 3.745$, $p = .035$, but no significant *Genotype* × *Time* interaction, $F_{(2,31)} = 1.512$, $p = .236$. Although the failure to detect an interaction suggested that CREmKI$^{-/-}$ mutants froze less during both pre-CS and CS periods of the test, I carried out follow-up univariate ANOVAs to see if the difference between genotypes was driven by freezing during the CS. These analyses revealed a significant effect of *Genotype* on freezing during the 1-min CS presentation, $F_{(2,31)} = 4.237$, $p = .024$, but not during the 2-min pre-CS period, $F_{(2,31)} = 1.778$, $p = .186$. During the CS, loxP control mice spent more time freezing than CREmKI$^{-/-}$ mice (p = .019). CREmKI$^{+/-}$ mice showed an intermediate level of freezing that did not differ significantly from either group (ps > .238).

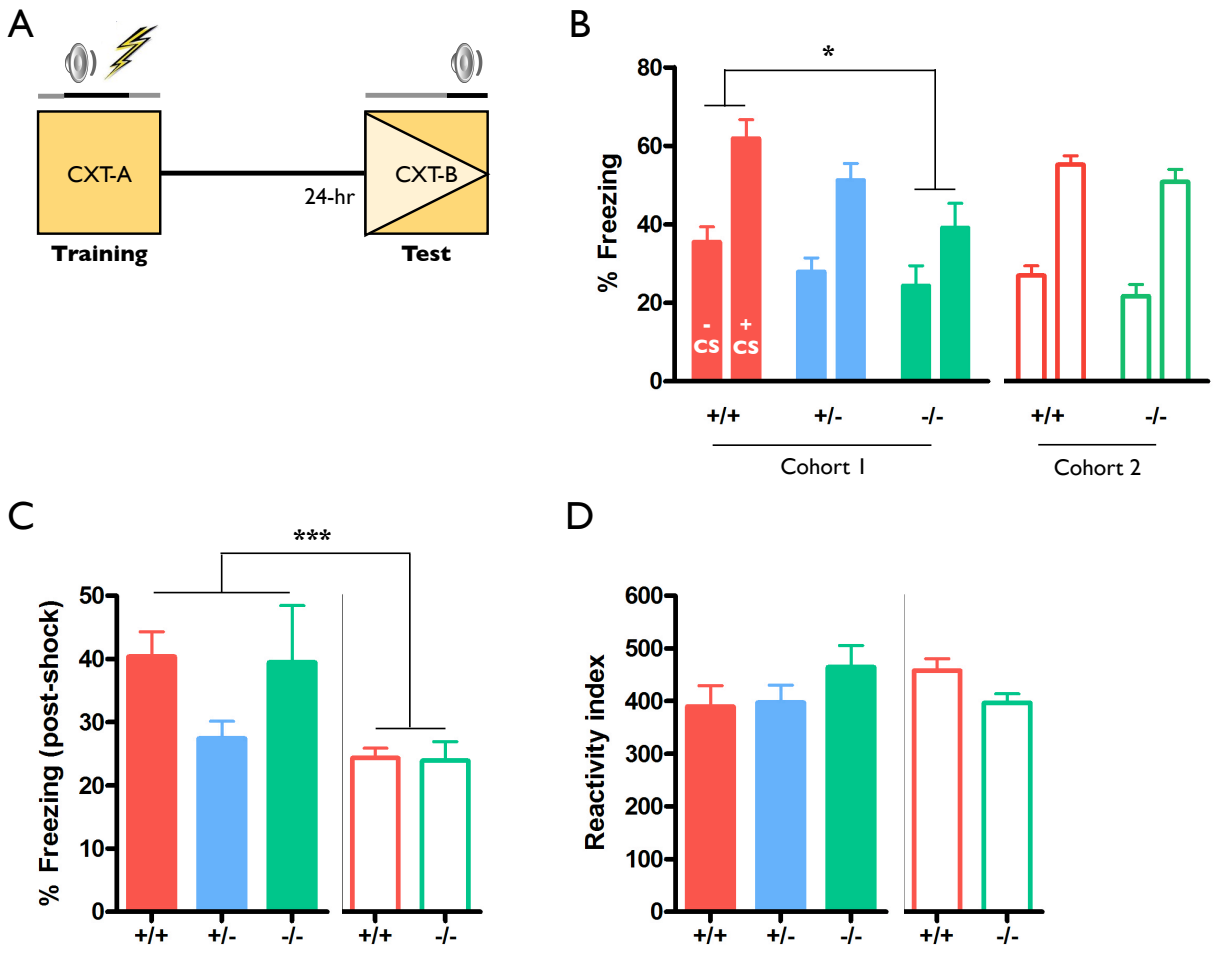

Figure 4.2: CREmKI$^{-/-}$ mice in the first cohort had a deficit in auditory fear memory

(A) During auditory fear conditioning a tone was paired with a foot-shock in a novel context (CXT-A). The memory test took place in a distinct context (CXT-B) 24 hours later and conditioned freezing was measured when the familiar tone was presented without the shock.

(B) Comparison of freezing behaviour during the memory test between genotype groups and cohorts. For each genotype, freezing is compared before the tone was presented (first bar, CS-) to freezing during the tone (second bar, CS+).

(C) Freezing assessed during the 30-s period after the shock was presented in CXT-A.

(D) Reactivity of the mice to the 2-s 0.5mA foot-shock.

*$p < .05$, ***$p < .001$.

4.4.3 Cohort 2

My ability to draw conclusions about the F1 generation of mice was limited by the low number of homozygous mutant offspring ($n = 7$) in this cohort. I repeated my experiments in F2 mice produced by wild-type × wild-type, heterozygous × heterozygous, and homozygous × homozygous crosses of the F1 parents (cohort 2[5]). I compared only loxP control and homozygous mutant mice from this cohort in the fear conditioning task, but some of the heterozygous mice underwent water maze training.

Comparably to control mice in cohort 1, homozygous and control mice in cohort 2 froze approximately 53% of the time during presentation of the CS, indicating a strong fear response to the tone (Fig. 4.2B). The mixed model ANOVA with the within-subjects factor of *Time* and between-subjects factor of *Genotype* showed a significant effect of *Time*, $F_{(1,58)} = 257.763$, $p < .001$, but no effect of *Genotype*, $F_{(1,58)} = 1.910$, $p = .172$, or *Genotype* × *Time* interaction, $F_{(1,58)} = 0.060$, $p = .807$. The results reflected increased freezing in both groups in response to the CS compared to freezing in the context without the CS.

4.5 CREmKI mutant mice exhibit typical motor learning on the rotarod

Motor learning in the rotarod task is affected by reductions in expression of CREB (Oliveira *et al.*, 2006) and BDNF (Boger *et al.*, 2011). Decreased CREB-mediated gene expression in cerebellar purkinje cells was also shown to impair the ability to stay on the rotarod (Brodie *et al.*, 2004), suggesting that baseline performance and learning of a motor skill could be impaired in CREmKI mutant mice. The rotarod task allowed me to investigate both of these possibilities.

LoxP control ($n = 15$), heterozygous ($n = 16$), and homozygous mutant mice ($n = 7$) had comparable overall performance on the rotarod and demonstrated a characteristic improvement across trials within a day and across days (Fig. 4.3A,B). A mixed model ANOVA with within-

[5] Although initially I only carried out the heterozygous × heterozygous cross of F1 parents to keep with the initial breeding strategy, there is no reason to suspect a systematic difference in the genotypes of wild-type and mutant offspring from the different F1 crosses. Therefore, I grouped all of the F2 mice into cohort 2 instead of carrying out separate analyses based on the parental background.

subjects factors of *Trial* (trial 1-10) and *Day* (day 1-8) and the between-subjects factors of *Cohort* and *Genotype* supported these observations, as only the main effects of *Trial*, $F_{(9,549)} = 12.908$, $p < .001$, and *Day*, $F_{(4,549)} = 16.620$, $p < .001$, were significant ($ps > .112$ for all other main effects and 2-, 3- and 4-way interactions).

4.6 The CREmKI mutation was associated with higher levels of anxiety in the open field

Bdnf$^{+/-}$ and *Bdnf*$^{met/met}$ mice, which have reduced BDNF expression in the brain, show increased anxiety-related behaviour in several tasks (Chen *et al.*, 2006; Li *et al.*, 2010). Decreased levels of BDNF are also implicated in anxiety-related disorders, including depression (Chen *et al.*, 2006; Duman *et al.*, 1997; Nibuya *et al.*, 1995, 1996) and PTSD (Berger *et al.,* 2010; Dell'osso *et al.,* 2009; Mahan & Ressler, 2011). Although the role of CREB in anxiety is less well-characterized, it has been proposed that the chronic administration of anti-depressants causes a sustained increase in CREB throughout the hippocampus (Nibuya *et al*., 1996), which results in increased expression of BDNF (Duman *et al.*, 1997; Nibuya *et al.*, 1995). I administered the OF test, in which mice explore a novel environment and anxiety is measured by comparing the amount of time spent in the central zones of the chamber compared to time spent in a peripheral zone (Fig 4.4A).

