A loss-of-function study of CRTTC1 in hippocampal neurons at basal state and during long-term synaptic plasticity

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**A LOSS-OF-FUNCTION STUDY OF CRTC1 IN
HIPPOCAMPAL NEURONS AT BASAL STATE AND
DURING LONG-TERM SYNAPTIC PLASTICITY**

LIM FANG YI AGNES

SCHOOL OF BIOLOGICAL SCIENCES

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LIM FANG YI AGNES

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of Doctor of
Philosophy

2019

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I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution.

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ABSTRACT

Encoding of long-term memory in neurons requires gene expression and protein synthesis. CREB, a well-characterised stimulus-induced transcription factor, is implicated in gene transcription supporting long-term plasticity. Several studies showed that CRTTC1 is an important co-activator for CREB activity. Although CRTTC1 is most abundantly expressed in the brain, its function during memory formation remains poorly understood. Studies have revealed that CRTTC1 could modulate long-term plasticity in the hippocampus, but extensive use of a conditional deletion strategy has never been performed. Hence, the overall aim of this project is to rigorously examine the impact of nuclear CRTTC1 translocation and transcription in regulating long-term memories. We investigated the role of CRTTC1 in hippocampal neurons by knocking out CRTTC1 protein expression using the lentiCRISPR/Cas9 system that we established. Our results indicate that basal loss of CRTTC1 reduced the number of inhibitory synaptic protein expression in hippocampal neurons, therefore leading to enhanced action potential firing and reduced mIPSCs. We report that the loss of excitatory synaptic protein expression only occurred in bicuculline-stimulated cultures. We also show that chemically-induced long-term potentiation and depression triggered CRTTC1 nuclear accumulation in hippocampal neurons. During long-term plasticity, loss of CRTTC1 perturbed the overall transcription of several CREB-mediated immediate early genes. In particular, the expression of an important regulator of excitatory-inhibitory balance in the hippocampal neurons, *Npas4*, is reduced. Hence, we speculate that CRTTC1 is crucial for the regulation of multiple CREB-mediated target gene transcription, including *Npas4* and that loss of *Npas4* expression perhaps contributes to the reduction in inhibition.

ABBREVIATIONS

14-3-3	soluble anchoring protein 14-3-3
AAV	Adeno-associated virus
AC	Adenylyl cyclase
AD	Alzheimer's disease
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP	Action potential
AP-1	Activator protein 1
Arc	Activity-regulated cytoskeleton-associated protein
Atf4	Activating Transcription Factor 4
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophin factor
Bic	Bicuculline
bZIP	Basic leucine zipper
CA	Comu Ammonis
CAMKs	Calcium/Calmodulin kinases
cAMP	Cyclic adenosine monophosphate
Cas9	CRISPR associated protein
CBD	CREB binding domain
CBP	CREB-binding protein
CHX	Cycloheximide
cLTP	Chemical long-term potentiation
CN	Calcineurin
CRE	cAMP response element
CREB	Cyclic-AMP responsive element-binding protein
CRISPR	Clustered regularly interspaced palindromic repeats
CRTC1	CREB regulated transcription coactivator 1
CsA	Cyclospoin A
DG	Dentate Gyrus
DHPG	Dihydroxyphenylglycine
DIV	Days <i>in vitro</i>
DNA	Deoxyribonucleic acid

DPT	Days post transduction
DSB	Double strand breaks
EGFP	Enhanced green fluorescent protein
Egr1/Zif268	Early growth response protein/ zinc finger protein 268
E-LTP	Early phase long-term potentiation
GAD67	Glutamic acid decarboxylase
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI-LTP	Glycine-induced long-term potentiation
GluA1	Glutamate receptor 1
GPCR	G-protein coupled receptor
GrDG	Granular layer of dentate gyrus
gRNA	Guide RNA
HBSS	Hank's Balanced Salt Solution
HD	Huntington's disease
HEK293T	Human Kidney Embryonic clone 293 expressing SV40 large T-antigen
HPRT1	Hypoxanthine guanine phosphoribosyl transferase-1
IEG	Immediate early genes
Indels	Insertions/deletions
KID	Kinase-inducible domain
KO	Knockout
KynA	Kynurenic acid
LTD	Long-term depression
LTF	Long-term facilitation
LTM	Long-term memory
L-LTP	Late phase long-term potentiation
MAP2	Microtubule associated protein 2
MARK	Microtubule affinity regulating kinase
MEF2	Myocyte enhancer factor 2
mEPSC	Miniature excitatory postsynaptic current
mGluR	Metabotropic glutamate receptor
mIPSC	Miniature inhibitory postsynaptic current
mRNA	Messenger RNA