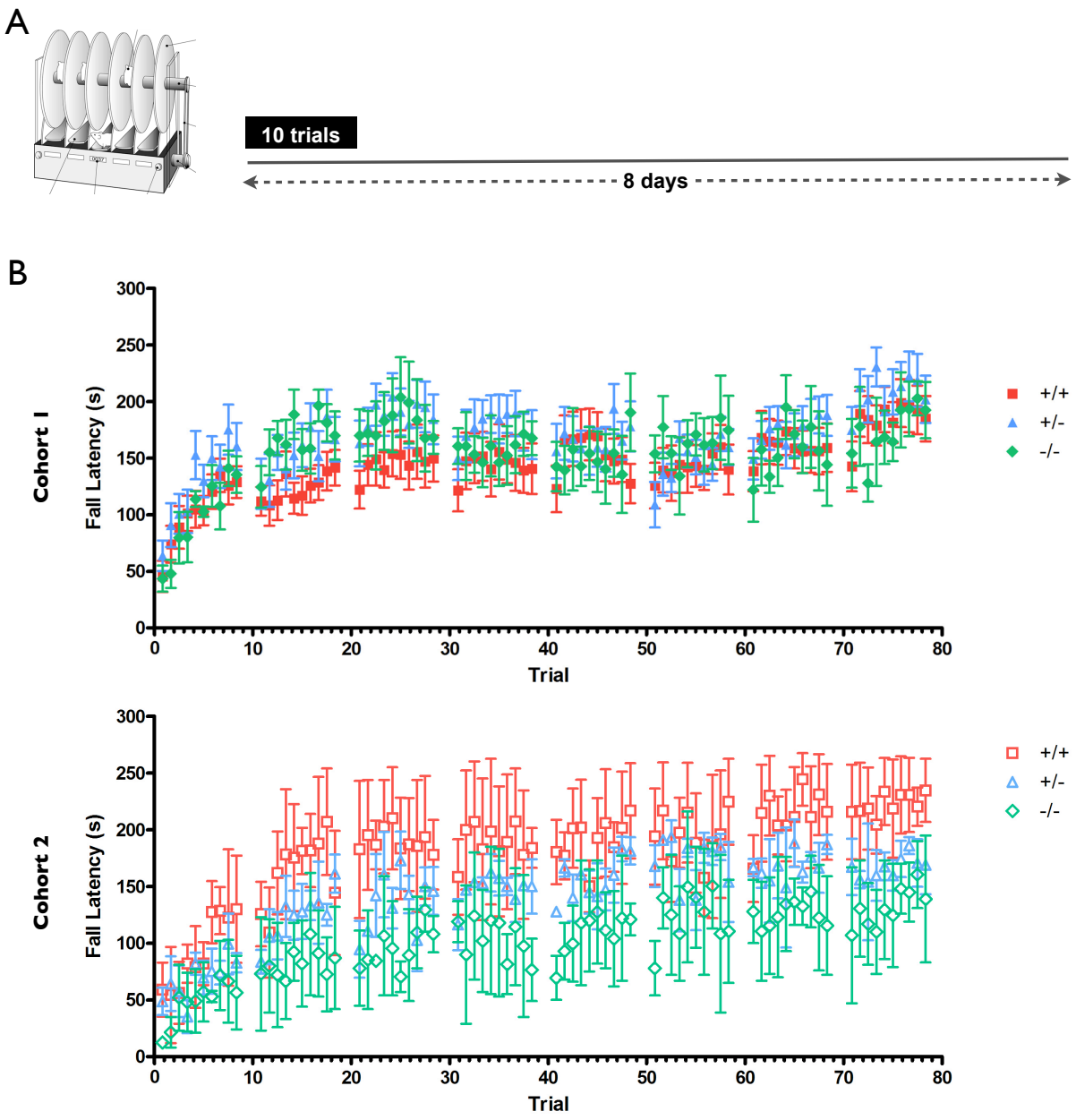

Figure 4.3: CREmKI mutant mice exhibit typical motor learning

(A) Diagram of the rotarod. The circular beam accelerated from 4-40 RPM over the course of each 5-min trial. Animals underwent 10 trials per day for 8 days (10-min inter-trial interval).

(B) Average latency to fall from the rotarod across trials within a day and across days.

4.6.1 Overall findings

I tested 36 mice from cohort 1 and 22 mice from cohort 2 in the OF. Mice in both cohorts displayed anxiety in the novel environment and spent most of their time near the walls of the chamber, as revealed by the mixed model ANOVA with the within-subjects factor of *Zone* (outer, middle, inner) and between-subjects factors of *Cohort* and *Genotype*. Overall, mice spent the most time in the outermost zone and the least time in the central zone of the open field (main effect of *Zone*, $F_{(1,53)} = 1830.001$, $p < .001$). Critically, the *Zone* × *Cohort* × *Genotype* interaction approached significance, $F_{(2,53)} = 2.711$, $p = .074$, suggesting that the *Cohort* variable modulated the amount of time that groups of mice spent in each zone (Fig. 4.4B,D). There were no other significant main effects or 2-, 3- or 4-way interactions (ps > .151).

All of the mice displayed similar levels of locomotor activity during the 15-minute session (Fig. 4.4C,E). The 2-way factorial ANOVA with factors of *Cohort* and *Genotype* showed no significant effects of *Cohort,* $F_{(1,49)} = 0.343$, $p = .561$, *Genotype*, $F_{(2,49)} = 1.945$, $p = .154$, or *Cohort* × *Genotype* interaction, $F_{(2,49)} = 0.340$, $p = .713$.

4.6.2 Cohort 1

I followed up the *Zone* × *Cohort* × *Genotype* interaction from the overall analysis by investigating differences across groups within each cohort. In cohort 1, all three groups of mice spent approximately 85% of the trial in the outermost zone and most of the remaining time in the middle zone (Fig. 4.4B). Supporting these observations, the mixed model ANOVA with the within-subjects factor of *Zone* and between-subjects factor of *Genotype* showed a significant effect of *Zone*, $F_{(1,32)} = 1127.223$, $p < .001$, but no effect of *Genotype*, $F_{(2,32)} = 1.330$, $p = .279$, or *Genotype* × *Zone* interaction, $F_{(2,32)} = 1.833$, $p = .176$.

4.6.3 Cohort 2

While the heterozygous and homozygous mutant mice in cohort 2 spent approximately 86% of the trial in the outermost zone, their loxP control littermates spent only 78% of the trial in this zone, spending more time in the middle and innermost zones than the mutant groups (Fig. 4.4D). The mixed model ANOVA showed that mice spent the most time in the outermost zone and the least time in the central zone of the open field (main effect of *Zone*, $F_{(1,18)} = 971.597$, $p < .001$),

but that the amount of time spent in each zone was dependent on genotype (*Genotype* × *Zone* interaction, $F_{(2,18)} = 3.854$, $p = .040$). The effect of *Genotype* was not significant, $F_{(2,17)} = 0.040$, $p = .961$. Tukey's *post-hoc* tests revealed that the loxP control mice spent less time than the mutant mice in the outermost zone (loxP vs. heterozygous comparison: $p = .052$; loxP vs. homozygous comparison: $p = .117$; heterozygous vs. homozygous comparison: $p = .989$) and more time in the middle zone (loxP vs. heterozygous comparison: $p = .040$; loxP vs. homozygous comparison: $p = .105$; heterozygous vs. homozygous comparison: $p = .977$).

4.7 The CREmKI mutation was associated with increased anxiety, but not spatial memory deficits, in the Morris water maze

The expression of BDNF is necessary for hippocampal LTP (Balkowiec & Katz, 2000, 2002; Korte *et al.*, 1995, 1996) and has been shown to directly modulate spatial memory in a variety of experimental tasks, including the Morris water maze (Kesslak *et al.,* 1998), contextual fear conditioning (Hall *et al.*, 2000; Liu *et al.*, 2004), the radial arm maze (Mizuno *et al.*, 2000), and the social recognition task (Suzuki *et al.*, 2011). CREB has also been extensively implicated in L-LTP (Barco *et al.*, 2002, 2005; Impey *et al.*, 1996) and learning and memory tasks dependent on the hippocampus (Bourtchuladze *et al.*, 1994; Gass *et al.*, 1998). However, this was the first time that the effect of CREB-mediated BDNF expression on spatial memory has been tested directly.

4.7.1 Strong protocol

Initially, I used a strong training protocol in which mice underwent 6 training trials per day for 6 days. Recent spatial memory was evaluated with probe tests on days 4 and 7 (probes 2 and 3), 24 hours after training. Remote memory was evaluated with a probe test on day 50, 6 weeks after the completion of training (Fig. 4.5A).

4.7.1.1 Training

Thirty-nine mice from cohort 1 and 30 mice from cohort 2 underwent the strong training protocol. Mice from all three groups demonstrated similar changes in performance over 6 days

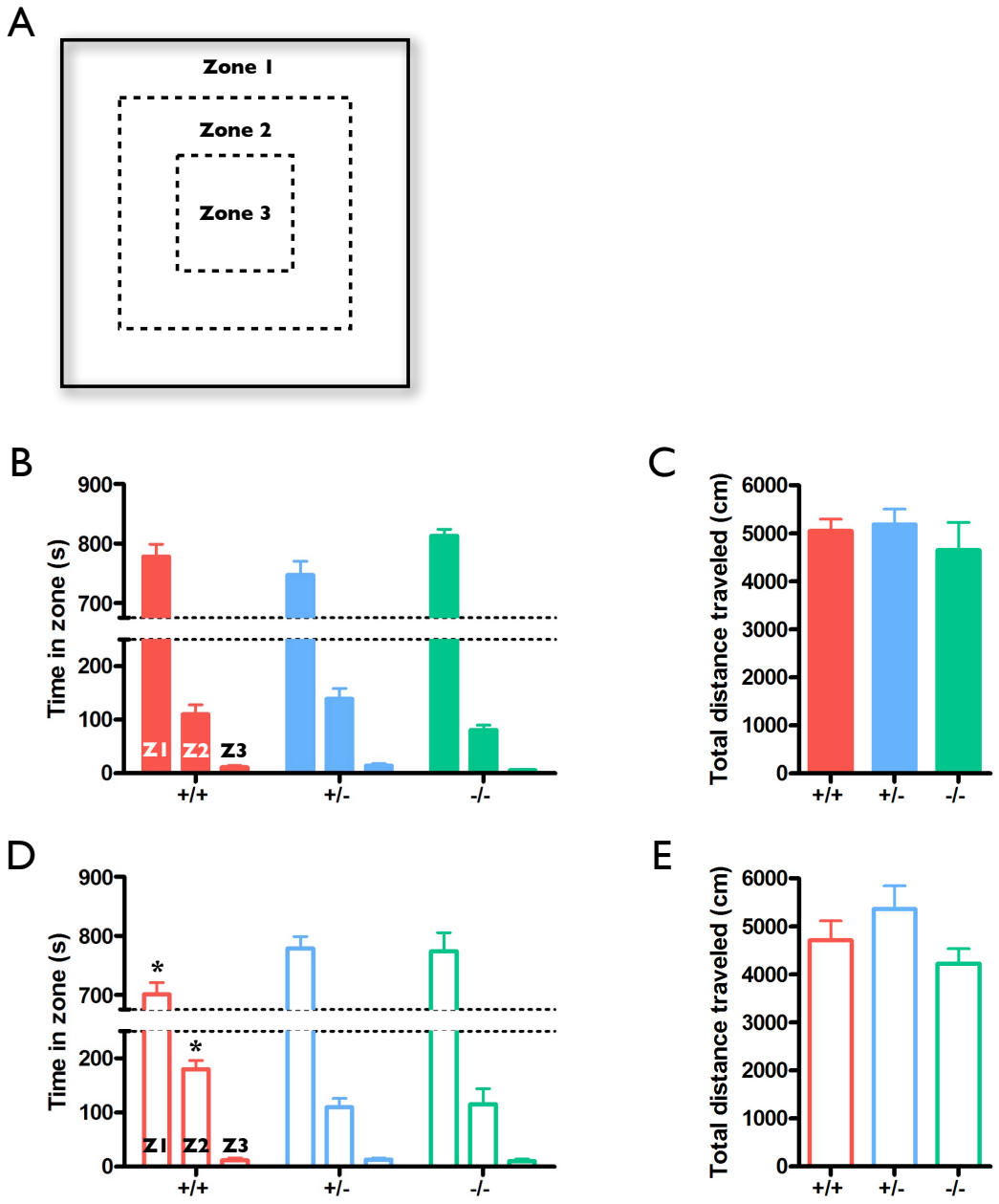

Figure 4.4: CREmKI mutant mice in cohort 2 showed an increase in anxiety-related behaviour

(A) Diagram of the open field chamber and its three virtual zones.