MWM	Morris water maze
NHEJ	Non-homologous end joining
NMDAR	N-methyl-D-aspartate receptor
NPAS4	Neuronal PAS domain protein 4
P[number]	Post natal day
p300	Histone acetyltransferase p300
pCREB	Phosphorylated CREB
PAK4	p21-activated kinase 4
PKA	Protein kinase A
PAM	Protospace adjacent motif
PFA	Paraformaldehyde
PoDG	Polymorph layer of dentate gyrus
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PSD95	Postsynaptic density protein 95
qPCR	Real-time polymerase chain reaction
SIK	Salt-inducible kinase
T7E1	T7 endonuclease 1
TTX	Tetradotoxin
TUJ1	Neuron-specific class III beta-tubulin
TX-100	Triton X-100
WT	Wildtype

1 INTRODUCTION

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1.1 Long-term memory

The highly interconnected circuitry of the human brain is made up of dense networks of synaptically-connected neurons capable of experience-dependent rewiring in response to a wide range of stimuli. A single neuron, on average, makes between 1000 to 7000 synaptic connections with other neurons (Russell, Hertz, & McMillan, 2013). Depending on the inputs it receives, a neuron can alter its connectivity within a neural network by either strengthening or weakening its synaptic connections (Holtmaat & Svoboda, 2009; Kasai et al., 2010).

Memory is regarded as the capacity to retain learned information, can be divided into two distinct groups based on the duration in which this information is stored: short- and long-term (Squire, 2016). Short-term memory, as the name implies, is the retention of information for a brief interval without any persistent neural changes (Jonides et al., 2008). Conversely, long-term memory formation involves an experience or event that can lead to a dynamic signalling cascade which produces long-lasting chemical, electrical, and structural changes in the brain (Bailey, Bartsch, & Kandel, 1996). One well-characterised differentiation between the two is that the latter is dependent on transcription and translation (Alberini, 2009; Kandel, 2004); hence a disruption to either RNA or protein synthesis before or immediately after training can impair long-term memory formation (Davis & Squire, 1984).

1.1.1 Transcription-dependent plasticity during encoding of long-term memories

Studies in invertebrates (snails and crabs) and mammals (mice and rats) involving the use of transcriptional inhibitors such as actinomycin D (ActD) blocked mRNA synthesis around the time of training robustly inhibits retention of long-term memory. These pioneering experiments demonstrated the necessity of mRNA synthesis during learning and memory (Brink, Davis, & Agranoff, 1966; Igaz, Vianna, Medina, & Izquierdo, 2002; Neale, Klinger, & Agranoff, 1973; Pedreira, Dimant, & Maldonado, 1996; Quevedo et al., 1999; Sangha, Scheibstock, & Lukowiak, 2003; Squire & Barondes, 1970; Thut

& Lindell, 1974; Wetzel, Ott, & Matthies, 1976). However, transcriptional inhibitors such as ActD can sometimes be toxic at high doses with known side effects (Rainbow, 1979; Wetzel et al., 1976). Besides ActD experiments, injections of adenosine analogue 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) or the RNA polymerase II inhibitor α -amanitin into the CA1 regions of the rat hippocampus also revealed the importance of transcription in establishing long-term spatial memory (Da Silva et al., 2008). In other studies looking at the temporal requirement for transcription, transcription-dependent long-term plasticity occurred at two time points: the first time point being at the time of training, and the second being 3 – 6 hours after training. These time points coincided with those of translation, indicating that multiple phases of transcription are often accompanied by translation (Igaz et al., 2002; Quevedo et al., 1999).

Apart from transcription, protein synthesis was also shown to be a critical contributor to long-term memory. Evidence from multiple publications showed that the infusion of translational inhibitors such as anisomycin and cycloheximide different brain regions (particularly in the amygdala and hippocampus) impaired long-term memory in association to their tasks measured (Artinian et al., 2008; Bourchouladze et al., 1998; Gafford, Parsons, & Helmstetter, 2011; Jarome & Helmstetter, 2014; Jobim, Pedrosa, Christoff, et al., 2012; Jobim, Pedrosa, Werenicz, et al., 2012; Nakajima, 1975; Parsons, Gafford, & Helmstetter, 2006; Rossato et al., 2007; Taubenfeld, Milekic, Monti, & Alberini, 2001). Besides somatic translation, local translation near the synapses is also critical for long-lasting synaptic plasticity (Cajigas, Will, & Schuman, 2010; Fernandez-Moya, Bauer, & Kiebler, 2014; Khlebodarova, Kogai, Trifonova, & Likhoshvai, 2018). The very first piece of evidence to illustrate the necessity of local protein synthesis in mediating plasticity came from Kang and Schuman in 1996 when they showed that local treatment of the neuropil with BDNF in the presence of anisomycin in hippocampal slices failed to induce BDNF-LTP (Kang & Schuman, 1996). In a different approach, Huber and colleagues showed that mGluR LTD also requires local protein synthesis in the neuropil region of the hippocampus (Huber, Kayser, & Bear, 2000).

At present, long-term potentiation (LTP) of CA3-CA1 synapses is widely accepted as a cellular mechanism to model memory formation in the mammalian hippocampus (Lynch, 2004; Neves, Cooke, & Bliss, 2008). It is important to note that LTP does not

model all forms of memory in the brain; the best described and well-studied form of potentiation is found in the CA3 – CA1 Schaffer-collateral connection in the hippocampus, an area critical for the formation of declarative memory (Kumar, 2011; Nicoll, 2017). In hippocampal slice studies, LTP can be separated into two physiological states: the early-phase (E-LTP) which is dependent on post-translational modifications of pre-existing proteins and a late-phase (L-LTP) requires transcription and translation of new proteins (Bliss & Collingridge, 1993; Goelet, Castellucci, Schacher, & Kandel, 1986; Kandel, 2004). E-LTP and L-LTP are thought to be cellular correlates for both short and long-term memories, respectively. Importantly, just like long-term memory formation, the addition of transcriptional and translational inhibitors actively blocks L-LTP, but not E-LTP, suggesting that L-LTP is transcription and translation-dependent (Govindarajan, Kelleher, & Tonegawa, 2006; Hagen & Manahan-Vaughan, 2013).

However, some forms of memories were thought to be transcription-independent – an inconsistent finding that may be attributed to slight differences in experimental setup or condition (Lee, Everitt, & Thomas, 2004; Merschbaecher, Hatko, Folz, & Mueller, 2016; Parsons, Gafford, Baruch, Riedner, & Helmstetter, 2006). Nevertheless, transcription is still an essential step since long-term memory requires *de novo* gene expression (Alberini & Kandel, 2015; Bekinschtein et al., 2007; Scharf et al., 2017). The requirement of new gene expression to encode long-term memories, therefore, underscores the importance of identifying and understanding not only the transcriptional response but also the identity and function of these new gene products and how they alter neuronal function during the experience-dependent rewiring of the brain.

1.1.2 Expression of Immediate Early Genes during learning and memory

Activity-dependent transcriptional regulation in neurons is a highly complex, orchestrated process that involves the interaction of a diverse set of transcriptional factors that may either act to activate or repress transcription, cofactors as well as other regulatory proteins during transcription (Alberini & Kandel, 2015). The expression of many activity-dependent immediate early genes (IEGs) during learning and memory are governed by a handful of master transcription factors that are responsive to neural activity such as cAMP Response Element Binding protein (CREB), Myeloid Elf-1-like factor (MEF), and Serum Response Factor (SRF) (Etkin et al., 2006; Nonaka, Kim,

Sharpy, et al., 2014; Ramanan et al., 2005; Rashid, Cole, & Josselyn, 2014; Sakamoto, Karelina, & Obrietan, 2011). These major transcription factors regulate the expression of many of these early and late IEGs. For example, *Arc* is transcribed during neuronal activity (Kim, Kim, & Um, 2018; Korb & Finkbeiner, 2011; Shepherd & Bear, 2011) and the mRNA is shuttled to distal dendrites and synapses upon activity-dependent transcription (Flavell & Greenberg, 2008; Steward, Farris, Pirbhoy, Darnell, & Driesche, 2015; Steward, Wallace, Lyford, & Worley, 1998). *Arc* is locally translated and has been shown to maintain synaptic resources during synaptic plasticity through the regulation of AMPA receptors expression (Shepherd et al., 2006). More recently, El-Boustani and colleagues showed that synapses with enriched *Arc* were able to trigger more AMPA receptor expression while weakening neighbouring synapses resulted in fewer AMPA receptors distribution (El-Boustani et al., 2018).

Another transcription factor regulated as an IEG is the zinc-finger protein, Zif268 (or *Egr1*) (Duclot & Kabbaj, 2017; Guzowski, Setlow, Wagner, & McGaugh, 2001). Its mRNA expression is induced by LTP and is necessary for memory formation (Jones et al., 2001; Penke et al., 2014). It was shown that Zif268 knockout impairs long- but not short-term memory, whereas the overexpression of Zif268 facilitates long-term memory (Jones, French, Bliss, & Rosenblum, 1999; Renaudineau, Poucet, Laroche, Davis, & Save, 2009). Another increasingly important IEG, neuronal Per-Arnt-Sim (PAS) homology factor 4 (*Npas4*) functions to maintain a homeostatic balance by modulating both excitatory and inhibitory synapses (Kim et al., 2010; Lin et al., 2008). While it has been shown that NPAS4 has an indirect impact on plasticity via *Bdnf* and *Narp* (Bloodgood, Sharma, Browne, Trepman, & Greenberg, 2013; Chang et al., 2010; Greenberg, Xu, Lu, & Hempstead, 2009), its exact role in the synaptic plasticity is far from being understood (Sun & Lin, 2016). Reduction in NPAS4 showed an impairment to fear memories and the re-expression of which restored this memory formation (Ploski, Monsey, Nguyen, DiLeone, & Schafe, 2011; Ramamoorthi et al., 2011).