(B, C) Between-genotypes comparisons of time spent in each of three concentric zones and total locomotor activity during the 15-min trial in cohort 1. Zone 1 (first bar; Z1) refers to the outermost zone of the open field, zone 2 (middle bar; Z2) refers to the middle zone, and zone 3 (third bar; Z3) refers to the central zone.

(D, E) Between-genotypes comparisons in cohort 2.

*Significant effect of *Genotype*, $p < .05$.

of training (Figs. 4.5B,E), as revealed by the mixed model ANOVA assessing trial duration, with the within-subjects factor of *Day* (day 1-6) and between-subjects factors of *Cohort* and *Genotype.* All mice gradually took less time to find the hidden platform and improved at a similar rate (main effect of *Day,* $F_{(3,201)} = 273.740$, $p < .001$, but no other main effects, *ps* > .317). There were also significant *Day* × *Cohort,* $F_{(3, 201)} = 4.193$, $p = .006$, and *Day* × *Genotype* interactions, $F_{(6, 201)} = 2.630$, $p = .016$. No other 2- or 3-way interactions were significant (*ps* > .166). Probing the significant interactions further revealed that mice in cohort 2 took less time to reach the platform on day 1 of training, $F_{(1,63)} = 4.995$, $p = .029$. Tukey's HSD *post-hoc* tests revealed that on day 2, homozygous mutant mice had reduced latency to find the platform compared to loxP control ($p = .030$) and heterozygous mice ($p = .083$; control vs. heterozygous comparison: $p = .675$). I found a non-significant trend for the opposite effect on day 6 of training, where the loxP control group took less time to reach the platform than the homozygous mutant group ($p = .077$; control vs. heterozygous comparison: $p = .982$; heterozygous vs. homozygous comparison: $p = .192$). Therefore, CREmKI$^{-/-}$ mice appeared to find the platform faster at the beginning of training, but the effect was reversed on day 6.

Mice in all three groups also exhibited a decrease in swim speed and time spent near the walls of the pool (thigmotaxis) over the course of training (Figs. 4.5C,D,F,G), as revealed by the mixed model ANOVA with the within-subjects factor of *Day* and between-subjects factors of *Cohort* and *Genotype*. Overall, there were significant effects of *Day* on swim speed, $F_{(4,255)} = 16.941$, $p < .001$, and thigmotaxis, $F_{(3,177)} = 162.449$, $p < .001$. However, both measures were modulated by *Cohort* and *Genotype*. Mice in cohort 2 had a higher average swim speed than mice in cohort 1 (main effect of *Cohort*, $F_{(1,63)} = 10.879$, $p = .002$), and there was a significant *Cohort* × *Genotype* interaction, $F_{(2,63)} = 4.376$, $p = .017$, for swim speed (*ps* > .080 for other main effects and 2- and 3-way interactions). In the analysis of thigmotaxis, there were significant *Day* × *Genotype*, $F_{(6,177)} = 3.553$, $p = .003$, and *Day* × *Cohort* × *Genotype* interactions, $F_{(6,177)} = 2.367$, $p = .035$. The effect of *Genotype*, $F_{(2,63)} = 2.732$, $p = .073$, and all other interactions approached significance (*ps* > .055) and only the effect of *Cohort* was definitively non-significant, $F_{(2,63)} = 1.236$, $p = .270$. Since these observations were difficult to interpret, I conducted additional analyses for each cohort separately.

In cohort 1, all of the mice demonstrated similar reductions in swim speed (main effect of *Day*, $F_{(5,180)} = 17.700$, $p < .001$) and thigmotaxis (main effect of *Day*, $F_{(3,100)} = 91.972$, $p < .001$)

over the course of training (Fig. 4.5C,D). All effects of *Genotype* and interactions were non-significant (*p*s > .082).

Although mice in cohort 2 showed higher overall swim speed than mice in cohort 1, they demonstrated a similar pattern of reduced swim speed over days (main effect of *Day*, $F_{(3,94)}$ = 4.548, p = .003; Figure 4.5F). All effects of *Genotype* and interactions were non-significant (*p*s > .115). Unlike cohort 1, the reduction in thigmotaxis during training in cohort 2 depended on genotype (Figure 4.5G). The mixed model ANOVA assessing thigmotaxis revealed a significant effect of *Day*, $F_{(3,68)}$ = 75.020, p < .001, and *Day* × *Genotype* interaction, $F_{(5,68)}$ = 3.782, p = .004, but no effect of *Genotype*, $F_{(2,27)}$ = 2.513, p = .100. To probe the interaction further, I conducted univariate ANOVAs to compare the groups on individual days. Tukey's HSD *post-hoc* tests showed that heterozygous mutant mice exhibited more thigmotactic behaviour on day 1 than the loxP control (p = .021) or homozygous mutant mice (p = .018; control vs. homozygous comparison: p = .917).

4.7.1.2 Probes

During probe tests, the platform was removed and spatial memory was assessed based on the amount of time that mice spent swimming in the region of the pool where the platform had been located during training (*target zone*, r = 20 cm) compared to three other equally sized zones (*left, opposite, and right zones*). Each probe test was analyzed with a separate mixed model ANOVA with the within-subjects factor of *Zone* and between-subjects factors of *Cohort* and *Genotype*. As expected, mice did not show a zone preference during probe 1 and spent little time (~4%) in the target zone corresponding to the platform (Fig. 4.6A,B). The mixed model ANOVA revealed that there were no significant main effects (*p*s > .091). There was a significant *Zone* × *Genotype* interaction, $F_{(5,157)}$ = 2.611, p = .027 (*p*s > .144 for all other 2- and 3-way interactions). This reflected increased time spent in the left and target zones by the homozygous mutant group (analyses not shown).

Mice in all groups showed a comparable bias for the target zone during the recent and remote probe tests (Fig. 4.6C-H). The mixed model ANOVAs revealed significant effects of *Zone* in all three tests (probe 2: $F_{(2,127)}$ = 79.556, p < .001; probe 3: $F_{(2,117)}$ = 90.154, p < .001; remote probe: $F_{(2,80)}$ = 21.618, p < .001), indicating a robust preference for the target zone. There was also a significant *Cohort* × *Genotype* interaction during probe 3, $F_{(2,63)}$ = 3.398, p = .040.

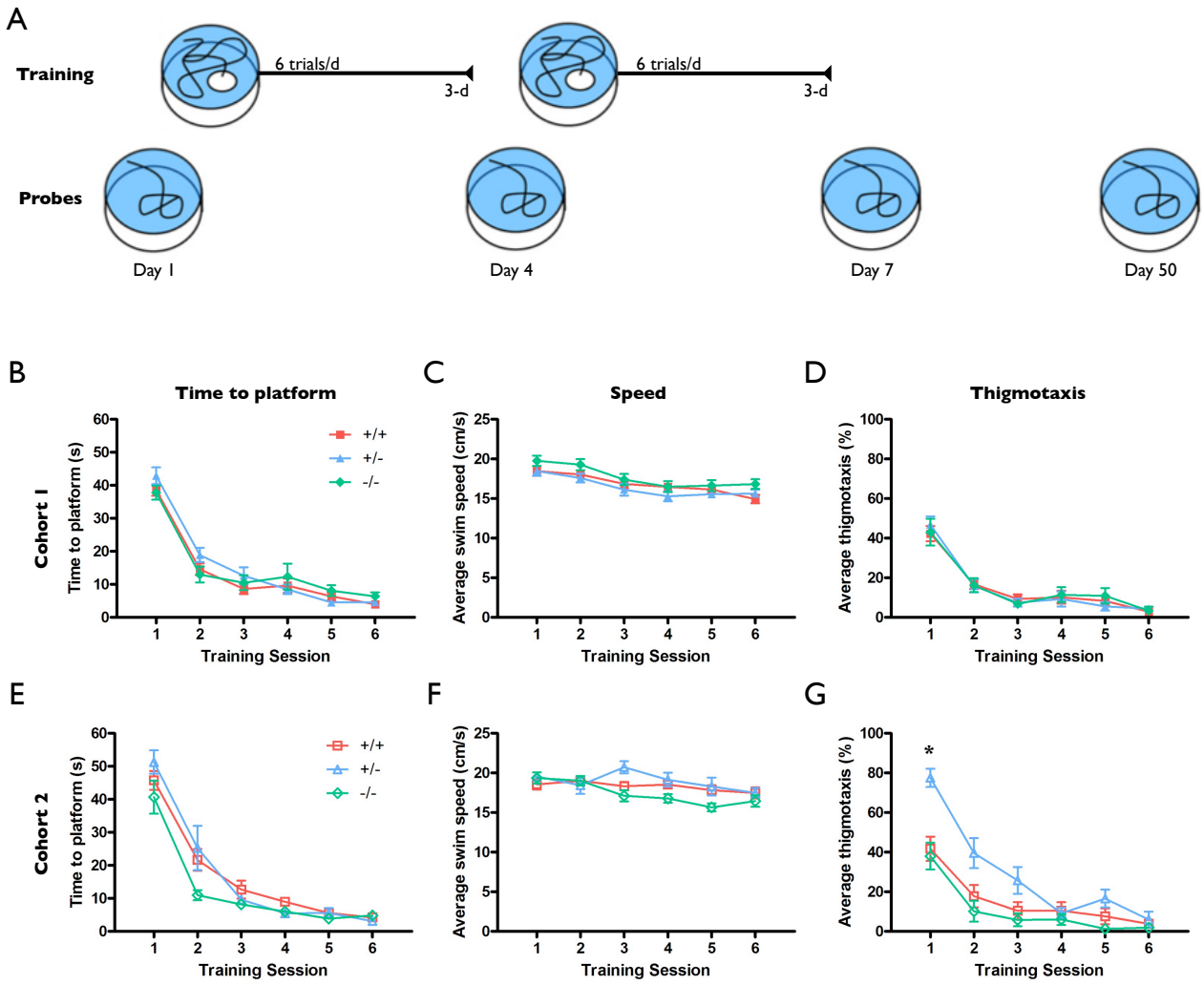

Figure 4.5. Water maze training – strong protocol

(A) Diagram of the strong water maze protocol.