The expression of IEGs, many of which encode transcription factors, are responsible for regulating gene expression. *Npas4* previously described is a transcription factor. Another member of the bZIP superfamily of transcription factor is the activating protein-1 (AP-1). This protein-protein complex is made up of either homodimers or heterodimers between members of Fos and Jun families (Hai & Hartman, 2001;

Herdegen & Leah, 1998). *cFos* expression typically correlates with the induction of neuronal activation as its expression peaks after training (Bertaina-Anglade, Tramu, & Destrade, 2000) and has been shown to be crucial for the formation of appetitive memory (Countryman, Orłowski, Brightwell, Oskowitz, & Colombo, 2005; Smith, Countryman, Sahuque, & Colombo, 2007). In contrast, c-Jun expression reportedly increases during both short- and long-term memories (Abraham et al., 1993; Wisden et al., 1990). It is also found to be upregulated in some instances of LTP and/or LTD (Cole, Abu-Shakra, Saffen, Baraban, & Worley, 1990; Worley et al., 1993).

1.1.3 CREB is a member of the bZIP family of transcription factors

One of the earliest identified transcriptional regulators of IEG expression to be implicated in learning and memory is CREB (Dash, Hochner, & Kandel, 1990; Kandel, Dudai, & Mayford, 2014; Yin & Tully, 1996) – a well-characterised and ubiquitously-expressed transcription factor in brain cells. It is essential for regulating gene transcription that supports learning and memory and belongs to a family of basic leucine zipper (bZIP) transcription factors that bind to highly conserved palindromic sequences TGACGTCA, which are referred as cAMP response elements (CRE). CRE elements are found upstream in the promoter regions to drive activity-dependent immediate early gene expression during memory formation (Comb, Birnberg, Seasholtz, Herbert, & Goodman, 1986; Montminy, Sevarino, Wagner, Mandel, & Goodman, 1986).

The CREB family is made up of three homologous members: CREB, cAMP Response Element Modulator (CREM), and activating transcription factor-1 (ATF-1). Both CREB and ATF-1 are ubiquitously-expressed throughout the body, whereas CREMs are mainly found in the neuroendocrine tissues (for CREM and ATF-1 reviews: (De Cesare & Sassone-Corsi, 2000; Lonze & Ginty, 2002; Montminy, 1997). The most abundant CREB isoforms, CREB α and CREB Δ , contain four highly conserved domains – the C-terminal basic leucine zipper domain (bZIP) that mediates the binding to specific regulatory sequences. The bZIP comprises two juxtaposed regions where one is enriched with basic amino acids, and the other has a leucine residue at every seventh position so that it creates a leucine-enriched surface that mediates dimerisation of the bZIP family of proteins. However, whether these proteins bind as homo-dimers or

hetero-dimers depends mainly on the sequences of their bZIP domains (Cristina M Alberini, 2009).

The N-terminus, on the other hand, contains a bipartite transcriptional activation domain: glutamine-rich Q1 and Q2 constitutive active domain (CAD) that flanks the kinase-inducible domain (KID) (Lonze & Ginty, 2002; Shaywitz & Greenberg, 1999; Yamamoto, Gonzalez, Biggs, & Montminy, 1988). These glutamine-enriched domains (Q1 and Q2) interact with TAF4 (TBD associated factor 4) for the recruitment of basal transcription machinery at the promoter region (Barco, Pittenger, & Kandel, 2003; Ferreri, Gill, & Montminy, 1994; Quinn, 1993; Xing & Quinn, 1994) whereas the KID contains the critical residue, serine 133 (S133), which when phosphorylated makes it a binding target for transcriptional coactivators required for CREB transcription of downstream target genes (Figure 1-1).

Figure 1-1: Domain organisation of CREB and family members

Schematic illustration showing domain structure of CREB and its family of transcriptional factors (adapted from Mayr & Montminy, 2001).

1.1.4 CREB signalling: calcium & cAMP-induced signalling

While phosphorylation at S133 is required for CREB to be active, S133 does not lead to the prediction of any transcriptional output (Giebler, Lemasson, & Nyborg, 2000; Kornhauser et al., 2002; Lau & Suh, 2017). In neurons, CREB phosphorylation is dynamically initiated by a wide variety of stimuli, including synaptic activity, depolarisation, stress, and hypoxia – all of which results in the intracellular increase of calcium and cAMP levels (Flavell & Greenberg, 2008). Early studies demonstrated that CREB can be activated in a Ca^{2+} -dependent manner, which is brought about by two pathways: first, the influx of calcium through voltage-sensitive calcium channels (VSCC) created from membrane depolarisation or glutamatergic NMDAR-mediated synaptic transmission allows the entry of calcium to interact with its binding protein, calmodulin (CaM) (Dash, Karl, Colicos, Prywes, & Kandel, 1991; Sheng, Thompson, & Greenberg, 1991). The binding of calcium and CaM activate CaMKII and CaMKIV to phosphorylate CREB *in vitro* and *in vivo* (Bito, Deisseroth, & Tsien, 1996; Kang et al., 2001; West et al., 2001). Second, calcium apart from activating CaMKs, is also capable of triggering the activation of Ras/ERK or Ras/MAPK to catalyse the phosphorylation of CREB at S133 (Bading & Greenberg, 1991; Sweatt, 2001; Dolmetsch, Pajvani, Fife, Spotts, & Greenberg, 2001; Rosen, Ginty, Weber, & Greenberg, 1994).

Alternatively, negative feedback regulation exists to limit calcium/CaM-induced CREB phosphorylation. It is thought that calcium/CaM signalling results in the induction of PP2B/calcineurin, and the subsequent activation of protein phosphatases PP1 and PP2A. These phosphatases inhibit CREB gene transcription by dephosphorylating CREB at S133 (Alberts, Montminy, Shenolikar, & Feramisco, 1994; Bito et al., 1996; Genoux et al., 2002; Hagiwara et al., 1992; Wadzinski et al., 1993). One such example is illustrated with extrasynaptic NR2B-containing NMDARs, which activates PP1 to initiate CREB shut-off (Hardingham, Fukunaga, & Bading, 2002). Additionally, CaMKII (Matthews et al., 1994) or CaMKIV (Ghosh, Ginty, Bading, & Greenberg, 1994) phosphorylation of CREB at S142 and S143 results in the dissociation of CREB dimers, thereby inhibiting CREB activity. The second pathway for inducing CREB activation is through the increase in intracellular cAMP levels via the stimulation of G-protein coupled receptors (GPCRs) (Kandel, 2004) (Figure 1-2) and the subsequent activation of

adenylyl cyclase (AC) (Poser & Storm, 2001). The activated AC then converts adenosine triphosphate (ATP) to the second messenger cAMP, which causes the release of PKA in the cytosol. PKA then translocates into the nucleus where it binds and phosphorylates CREB. It was later discovered that cAMP activity not only regulated PKA but also resulted in the phosphorylation of S133 on CREB which was critical for downstream transcription activation of CREB genes (Gonzalez & Montminy, 1989; Yamamoto et al., 1988). Together, these Ca^{2+} and cAMP signalling mechanisms represent important signalling measures of controlling the kinetics of CREB transcriptional activity in the contribution of mediating CREB-dependent gene expression.

Figure 1-2: Mechanism of CREB activation and CRTC nucleocytoplasmic shuttling

When a signal (for example, a hormone stimulus) arrives at the cell surface, it binds to GPCR and triggers the activation of AC to catalyse the conversion of ATP to cAMP. A rise in cellular levels of cAMP stimulates PKA signalling. Binding of cAMP to regulatory (R) subunits of PKA results in the liberation of catalytic subunits, during which a conformational change causes PKA to become active. Active PKA catalytic subunits then shuttle into the nucleus via passive diffusion and phosphorylate CREB on S133. Nuclear translocation of CRTC determines the transcriptional status (adapted from Altarejos & Montminy, 2011).

1.1.5 CREB function in long-term memory

Studies from the invertebrate *Aplysia californica* have advanced our knowledge of CREB in short- and long-term memory. When a stimulus such as a mild tail shock is delivered to the siphon area of an *Aplysia*, the animal learns by withdrawing its gill as a form of defensive reflex (Hawkins, Cohen, Greene, & Kandel, 1998). The duration of this response, however, is analogous to long-term memory in mammals (Mayford, Siegelbaum, & Kandel, 2012). It was discovered that a single shock lasting for as short as few minutes can induce short-term sensitisation of the gill requiring post-translational modifications, whereas multiple shocks at spaced intervals induced long-term sensitisation lasting up to weeks, is dependent on *de novo* gene expression (Kandel, 1989).

Both short- and long-term sensitisation were later reproduced *in vitro* using applications of serotonin, a neurotransmitter found to mediate sensitisation (Brunelli, Castellucci, & Kandel, 1976). A single application of serotonin failed to recapitulate long-term sensitisation (Frost, Castellucci, Hawkins, & Kandel, 2006; Montarolo et al., 1986), while repeated applications of serotonin produced long-term sensitisation that lasts for days (Martin et al., 1997; Michael et al., 2002). Both short- and long-term sensitisation induced by serotonin were found to have depended on the cAMP/PKA signalling pathway with the exception of only long-term sensitisation requiring both transcription and translation (Castellucci et al., 1986; Montarolo et al., 1986).