(B-D) Changes in latency to reach the platform, average swim speed, and average thigmotaxis (time spent within 5-cm of the outer walls of the water maze) over the course of training in cohort 1.

(C-G) Changes in latency to reach the platform, average swim speed, and average thigmotaxis over the course of training in cohort 2.

*Significant effect of *Genotype*, $p < .05$.

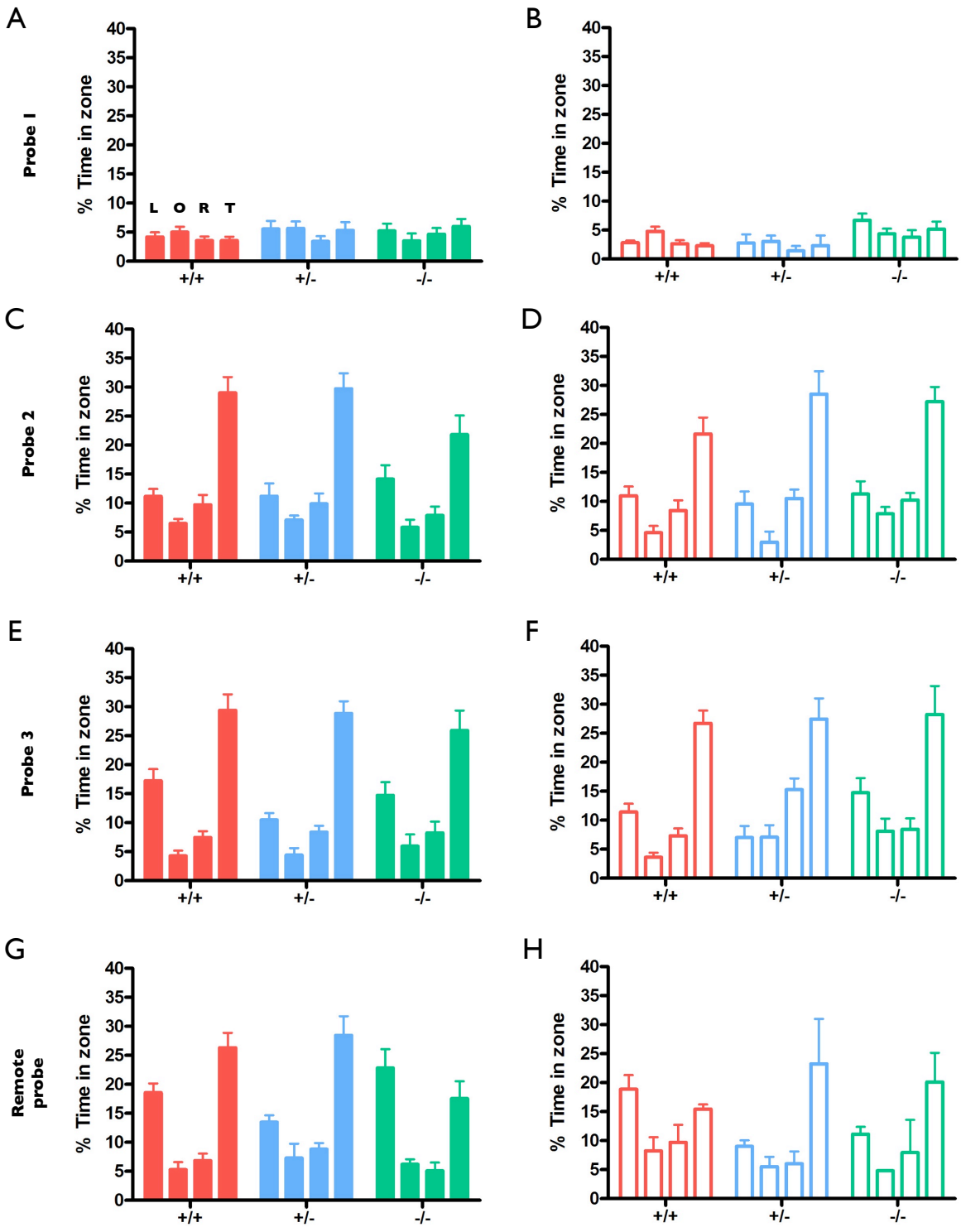

Figure 4.6: CREmKI mutant mice did not have recent or remote spatial memory impairments
(A, C, E, G) Between-genotypes comparisons in cohort 1 of time spent in the target zone (T; *r* =20 cm) corresponding to the location of the platform during training and time spent in three other equally sized zones (left, L; opposite, O; right, R).
(B, D, F, H) Between-genotypes comparisons in cohort 2 of time spent in the target zone compared to the left, opposite, and right zones.

The interaction appeared to be driven by an increased preference for the target zone in CREmKI$^{+/+}$ mice in cohort 1 compared to this group of mice in cohort 2, $F_{(1,33)} = 4.943$, $p = .033$. Furthermore, mice in cohort 1 spent more time in the virtual zones than mice in cohort 2 during the remote probe (main effect of *Cohort*, $F_{(1,42)} = 9.344$, $p = .004$). There were no other significant main effects or 2- or 3-way interactions (ps > .078). Therefore, mice in cohort 1 appeared to develop a more robust preference for the target zone than mice in cohort 2.

4.7.2 Weak protocol

While my findings suggested that CREmKI mutant mice did not perform differently from loxP control littermate mice in the water maze, I could not rule out the possibility that the strong protocol was not sensitive enough to detect performance deficits in these mice. To investigate this possibility, I administered a weak protocol that involved only 3 days of training with 3 trials per day. Probe tests were administered before training on day 1 (probe 1) and on day 4, 24 hours after the last training trial (probe 2). I suspected that if mutant mice had a deficit in the water maze it would be apparent in probe 2 so long as the training was sufficient for the control group to learn (Fig. 4.7A).

4.7.2.1 Training

A total of 13 mice from cohort 2 underwent the weak protocol. The level of performance reached by the final day of weak training was considerably worse than following strong training, as the average latency to reach the platform on day 3 was approximately 15 s (compared to ~5 s after strong training). The mixed model ANOVA with the within-subjects factor of *Day* and between-subjects factor of *Genotype* revealed that, overall, mice showed a reduction in latency to reach the platform over the course of training (main effect of *Day*, $F_{(1,13)} = 8.793$, $p = .007$; Fig. 4.7B). Although the main effect of *Genotype* was not significant, $F_{(2,10)} = 0.862$, $p = .452$, latency to reach the platform over days was modulated by genotype (*Day* × *Genotype* interaction, $F_{(3,13)} = 3.563$, $p = .048$). Tukey's HSD tests revealed that on day 1 the homozygous mutant mice took less time to reach the platform than heterozygous mice ($p = .027$), but not loxP control mice ($p = .416$; control vs. heterozygous comparison: $p = .246$). Therefore, CREmKI$^{-/-}$ mice appeared to perform better than their littermates at the outset of training, but all mice reached a similar level of performance before the second probe test.

Separate mixed model ANOVAs were used to assess the secondary measures of swim speed and thigmotaxis over the course of training (Fig. 4.7C,D). Contrasting the decrease in swim speed over days during strong training, there was only a trend towards decreased swim speed over the course of weak training (main effect of *Day*, $F_{(2,20)} = 2.861$, $p = .082$; ps > .517 for other main effect and interaction). Finally, my analysis of thigmotaxis revealed that all animals spent less time near the walls of the water maze over the course of training (main effect of *Day*, $F_{(2,20)} = 5.405$, $p = .013$; ps > .374 for other main effect and interaction).

4.7.2.2 Probes

Consistent with the performance of mice after strong training, my findings showed that mice in all three groups displayed similar preferences for the target zone during probe 2 (Figure 4.7E,F). For probe 1, the mixed model ANOVA with the within-subjects factor of *Zone* and between-subjects factor of *Genotype* revealed that mice did not have a baseline preference for any of the zones (main effect of *Zone*, $F_{(3,30)} = 0.990$, $p = .411$; ps > .166 for other main effect and interaction). In probe 2, mice had an overall preference for the target zone (main effect of *Zone*, $F_{(2,16)} = 27.510$, $p < .001$), but there was no significant effect of *Genotype*, $F_{(2,10)} = 0.370$, $p = .700$, or *Day* × *Genotype* interaction, $F_{(3,16)} = 0.418$, $p = .751$. Therefore, mice in all groups showed a comparable bias for the target zone after strong and weak training protocols.