Subsequently, microinjection of CRE binding sites into the nucleus of *Aplysia* sensory neurons competed with endogenous CRE binding, thereby blocking long-term facilitation (LTF; the synaptic strengthening thought to underlie sensitisation) induced by multiple pulses of serotonin (Dash et al., 1990). It was this study that showed CRE-dependent gene expression is essential for LTF but not short-term facilitation (STF). Moreover, it was found that calcium influx and activation of cAMP pathways triggered CREB phosphorylation (Dash et al., 1991; Sheng et al., 1991) which are all critical molecular drivers of CREB-dependent gene transcription leading to the induction of LTF in *Aplysia* (Kaang, Kandel, & Grant, 1993; Kandel, 2004).

Genetic studies in other animal models also revealed CREB's importance in learning and memory. The study of CREB mutants was first demonstrated in *Drosophila* using genetic methods to manipulate CREB activity. Dominant negative CREB was generated to conditionally express the inhibitory isoform of CREB (dCREB2-b) (Yin et al., 1994). Expression of the CREB repressor was placed under the control of a heat-shock (hs) promoter (hs-dCREB2-b) to examine whether disruption to CREB activity affects long-term memory formation in flies (Yin et al., 1994). When flies were trained to associate one of two odours coupled to shock, this group of researchers found that heat-shock induced flies expressing the CREB repressor were unable to form long-term memory (Yin et al., 1994).

On the contrary, flies that were not induced by heat-shock retained their normal long-term memory for the task (Yin et al., 1994). This study suggested that CREB-induced transcription is necessary for long-term memory in *Drosophila*. The same group then tried to recapitulate the experiment as above with a CREB activator isoform instead (dCREB2-a) (Yin, Del Vecchio, Zhou, & Tully, 1995). In this setup, flies were either spaced trained in which there is a 15 minute of rest interval for every ten sessions of training, or massed trained where there are zero intervals between training sessions. As expected, the conditional induction of CREB activator allowed these flies to form long-term memory following massed-training. A comparison between spaced and massed training revealed that there was no discernible difference in the strength of the long-term memory developed (Yin, Del Vecchio, Zhou, & Tully, 1995), suggesting that the enhancement of CREB expression alone does not produce strengthened memory but rather induced a memory with less training.

In *Aplysia*, not only does blocking the activity of CREB repressor (ApCREB2) showed an increased in the expression of CREB activator (ApCREB1), LTF was also induced at the sensory-motor synapse with a single application of serotonin as opposed to five spaced applications (Bartsch et al., 1995). These loss- and gain-of-function studies indicated that CREB served as a molecular switch for long-term memory. Findings from *Aplysia* and *Drosophila* led to the question of how different CREB isoforms can contribute to long-term memory. Hence, mice lacking brain-specific CREB isoforms were generated (in which two of three isoforms α and Δ were ubiquitously deleted), and

these mice showed deficits in learning and memory as well as neurodegeneration (Bourtchuladze et al., 1994; Mantamadiotis et al., 2002); the deletion of all isoforms in mice, however, is proven to be embryonically lethal (Rudolph et al., 1998). CREB ^{α/Δ} knockout mice retaining only the β isoform develops into adulthood; the deletion of CREB ^{α/Δ} results in the upregulation of CREB β but also CREM activator (τ) and repressor (α and β) isoforms (Blendy, Kaestner, Schmid, Gass, & Schutz, 1996).

Despite these upregulation, these CREB ^{α/Δ} mice were later established to have virtually non-existent CRE-DNA binding (about > 90 % were abolished) (Pandey, Mittal, & Silva, 2000; Walters & Blendy, 2001) and their CREB protein expression was reduced by 80-90% relative to basal expression (Walters, Kuo, & Blendy, 2003). Conversely, overexpression of CREB has led to an increased in cognitive tasks and enhanced the strength of new memories without compromising memory precision (Barco, Alarcon, & Kandel, 2002; Suzuki et al., 2011). Furthermore, Yiu et al. showed that neurons overexpressing CREB are selected in memory trace to improve memory formation (Yiu et al., 2014).

Studies using hippocampal slices from CREB ^{α/Δ} knockout mice also revealed an impairment of long-term potentiation (LTP) in these mice (Bourtchuladze et al., 1994). LTP is a type of activity-dependent synaptic paradigm for studying learning and memory *in vitro* and *in vivo* (Nicoll & Malenka, 1999; Sigurdsson, Doyère, Cain, & LeDoux, 2007). LTP is divided into two phases: the early phase (or E-LTP) and the late phase (or L-LTP). E-LTP mostly involves post-translational modifications of proteins and quickly decays over time even under high frequency trains of stimulation (HFS) whereas L-LTP is believed to be dependent on both transcription and translation with the potential to persist for hours and even weeks (Frey, Krug, Reymann, & Matthies, 1988; Osten, Valsamis, Harris, & Sacktor, 1996; Tsokas, 2005).

There are, however, contrasting reports indicating the insufficiency of conditional disruption of all CREB isoforms to impair LTP and long-term depression (LTD) (Balschun et al., 2003). Moreover, in another separate report, a conditional mouse model in which functions of all CREB subfamilies were inhibited in CA1 neurons of the dorsal hippocampus showed the disruption to forskolin-induced and dopamine-regulated

potentiation (Pittenger et al., 2002). Despite these discrepancies, the nature of disruption in classical knockout models and genetic manipulations do have drawbacks and these can be explained by the potential for developmental compensation for the deleted gene through the upregulation of other related transcriptional regulators (Cole & Josselyn, 2008) or that some forms of memories and LTP are just CREB-independent (Alberini, 2009). Therefore, the conclusion that CREB is a crucial mediator of long-term memory still holds true.

1.1.6 CREB coactivators

Canonically, CREB activation in neurons is most commonly viewed through the phosphorylation at S133. However, CREB phosphorylation can also occur on other residues (*e.g.* S129, S121) found at the kinase-inducible domain (KID) (Moens, 2007). The long-held dogma in the field of CREB transcription suggests that phosphorylation of S133 is necessary and sufficient for CREB mediated transcription of CRE target gene (Giebler et al., 2000; Kornhauser et al., 2002; Sun, Means, LeMagueresse, & Means, 1995). However, in recent years, several papers have shown that S133 phosphorylation while critical for CREB activity, does not always correlate to CREB-mediated transcription (Briand, Lee, Lelay, Kaestner, & Blendy, 2015; Impey et al., 1996; Mayr & Montminy, 2001; Shaywitz & Greenberg, 1999), suggesting that other mechanisms exist for driving CRE-dependent transcriptional mechanisms during plasticity as well as memory formation.

Since CREB respond to a wide range of stimuli, different stimuli recruit different cofactors or coactivators that can alter CREB transcriptional activity (Conkright et al., 2003; Goodman & Smolik, 2000; Mayr & Montminy, 2001; Sreaton et al., 2004; Vo & Goodman, 2001). CREB-binding protein (CBP) and its paralogue p300 is a CREB coactivator that is recruited to promoter regions containing CRE sequences by activated CREB (Chrivia et al., 1993; Montminy & Bilezikjian, 1987; Parker & Montminy, 1996). Both CBP and p300 binds to the KID on CREB via their KIX (KID interaction) domains and triggers transcription by functioning as scaffolding proteins (recruiting other components of the transcriptional machinery) and as histone acetyltransferases (reducing the affinity of histones to DNA, rendering the accessibility of transcription factors to the DNA) (Bannister & Kouzarides, 1996; Nakajima et al., 1997; Vo &

Goodman, 2001). Phosphorylation sites within the KID domain not only modulate transcriptional responses to signals but may also promote S133 CREB activation, except that the biological contexts to which these accessory sites can regulate CREB activity have yet to be clearly defined. As such, future gain- or loss-of-function studies at these sites could provide further insight into this process.

1.2 CREB regulated transcriptional co-activator 1 (CRTC1)

The notion that CREB can be activated independently of S133 phosphorylation led to the discovery of CREB-regulated transcriptional coactivators (CRTCs) (Conkright et al., 2003; Iourgenko et al., 2003). Iourgenko and colleagues performed an *in vitro* screen to isolate cellular factors that could enhance CREB transcription. Their studies isolated CRTC family of proteins that undergo nuclear import to drive CRE-dependent target gene expression even in the absence of phosphorylation at S133 (Conkright et al., 2003; Nonaka, Kim, Fukushima, et al., 2014). The CRTC family of coactivators consists of three members in mammals: CRTC1, CRTC2 and CRTC3, with CRTC1 being most abundantly expressed in the brain, particularly in the hippocampus and cortex (Kovacs et al., 2007; Li, Zhang, Takemori, Zhou, & Xiong, 2009; Watts, Sanchez-Watts, Liu, & Aguilera, 2011).

Much of the research in the CRTC family in the brain has focused mainly on CRTC1 even though CRTC2 and CRTC3 share 32% homology with CRTC1 (Deisseroth, Mermelstein, Xia, & Tsien, 2003); mRNA expression profile reveals lower expression of CRTC2 in the brain in relation to CRTC1, but a much higher expression in the liver as well as the β -islet of the pancreas (Watts et al., 2011; Data not shown). Meanwhile, CRTC3 is found in trace amounts in the brain, but it appears to have a similar distribution as CRTC2 (Than, Lou, Ji, Win, & Kaplowitz, 2011), although it can be found in adipocytes as well (Prats-Puig et al., 2016).