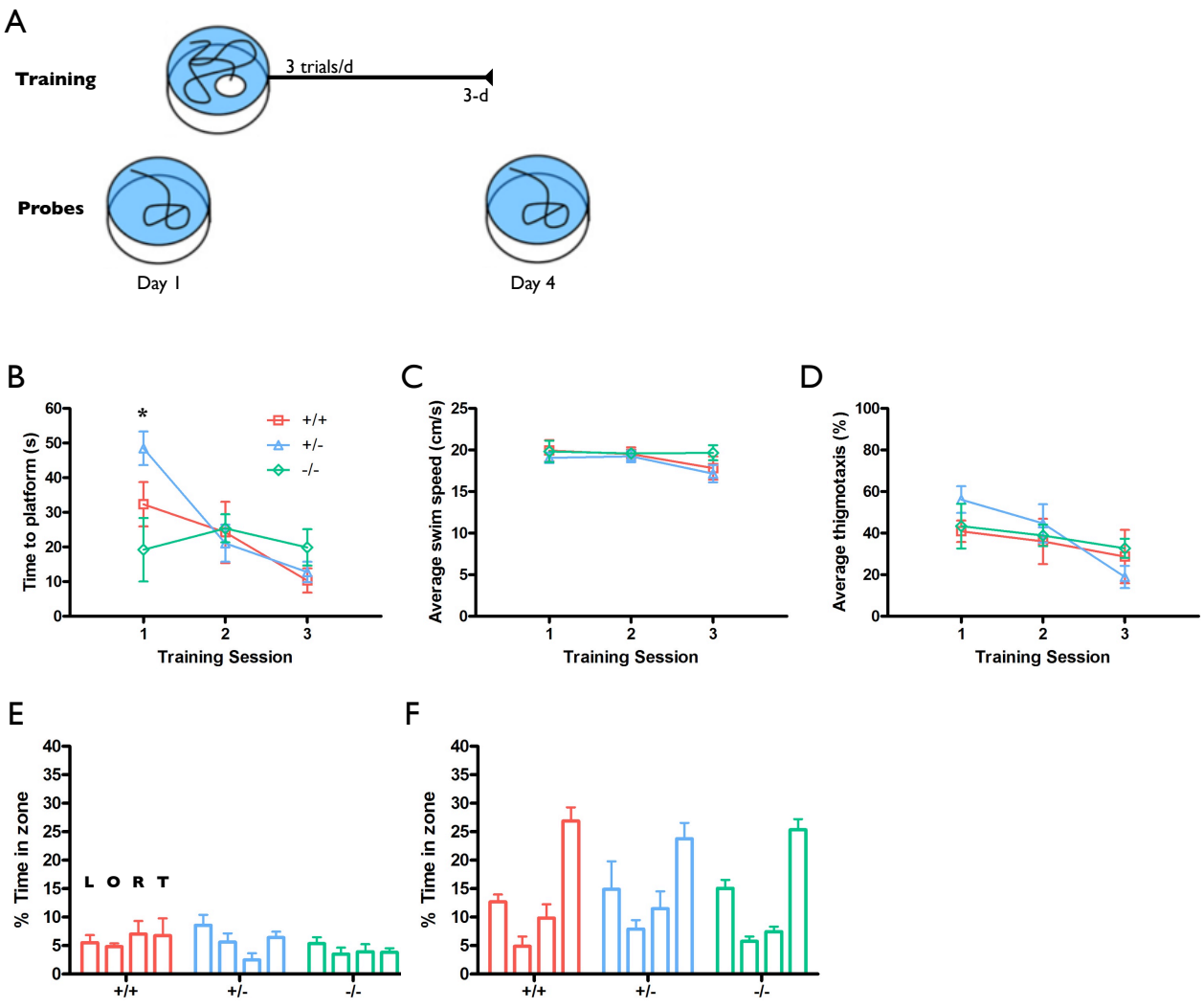

Figure 4.7: Water maze results – weak protocol

(A) Diagram of the weak water maze protocol.

(B-D) Changes in latency to reach the platform, average swim speed, and average thigmotaxis over the course of weak training in cohort 2.

(E, F) Between-genotypes comparisons in cohort 2 of time spent in the target zone (T; r =20 cm) and time spent in three other equally sized zones (left, L; opposite, O; right, R).

*Significant effect of *Genotype*, $p < .05$.

Chapter 5
Discussion

5.1 Summary of findings

The transcription factor CREB and its effector protein BDNF are two of the key molecular modulators of development, synaptic and structural plasticity, and behavioural processes in the nervous system. A crucial interface between CREB and BDNF resides at promoter IV of the *Bdnf* gene in the mouse, where CREB binds to a CRE-like consensus sequence in a necessary step for the initiation of transcription (Hong *et al.*, 2008; Tao *et al.*, 1998). Of the eight promoters in the *Bdnf* gene, promoter IV is the most responsive to experience-dependent changes in neuronal activity (Aid *et al.*, 2007). In this study, I used a transgenic mouse in which this critical interaction between CREB and BDNF is disrupted in a brain-wide, neuron-specific manner from the time of conception. The disruption of this interaction in CREmKI$^{-/-}$ mutant mice is associated with impaired inhibitory synapse development in the visual cortex, resulting in adult mice with abnormal physiological responses to electrical and sensory stimulation (Hong *et al.*, 2008). My study investigated the behavioural phenotype of adult CREmKI mutant mice and addressed the broader and more theoretically salient question of how CREB and BDNF interact to influence behavioural processes.

My investigation showed that disruption of the CREB-mediated expression of promoter IV-derived BDNF protein had differential effects on consecutive generations of CREmKI mutant mice, revealing subtle effects on memory- and anxiety-related behaviours. Since CREB and BDNF regulate memory consolidation, I hypothesized that auditory fear memory, spatial memory, and learning of a motor skill, all of which are protein synthesis-dependent, would be disrupted in the CREmKI mutant mouse. However, there was no such consistency in my findings. Neither homozygous nor heterozygous CREmKI mutants showed deficits in motor learning on the rotarod or spatial memory assessed in the water maze. CREmKI mutant mice showed a subtle, gene dosage-dependent impairment in memory 24 hours after auditory fear conditioning that could not be accounted for by differences in pain sensitivity or their baseline reactions to the shock. This finding applied only to the first generation of mice, while mutant and control mice in the F2 generation performed almost identically on the memory test.

Given the roles that CREB and BDNF play in mediating responses to acute and chronic stress, I also hypothesized that CREmKI mutant mice would demonstrate an increase in anxiety-related behaviour in the OF test. My results were somewhat consistent with this hypothesis, as F2 generation heterozygous and homozygous mutants demonstrated a modest, but significant reduction in exploratory behaviour that could not be explained by differences in locomotor activity. Interestingly, mice in the F1 generation showed no evidence of differences in exploratory behaviour. Consistent with this observation in F2 mutants is the higher level of thigmotaxis, albeit only in heterozygous mice, on the first day of water maze training. Taken together, my results demonstrate a double dissociation between memory- and anxiety-related deficits in two generations of CREmKI mutant mice. In the F1 generation, CREmKI mutants showed a subtle and selective impairment in auditory fear memory, but normal levels of anxiety. In the F2 generation, CREmKI mutants showed a heightened level of anxiety-related behaviour but normal memory capacities.

One important contraindication poses a major challenge to the interpretation of my findings; that is, the practice of separately assessing the behaviour of two generations of transgenic mice that carry the same focal mutation. I feel strongly that breaking up experimental data into separate subsets is not justified under most circumstances and must be avoided to reduce spurious findings. The replicability of experiments is crucial for establishing valid conclusions and furthering science. I had no *a priori* expectation that the cohorts would behave differently, nor did I initially intend to analyze them separately. However, after conducting the study I believe that there is a real likelihood that behavioural differences exist between the two generations of mice and that these differences are informative and relevant to understanding the role of the CREB-BDNF interaction in behaviour. There is a true, quantifiable difference in genetic background between the two cohorts of mice. Both the F1 and F2 generation of mice had a mixed C57B/6 × 129sv background, but the contribution of the 129sv parental strain was greater in the genetic background of the F2 generation. Furthermore, I only evaluated the cohorts separately when statistically justified by an interaction between cohort and genotype.

Is there a real likelihood that the difference in genetic background between the cohorts could lead to different patterns of behaviour in CREmKI mutant mice? I believe that the answer is yes. Although few experiments have investigated differences in mouse strains that are relevant to my study, there are measurable differences in pure C57B/6 and 129sv strains. Based

on 3-dimensional MRI, C57B/6 mice had increased cortical and striatal volume and a larger corpus callosum than 129sv mice, while 129sv mice showed increased hippocampal and cerebellar volume and body weight (Chen *et al.*, 2006b). In that study, volume of the amygdaloid region was similar in C57B/6 and 129sv strains. C57B/6 mice also undergo increased and longer lasting LTP compared to 129sv mice following stimulation of the Schaffer collateral pathway of the hippocampus (Nguyen *et al.*, 2000). Several behavioural studies have shown that both C57B/6 and 129sv mice demonstrate similar acquisition and spatial memory retention in the water maze (Holmes *et al.*, 2002; Nguyen *et al.*, 2000; Rogers *et al.*, 1999). The two strains also show similar cued and contextual fear memory (Nguyen *et al.*, 2000) and retention of memory for a familiar scented food in the social transmission of food preference task (Holmes *et al.*, 2000). Interestingly, all of these studies showed that 129sv mice had a significant reduction in exploratory behaviour in the OF compared to C57B/6 mice (Holmes *et al.*, 2002; Nguyen *et al.*, 2000; Rogers *et al.*, 1999), which resulted in deficits in another spatial memory task, the Barnes maze, in two of these studies (Holmes *et al.*, 2002; Nguyen *et al.*, 2000). Although the difference in genetic background between the cohorts is not comparable to differences between two completely different strains of mice, it is clear that the C57B/6 and 129sv genetic backgrounds have distinct effects on mouse development and behaviour.

One possibility is that genetic background interacted with the highly complex regulation of BDNF in the brain. There is a wealth of evidence that differences in *Bdnf* gene regulation and dosage cause differential effects on developmental, physiological, and behavioural processes. While $BDNF^{+/-}$ mice demonstrate normal neuronal morphology and responses to MD (Bartoletti *et al.*, 2002), mice overexpressing BDNF in the visual cortex (Huang *et al.*, 1999) and mice in which BDNF signaling is blocked by an inducible TrkB mutation (Kaneko *et al.*, 2008b) show abnormal ocular dominance plasticity and recovery from MD. $BDNF^{klox/klox}$ mice, in which BDNF expression is restricted to cell soma, show marked deficits in their responses and recovery from MD (Kaneko *et al.*, 2012). They also show developmental deficits in dendritic spine pruning in the hippocampus (An *et al.*, 2008) and visual cortex (Kaneko *et al.*, 2012). Therefore, both the level and localization of BDNF expression influences nervous system development. Similarly, the impairment of BDNF knockout mice in LTP is directly related to the magnitude of gene knockdown (Korte *et al.*, 1995). In the OF and EPM tests of anxiety, $BDNF^{+/Met}$, $BDNF^{Met/Met}$ and $BDNF^{+/-}$ mice demonstrate increased levels of anxiety-related behaviour

compared to wild-type mice (Chen *et al.*, 2006a). Increases in anxiety-related behaviour were greatest in $BDNF^{+/-}$ mice and smallest in $BDNF^{+/Met}$ mice, which corresponds to differences among these mutants in the magnitude of BDNF knockdown ($BDNF^{+/-}$ mice have a ~50% reduction in total BDNF, whereas $BDNF^{Met/Met}$ and $BDNF^{+/Met}$ mice have ~30% and ~20% reductions, respectively, in activity-dependent BDNF secretion). A similar pattern of results was observed in contextual fear conditioning. It is plausible that the magnitude of gene knockdown can account for why some transgenic lines, and not others, display deficits in behavioural tasks, and that there is an optimal range of BDNF expression in the brain for appropriate behavioural responses. Since the expression of native and activity-dependent levels of BDNF, and even of transcripts from distinct *Bdnf* promoters, is highly variable among brain regions, it is also likely that this optimal range differs in different brain areas (Aid *et al.*, 2007; An *et al.*, 2008; Kolbeck *et al.*, 1999; Timmunsk *et al.*, 1993). To reinforce this point, VP16-CREB mice, which have elevated levels of BDNF expression in the brain (Barco *et al.*, 2005), demonstrate an enhancement in cued fear memory (Viosca *et al.*, 2009a), but have deficits in spatial memory in the water maze (Viosca *et al.*, 2009b), perhaps due to the fact that these tasks are subserved by different brain areas. Given the highly complex temporal and spatial regulation of BDNF in the brain, there is potential for differences in animal background genetics to interact with levels of BDNF expression, influencing behavioural results.

It is also plausible that differences in genetic background led to differential expression of genetic compensatory mechanisms in the cohorts of CREmKI mice. Hong *et al*. (2008) showed that the activity-dependent expression of *Bdnf* exons I and III was upregulated in $CREmKI^{-/-}$ mice *in vitro* and *in vivo*. Since all of the *Bdnf* promoters code for an identical protein, this could be a direct source of compensation for the CREmKI mutation. Furthermore, visual cortex expression of *Bdnf* exon IV was markedly, but incompletely attenuated in $CREmKI^{-/-}$ mice. While exposure to light induced a 20-fold increase in *Bdnf* exon IV transcripts in light-deprived control mice, this manipulation still induced a 5-fold increase in expression in $CREmKI^{-/-}$ mice, leaving the possibility that the residual exon IV mRNA expression is sufficient to prevent deficits in behaviour. The expression of BDNF exon IV can indeed be induced independently of activation by CREB at two other regulatory sites just upstream of *Bdnf* promoter IV, albeit the magnitude of BDNF expression is dramatically (~90%) attenuated (Tao *et al.*, 1998, 2002). Several IEGs, including *c-fos*, *Np-2,* and *Arc* were also upregulated in $CREmKI^{-/-}$ mice (Hong *et*

al., 2008). The proto-oncogene *c-fos* is expressed in neurons in response to a wide variety of extracellular stimuli and regulates many synaptic responses (Greenberg *et al.*, 1986; reviewed in Greer & Greenberg, 2008). Neuronal pentraxins (NPs) contribute to excitatory synaptogenesis in the developing brain by recruiting AMPA receptors to immature synapses (Bjartmar *et al.*, 2006; Koch & Ullian, 2010; Xu *et al.*, 2003). The upregulation of *Arc* is particularly noteworthy because Arc protein expression is regulated by BDNF in learning and memory processes. Arc is a cytoplasmic protein that regulates synaptic strength by promoting AMPA receptor endocytosis (Chowdhury *et al.*, 2006; Waung *et al.*, 2008). Suppression of Arc expression by infusion of antisense OGNs or transgenic knock-out impairs L-LTP but not E-LTP in the Schaffer collateral pathway, and impairs LTM but not STM in the water maze, fear conditioning, CTA and object recognition tasks (Guzowski *et al.*, 2000; Plath *et al.*, 2006; reviewed in Tzingounis & Nicoll, 2006). The activity-dependent secretion of BDNF during LTP leads to Arc protein expression, which is thought to contribute to consolidation processes by regulating actin polymerization at the synapse (Messaoudi *et al.*, 2007; reviewed in Bramham *et al.*, 2008). Therefore, it seems almost certain that compensatory BDNF expression, or compensation by other activity-dependent genes, would obscure the full functional effect of disrupting the CREB-BDNF interaction. To what extent, if any, these mechanisms are differentially modulated by differences in background genetics is unclear, but should be considered a possibility.

I believe that the double dissociation in memory- and anxiety-related behaviours observed between two cohorts of mice is informative because it highlights a range of effects that abnormal activity-dependent regulation of BDNF can have on behaviour. Although I did not consider the effects of genetic background on CREB expression and compensatory mechanisms, such effects have been shown to modulate learning and memory processes (Balschun *et al.*, 2003; Gass *et al.*, 1998). However, CREB expression and phosphorylation are not altered by the CREmKI mutation, so I would not expect these effects upstream of BDNF.

5.2 A deficiency in CREB-mediated BDNF expression does not cause overt phenotypic abnormalities

CREmKI mutant mice were viable, born in the expected Mendelian ratios, and physically indistinguishable from control littermates, consistent with the observations of Hong *et al.* (2008). I did not observe any gross abnormalities in brain morphology that could account for behavioural differences in my study. CREmKI mutant mice also showed normal levels of locomoter activity, consistent with findings in $BDNF^{Met/Met}$ mice, which also have a deficit specific to the activity-dependent secretion of BDNF (Chen *et al.*, 2006a; Li *et al.*, 2010). Contrastingly, $BDNF^{+/-}$ mice are hyperactive, suggesting that this trait is modulated by basal levels of BDNF expression (Kernie *et al.*, 2000). I also observed sexual dimorphism in the regulation of body weight in CREmKI mutant mice, as male heterozygotes were larger than their littermates at 3 months of age, and female homozygotes were smaller than their littermates. Although obesity has been observed in $BDNF^{+/-}$ mice (Boger *et al.*, 2011; Kernie *et al.*, 2000), this does not help explain the sexually dimorphic effect in my study. It is possible that a reduction in BDNF levels led to differential interactions with sex hormones in males and females, but I did not investigate this possibility. There were no differences between male and female mice in any of the behavioural measures investigated in my study (data not shown).

5.3 Deficient CREB-mediated BDNF expression does not cause impairments in motor or spatial learning

There was no indication that disruption of the CREB-BDNF interaction affected two forms of learning that require protein synthesis for memory consolidation. I showed that CREmKI mutant mice had typical overall levels of performance and demonstrated a characteristic learning curve in a rotarod task that assessed the ability to learn a motor skill over a period of 8 days. This finding is perhaps surprising given that mice with abnormal regulation of CREB activation by CBP have a deficit in learning this task (Oliveira *et al.*, 2006), and mice that exhibit decreased CREB phosphorylation in the cerebellum have severely impaired baseline performance in this task (Brodie *et al.*, 2004). Unlike mice in which CREB expression is disrupted, the regulation of CREB is normal in CREmKI mice, but the ability of CREB to regulate its target genes is not.

Since the disruption of CREB expression in the previous studies would have presumably led to deficient expression of numerous CREB target genes, it appears that some CREB target other than BDNF modulated performance on the rotarod. This interpretation is supported by the fact that BDNF is not endogenously expressed by striatal MSNs, so CREB-mediated expression of BDNF would have to occur at some other site, such as the SN or cortex, where it is not clear if CREB is involved in motor learning. It is also possible that the disruption of CREB-mediated BDNF expression simply does not have a pronounced effect on motor learning in young adult (~3-month-old) mice. Striatal levels of BDNF, performance on the rotarod, and the integrity of dopaminergic neurons in BDNF-deficient mice decline steeply with age (Baker *et al.*, 2005; Boger *et al.*, 2011). However, I did not address the behaviour of aged mice in my study.

I did not observe any indication of spatial memory impairments in CREmKI mutant mice in the water maze in spite of an extensive body of evidence that has implicated both CREB and BDNF in the consolidation of hippocampal LTP and memory processes. CREmKI mice demonstrated an improvement in latency to reach the hidden platform throughout training, and showed a strong bias for the target zone in recent (24-hr) and remote (6-wk) memory tests. A ceiling effect during training could not account for my findings, as using a weaker training protocol previously shown to undertrain wild-type mice (see Sekeres *et al.*, 2010) also revealed that mutant mice learned at a similar rate to control littermates. It is possible that the CREB-mediated component of BDNF expression driven by promoter IV is not needed for spatial learning and memory. However, both CREB and BDNF are upregulated in the hippocampus in an activity-dependent manner following spatial learning (Falkenberg *et al.*, 1992; Kesslak *et al.*, 1998; Mizuno *et al.*, 2000; Viosca *et al.*, 2009b), and disruption of the activity-dependent increase in BDNF impairs spatial task performance (Mizuno *et al.*, 2000). While the role of CREB-mediated expression of *Bdnf* promoter IV has not been specifically investigated, promoter IV-derived BDNF accounts for the majority of activity-dependent BDNF expression in the brain and hippocampus (Aid *et al.*, 2007; Timmunsk *et al.*, 1993).

Perhaps a more likely explanation for the null finding in my study is that, while promoter IV-derived BDNF levels are important for spatial memory, the incomplete knockdown and compensatory gene expression in CREmKI mutant mice minimized the effect of its disruption. This interpretation is consistent with mixed results from studies in both CREB- and BDNF-deficient mice where gene knockdown is incomplete. $CREB^{\alpha\delta-/-}$ mice are the best-studied

example of CREB-deficient mice in the water maze, and have demonstrated deficits in spatial memory in some studies (Balschun *et al.*, 2003; Bourtchuladze *et al.*, 1994; Kogan *et al.*, 1997; Sekeres *et al.*, 2010) but not others (Gass *et al.*, 1999; Graves *et al.*, 2002). Moreover, $CREB^{NesCre}$ mice had a deficit in water maze learning, while $CREB^{CaMKCre7}$ mice, which have a more spatially and temporally restricted mutation in CREB, did not (Balschun *et al.*, 2003). Mice with more extensive CREB knockdown, such as KCREB and $CREB^{comp}$ mice, tend to have a robust impairment in spatial memory in the water maze (Balschun *et al.*, 2003; Pittenger *et al.*, 2002). $BDNF^{+/-}$ mice appear to be impaired in spatial learning and memory (Linnarsson *et al.*, 1997), but $TrkB^{+/-}$ mice are not (Minichiello *et al.*, 1999). These findings indicate that the magnitude of disruption in CREB and BDNF function needed to induce spatial memory impairments may not always be achieved in transgenic mouse models.

The only notable difference observed in CREmKI mutants was an increase in thigmotaxis during the first day of water maze training. This effect was pronounced but limited to heterozygous mice in cohort 2, and was only apparent on the first day of the strong training protocol. Increased thigmotaxis can be associated with a reduction in exploratory behaviour due to the stress of being placed in a large pool of water with no apparent means of escape (Minichiello *et al.*, 1999). Alternatively, thigmotaxis can be used as a simple search strategy for mice placed in a novel environment (Gallagher *et al.*, 1993; Garthe *et al.*, 2009; Stone *et al.*, 2011). As mice become familiarized with the water maze over the course of training, they tend to adopt more localized approaches to finding the hidden platform, such as swimming directly in its direction or conducting a focal search in an area proximal to the platform. While in my study the increase in thigmotaxis was not persistent and did not appear to detract from water maze learning, persistent thigmotaxis has been suggested to account for apparent spatial memory impairments in other studies, either due to use of an ineffective search strategy (Balschun *et al.*, 2003) or increased anxiety (Minichiello *et al.*, 1999).

5.4 Modulation of fear memory by CREB-dependent BDNF expression

CREmKI mutant mice demonstrated impairments in memory 24 hours after auditory fear conditioning that could not be accounted for by differences in pain sensitivity or immediate

behavioural responses to the foot-shock. The impairment was also greater in the homozygous mutants than in heterozygous mutants, suggesting that it was gene dosage-dependent. However, this impairment was only observed in mice from cohort 1.

The finding that CREmKI mutant mice had a deficit in auditory fear memory is intriguing because BDNF is strongly implicated in the extinction of cued fear memories, but its role in the acquisition and consolidation of these memories is unclear. $BDNF^{+/-}$ mice do not appear to exhibit deficits in cued fear memory (Chen *et al.*, 2006a; Liu *et al.*, 2004). Adult mice with a conditional knock-out of BDNF throughout the brain under the control of the tTa/Tet-Op inducible system also had normal auditory fear memory, but juvenile mice had a slight impairment (Monteggia *et al.*, 2004). Viral-vector mediated disruption of BDNF in the LA induced deficits in auditory fear memory in some studies (Ou *et al.*, 2010; Rattiner *et al.*, 2004), but not others (Choi *et al.*, 2010; Heldt *et al.*, 2007). Interestingly, $BDNF^{+/Met}$ and $BDNF^{Met/Met}$ mice, which are perhaps the closest previously tested analog of the mice used in my study because of their impairment in activity-dependent but not native secretion of BDNF, show intact memory but delayed extinction in cued fear conditioning (Chen *et al.*, 2006a; Soliman *et al.*, 2010) and CTA (Yu *et al.*, 2009). Therefore, my finding of an auditory fear memory impairment in CREmKI mutants is somewhat surprising, especially given the nature of this subtle mutation and the fact that the memory impairment did not extend to other memory tasks.

Of course, reductions in CREB have been repeatedly implicated in auditory fear memory deficits (Bourtchuladze *et al.*, 1994; Gass *et al.*, 1998; Kida *et al.*, 2002; Pittenger *et al.*, 2002; Rammes *et al.*, 2000), and studies in my lab have shown that viral vector-mediated CREB overexpression is highly effective at enhancing cued fear memory (Han *et al.*, 2007, 2009; Josselyn *et al.*, 2001). An appealing explanation for my finding is that disruption of the ability of CREB to regulate BDNF in the amygdala impaired memory in a task heavily dependent on CREB. However, I cannot discount the possibility that abnormal regulation of BDNF in some other area of the brain, such as the prelimbic cortex (Choi *et al.*, 2010), led to the deficit in fear memory. Nevertheless, it is interesting that past studies in which BDNF expression was more extensively disrupted did not produce cued fear memory deficits. This points to the importance of CREB-mediated signaling in the deficit observed in my study.

It appears that the CREB-mediated expression of *Bdnf* promoter IV plays a role in the consolidation, but not acquisition of conditioned fear memories. The CREB-BDNF interaction could modulate memory consolidation through the regulation of inhibitory synapses, which would be consistent with its developmental role observed in CREmKI mutants (Hong *et al.*, 2008). Evidence has shown that memory consolidation following cued fear conditioning is accompanied by downregulation of inhibitory synapse markers in the BLA (Heldt & Ressler, 2007) and increased internalization of the GABA α1 receptor subunit (Chhatwal *et al.*, 2005; Ressler *et al.*, 2002). Coincidentally, the application of BDNF to cultured neurons from the mouse hippocampus or amygdala results in a similar increase in GABA α1 subunit internalization (Mou *et al.*, 2011), as well as increased internalization of the GABA β2/3 receptor subunit (Cheng & Yeh, 2003), and increased surface expression of the GABA δ subunit at inhibitory synapses (Joshi & Kapur, 2009). Furthermore, GABA α1 subunit internalization, which is associated with the consolidation of fear memories, was impaired following the inducible deletion of TrkB in the forebrain of mice by 1NMPP1, and depended on PKA and PKC signaling in the hippocampus, and PKC in the amygdala (Mou *et al.*, 2011). This suggests that both CREB- and BDNF/TrkB-mediated signaling pathways are involved in inhibitory synapse regulation during memory consolidation.

The modest deficit in auditory fear memory in CREmKI mice might be accounted for by the expression of other CREB target genes, or BDNF protein derived from other transcripts, contributing to memory consolidation. Recently, the IEG *Homer1a* (*H1a*) has garnered interest for its putative role in memory consolidation. *H1a* is a CREB target gene (Naidoo *et al.*, 2012) that is also regulated by BDNF in an activity-dependent manner (Mahan *et al.*, 2012). It has been previously implicated in the regulation of dendritic spine morphology through modulation of AMPA and NMDA receptor-mediated currents at excitatory synapses (Sala *et al.*, 2003). It was recently observed that cued fear conditioning results in two types of epigenetic changes in the LA, demethylation of H3 histones and subsequent acetylation, which transform chromatin to an active state and promote gene transcription (Maddox & Schafe, 2011). Infusions of a histone methyltransferase inhibitor or a histone deacetylase inhibitor into the LA prior to fear conditioning caused impairments or enhancements in fear memory, respectively (Monsey *et al.*, 2011), and similar effects were observed on re-consolidaton (Maddox & Schafe, 2011). Interestingly, the upregulation of *H1a* expression that occurs *in vivo* following fear conditioning

could be replicated in cultured neurons from the hippocampus or amygdala by bathing them in BDNF (Mahan *et al.*, 2012). Chromatin immunoprecipitation assays revealed decreased methylation of H3 histones of the *H1a* promoter in the amygdala and increased acetylation of H3 histones in the hippocampus, both *in vitro* following BDNF application and *in vivo* following fear conditioning. *H1a* expression was also dependent on MAPK signaling both *in vitro* and *in vivo*, implicating an important downstream target of both CREB and BDNF in memory consolidation.

Finally, it is likely that compensatory gene expression in CREmKI mutant mice would have resulted in a partial rescue of fear memory impairment. Under normal circumstances in mice, *Arc* mRNA is upregulated in the LA immediately following auditory fear conditioning (Maddox & Schafe, 2011; Ploske *et al.*, 2008), and following successful memory retrieval (Han *et al.*, 2007, 2009). Mice injected with antisense OGNs that suppress *Arc* expression had impairments in both LTM consolidation (Plath *et al.*, 2006; Ploske *et al.*, 2008) and reconsolidation of an old fear memory (Maddox & Schafe, 2011). The upregulation of *Arc* in CREmKI mutant mice may thus have improved memory consolidation in mice that would have otherwise been severely impaired.

5.5 Modulation of anxiety-related responses by CREB-dependent BDNF expression

Interestingly, I observed that CREmKI mutant mice showed a reduction in exploratory behaviour in the OF. This is suggestive of an aversion to open spaces due to increased levels of anxiety (Berton *et al.*, 2006). As noted previously, this result could not be accounted for by a reduction in locomoter activity in mutant mice. Furthermore, the increase in anxiety-related behaviour was restricted to cohort 2, and thus does not appear to be related to deficits in auditory fear learning shown in cohort 1.

The finding that mice with disrupted CREB-mediated regulation of BDNF showed a reduction in exploratory behaviour is not surprising in light of the fact that several lines of CREB and TrkB transgenic mice exhibit persistent thigmotaxis in the water maze (Balschun *et al.*, 2003; Gass *et al.*, 1998; Minichiello *et al.*, 1999). As noted, above, heterozygous mice in cohort

2 also demonstrated an increase in thigmotaxis on the first day of water maze training. However, thigmotaxis may reflect the use of an ineffective search strategy rather than anxiety-related behaviour (Garthe *et al.*, 2009; Stone *et al.*, 2011), even though task demands in the OF and the first trial of the water maze are not necessarily different. Furthermore, the water maze clearly presents a more stressful environment for mice due to the threat of sinking. My results are more directly supported by two previous studies in which $BDNF^{+/-}$ and $BDNF^{Met/Met}$ mice showed decreased exploratory behaviour in the OF and spent less time in the open arms of the EPM (Chen *et al.*, 2006a; Li *et al*., 2010).

A more curious finding in my study is evidence of a double dissociation in fear-related learning and anxiety-related behaviour in cohorts 1 and 2. Both types of behaviours have similar features and are mediated by overlapping neural correlates (reviewed in Davis *et al.*, 2010). Under normal circumstances, fear is an acute, short-lived response induced by an imminent danger. Anxiety can be conceptualized as a prolonged fear response that occurs when a threat is anticipated based on past experience or following a dangerous or traumatic situation that has already passed (Fanselow, 1986). Therefore, two key features distinguish fear and anxiety: the duration of the response, and the presence or absence of immediate danger. In rodents, responses to stress are mediated by the hypothalamic-pituitary-adrenal (HPA) axis and its regulation of the glucocorticoid, corticosterone (reviewed in Diorio & Meaney, 2007; McEwen *et al.*, 1986). The termination of stress responses is ensured by homeostatic feedback to glucocorticoid receptors in the HPA axis and hippocampus (Weaver *et al.*, 2004). A critical modulator of the behavioural response to stress is the periventricular nucleus (PVN) of the hypothalamus, which secretes corticosterone-releasing factor (CRF) to the CeA (Davis *et al.*, 2010). The CeA projects to a region of the extended amygdala called the bed nucleus of the stria terminalis (BNST), which projects to the locus coeruleus (LC). The LC then triggers the behavioural stress response by secreting norepinephrine (Diorio & Meaney, 2007). The elevation of CRH levels in response to stress is associated with decreased expression of the α-subunit of the GABA receptor in the amygdala (Caldji et al., 1998), and a reduction in BDNF protein levels in the amygdala and hippocampus (Liu *et al.*, 2000).

Distinct regions of the amygdala appear to mediate fear and anxiety, perhaps explaining how defective regulation of BDNF in CREmKI mutants might have affected one response and not the other. Davis *et al.* (2010) stipulated that the BLA responds directly to stressful situations

by innervating the CeA and sending glutamatergic projections directly to the lateral BNST. It is believed that activation of the medial nuclei of the CeA (CeA_M) drives acute fear responses, whereas direct activation of the BNST by the BLA or indirect activation through the lateral nuclei of the CeA (CeA_L), which release CRF, drives prolonged fear responses. Consistent with this interpretation, rats that received infusions of an AMPA receptor antagonist to the BNST prior to a FPS test in which the CS was presented for a prolonged period (8-min) showed a heightened startle response at the onset of the CS, but a depressed prolonged response relative to animals that received infusions into the CeA (Meloni *et al.*, 2006). Animals that received infusions into the BLA showed depression of both immediate and prolonged startle responses (Walker & Davis, 1997). It has also been shown that enhancement of FPS by intracerebroventricular (i.c.v.) infusion of CRF is blocked in rodents with BNST, but not CeA lesions (Lee & Davis, 1997). Both CRF- and light-enhanced startle paradigms, which induce prolonged startle responses, but not FPS, which induces an acute response, are sensitive to pre-test i.c.v. infusions of CRF antagonists (de Jongh *et al.*, 2003). The possibility that the differential regulation of BDNF expression in two parallel pathways of the amygdala might have distinct effects on fear and anxiety is intriguing and certainly warrants further investigation.

5.6 Limitations

Using the CREmKI mouse allowed me to investigate an interaction between CREB and BDNF that cannot be isolated using whole-gene or promoter knock-out approaches that induce a more complete knockdown of gene expression. However, this approach has several important disadvantages, two of which I have already discussed in detail above. Firstly, residual promoter-IV driven BDNF expression may have been sufficient to prevent a complete disruption of physiological and behavioural effects. Secondly, compensatory mechanisms identified previously by Hong *et al.* (2008), such as increased *Bdnf* promoter I and III-driven expression, as well as increased *c-fos*, *Np-2,* and *Arc* levels, may have contributed to a recovery of function in CREmKI mice. To what extent, if any, these factors masked behavioural deficits in CREmKI mutant mice is not known. Finally, behavioural deficits in CREmKI mice could be reflective of developmental abnormalities in these mice. The CREmKI mutation causes a developmental deficit in inhibitory synapse formation, and inhibitory synaptic transmission is critically

modulated during memory consolidation (Chhatwal *et al.*, 2005; Heldt & Ressler, 2007; Mou *et al.*, 2011). This raises the possibility that abnormal inhibitory signaling in the brain, rather than a deficit in CREB-mediated gene expression, could be responsible for my experimental findings. However, abnormal inhibitory synapse development has only been shown in the visual cortex of CREmKI$^{-/-}$ mice *in vivo* (Hong *et al.*, 2008), so these findings may not generalize to the brain areas critical for the behaviours investigated in my study.

Another important limitation in my study was the repeated-measures design. Given the exploratory nature of my study, the breadth of behaviours that I wanted to assess, and the limited availability of mice at the outset of the experiment, I saw it to my advantage to use the same animals in a battery of behavioural tasks. However, the major disadvantage of this design is that once animals have completed the first behavioural task they are no longer naïve, eliminating an important component of experimental control in my study. To compound the problem, not all animals underwent all of the behavioural tasks. To better conceptualize the potential problems that this inconsistency generates, consider the fact that a subset of the animals underwent an extensive rotarod training protocol prior to completing the water maze, while another did not. Rotarod training is associated with long-lasting synaptic and structural changes in striatal MSNs and results in considerable improvements in balance and coordination (Xu *et al.*, 2009; Yang *et al.*, 2009; Yin *et al.*, 2009). Although the water maze does not require mice to learn a new skill, it is conceivable that mice that underwent rotarod training would have a performance advantage in this task. After all, these mice had been extensively trained to avoid falling from a rapidly rotating beam so they were likely to be less affected by fatigue, and perhaps less anxious, when suddenly forced to swim to safety in the water maze.

It is unclear how, and to what extent, the potentially traumatizing experience during fear conditioning might have affected subsequent OF and water maze tasks. I certainly observed a high overall level of anxiety in the OF in control mice, as time spent outside of the central zone was essentially at ceiling in all groups. Although I think that this is more likely to reflect a lack of sensitivity of this task, the mice may have been showing a heightened anxiety-related response as a result of the previous aversive experience. It is also possible that exposure to multiple behavioural tasks resulted in reduced anxiety in my mice, as previous studies have demonstrated evidence of habituation in rodents re-exposed to an open chamber (Platel & Porsolt, 1982) or the EPM (Dawson *et al.*, 1994), though there are conflicting findings (see File, 1990; Lister, 1987).

Therefore, the potential pitfalls of the repeated-measures design are numerous. Of course, I made sure to give mice several days of recovery between tasks, and to maintain consistency in the order of task administration. Differences among subsets of mice were not evident when I was conducting experiments, and there is little reason to believe that motor learning would affect spatial learning ability.

Since most of the animals underwent the fear conditioning, OF, and water maze tasks and only a small subset of mice underwent rotarod training as well, I did not carry out statistical comparisons between groups of mice undergoing different protocols due to an obvious lack of statistical power. Low statistical power was another potential limiting factor in my study, as it would lead to a reduced likelihood of detecting a true significant effect. While this may have warranted the use of a power analysis to estimate the number of animals needed for each behavioural experiment prior to the study, there are also problems with utilizing this approach. The use of many subjects in order to obtain desirable power may not be necessary to achieve a statistical effect, leading to the wasteful use of animals. Power analysis also depends on the variance in the sample being used, necessitating an estimate that is typically based on past findings or a preliminary study. Since my experiment was novel and exploratory, the variance of the mice for a given behavioural measure could not be reliably predicted. Also, having adequate statistical power would not circumvent the pitfalls of using a repeated-measures design.

Finally, it is important to re-iterate that the interpretability of my findings is significantly hampered by the fact that I cannot explain the genotypic difference between cohort 1 and cohort 2 in a quantitative manner. There is also no definite quantification of the proportion of parental strains in the genetic background of CREmKI mice used in the original study (Hong *et al.*, 2008), which compounds the issue. I have no evidence to explain how a difference in genetic background between cohorts might have affected the physiology or behaviour of CREmKI mice, which calls the validity of my findings into question, especially where I analyzed the two cohorts separately. I believe that it was useful to analyze the cohorts separately when it was statistically justified in order to demonstrate potential effects of the CREB-BDNF interaction on behaviour; however, until it is possible to definitively state the effect of genetic background on behaviour in these mice, my results must be treated with caution and cannot be generalized beyond this study.

5.7 Future directions

Evidently, it would be useful for future iterations of this study to make some methodological adjustments. In the immediate future, my lab plans to incorporate a cross with wild-type mice from a pure C57B/6 line into our breeding strategy for CREmKI mice. We believe that this will allow us to generate experimental mice that better resemble those initially used by Hong *et al.* (2008). It would be useful to replicate the initial *in vivo* findings to ensure that CREB binding at *Bdnf* promoter IV is indeed disrupted in our CREmKI mutants and to determine whether developmental abnormalities in activity-dependent BDNF secretion and inhibitory synapse formation extend beyond the visual cortex. Once we have validated the mouse model ourselves, we can be more certain that these mice have a disruption of CREB-mediated BDNF expression. The final step will be to test these mice in a between-subjects design, perhaps initially focusing on tasks that seemed to be impaired in this experiment, such as auditory fear conditioning and the OF task. It might also prove fruitful to employ a task that has a higher ceiling for anxiety-related effects, such as the EPM or an acute stress manipulation. Finally, since aging mice show a reduction in BDNF expression, it might also be useful to examine performance in mice at various ages.

In this study, I asked the question of whether disrupting the CREB-BDNF interaction would affect behaviour in adult mice. The mice used in this study were not well suited to answer this question due to their developmental abnormalities. A more ideal approach would be to induce the CREmKI mutation in fully developed adult mice just prior to the behavioural experiments. Generating a mouse with an inducible mutation of this kind would be exceedingly difficult using conventional methods, since the mutation is highly localized. A more practical, though still challenging approach, would be to generate a mouse in which promoter IV of the *Bdnf* gene is floxed and can be excised in an inducible manner through the expression of Cre-recombinase. Such mice would not show developmental abnormalities and presumably, would show minimal compensatory effects once the mutation was induced. I also believe that a mouse in which the function of the entire promoter is disrupted would be more informative from a behavioural standpoint, since it is not clear to what extent the residual expression of *Bdnf* promoter IV is able to rescue behavioural deficits in CREmKI mutants. Hong *et al.* (2008) did generate such mutants, showing that they had similar, but slightly more severe, physiological abnormalities compared to CREmKI mutants. The vast proportion of activity-dependent *Bdnf*

promoter IV expression is regulated by CREB, so any behavioural deficits in these mice could still be informative about the roles of CREB-mediated transcription in behaviour.

A long-term goal of this project is to not only demonstrate that disrupting an interaction between CREB and *Bdnf* promoter IV has important behavioural consequences, but to elucidate the role that this interaction plays in behaviour. One question that we might address in our lab is to what extent does CREB elicit its effects on learning and memory by regulating *Bdnf* promoter IV. The studies by Han *et al.* (2007, 2009) in our lab revealing that neurons in the LA that overexpress CREB are preferentially incorporated into a fear memory trace has generated considerable interest. Both increased spine density (Marie *et al.*, 2005) and enhanced excitability (Zhou *et al.*, 2009) are plausible explanations for the apparent 'CREB advantage' of this population of virally transfected neurons. However, the identities of CREB effector genes that lead to the memory enhancement remain to be determined. Given its involvement in LTP and memory, BDNF is a likely candidate. The CREmKI mouse provides an ideal opportunity to address this question. If the CREB-mediated expression of *Bdnf* promoter IV drives enhancements in memory in mice infected with CREB, then one would predict that the infusion of HSV-CREB vector into the LA or hippocampus would not induce memory enhancements in CREmKI mutants as it does in wild-type mice. This would be a theoretically interesting question even if future attempts to replicate behavioural deficits in CREmKI mice are unsuccessful, since it is a CREB-mediated enhancement of memory that is of primary interest.

5.8 Conclusion

In this thesis, I have investigated one of the ways in which neural circuits in the brain are modulated by experience. My primary finding was that disrupting the capacity of the mouse brain to express BDNF through an experience-dependent mechanism mediated by CREB affected memory- and anxiety-related behaviour. While understanding the CREB-BDNF interaction is only one tiny step toward developing a holistic understanding of how experience modulates future behaviour by effecting changes in the brain, I believe that it is a very important step. CREB and BDNF are each implicated in a vast repertoire of cellular and behavioural processes that shape us into who we are. They are also linked to many debilitating cognitive

ailments and diseases. It will be crucial for future studies to continue to shed light on the mechanism and effects of the CREB-BDNF interaction so that the molecular events underlying these conditions can one day be fully understood.

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