1.2.1 Structure of CRTC1 and regulatory mechanism in neurons

CRTC members are evolutionarily conserved across different species such as *Drosophila melanogaster* and *Caenorhabditis elegans*. Despite different expression profiles in various tissues and organisms, the CRTC family of proteins share strong

homology in several conserved regions: N-terminal coil-coil CREB-binding domain (CBD), central regulatory (REG) domain, and a 200-amino acid C-terminal transactivation domain (Altarejos & Montminy, 2011). Unlike coactivators CBP/p300 where interaction occurs directly with phosphorylated S133 within the KID domain of CREB, CRTCs bind to its bZIP domain to facilitate CREB dimerisation and DNA binding.

The bZIP domain-binding CRTC family of transcriptional coactivators can dramatically upregulate CREB-target gene expression in the absence of phosphorylation at S133 (Li, Zhang, Takemori, Zhou, & Xiong, 2009; Liang et al., 2016; Parra-Damas, Rubió-Ferrarons, Shen, & Saura, 2017). CRTCs-mediated activation of CREB is stimulus-dependent since its nuclear localisation is dependent on the phosphorylation statuses (Bittinger et al., 2004; Sreaton et al., 2004). In hippocampal excitatory neurons, CRTC1 is localised mainly in the soma, dendrites and spines of electrically-silent excitatory neurons where it is tethered to 14-3-3 ϵ cytoplasmic anchoring proteins (Ch'ng et al., 2012). Upon NMDAR-mediated synaptic stimulation, increases in intracellular calcium trigger the dephosphorylation of the said coactivator by PP2B/calcineurin. Additionally, cAMP/PKA inhibits the activity of members of the AMP-activated protein (AMPK) family (including the salt-inducible kinases, SIKs), microtubule affinity regulating kinases (MARKs) from phosphorylating CRTC1, resulting in the dissociation of 14-3-3 ϵ and a subsequent nuclear translocation (Figure 1-3).

In non-neuronal cells, basal CREB transcription is low, and the presence of CRTC1 in the nucleus dramatically enhances CREB-driven transcription by more than several hundred folds (Conkright et al., 2003). In cultured cortical neurons, efficient CREB transcription requires association with CRTC1 (Parra-Damas, Rubió-Ferrarons, et al., 2017). Previous mass spectrometry data revealed a complex CRTC1 phosphorylation profile of at least 50 phosphorylated residues, among which S151 was thought to be the determinant of its subcellular localisation (Altarejos et al., 2008). While calcineurin-mediated dephosphorylation of S151 did not always correspond to CRTC1 nuclear translocation, one group established the involvement of two additional regulatory phosphorylation sites: S64 and S245 (Ch'ng et al., 2015). These sites seem to confer a specific binding affinity of each CRTC member and 14-3-3 (Sonntag et al., 2017).

Despite the complex nature of post-translational modifications, we still do not have a complete understanding of the underlying cellular and molecular mechanisms of its synapse-to-nucleus signalling but what is clear is that CRTTC1 serves as a transducer of various synaptic signals in the nucleus for the regulation of CREB-driven gene expression during long-term plasticity.

Figure 1-3: Domain organisation of CRTTC1

CRTTC1 co-activator is a serine/threonine-rich protein with CBD region – a coiled-coil domain that allows binding to CREB bZIP domain; the nuclear localisation signal, NLS; the regulatory domain consisting of multiple phosphorylation sites; the nuclear export signal, NES and the transactivation domain, TAD (adapted from Saura & Cardinaux, 2017).

1.2.2 CRTTC1 function in synaptic plasticity

Several studies have demonstrated the importance of CREB-dependent transcription during synaptic plasticity (Benito & Barco, 2010; Cohen & Greenberg, 2008; Kandel, 2004; Silva, Kogan, Frankland, & Kida, 1998; Yin & Tully, 1996) but there are now emerging evidence to support the involvement of CRTTC1 in synaptic plasticity, including the maintenance of hippocampal-dependent L-LTP in mouse hippocampal slices (Kovacs et al., 2007; Uchida et al., 2017; Y. Zhou et al., 2006).

Congruent with its role in the maintenance of L-LTP, the presence of nuclear CRTTC1 facilitates the induction of L-LTP in hippocampal slices while the dominant negative form of CRTTC1 suppresses it (Wu, Zhou, & Xiong, 2007; Zhou et al.,

2006). In behavioural experiments, increased in CRTTC1 or CREB function in mice hippocampal region is adequate to enhance memory stabilisation as well as memory retrieval without any effect on memory quality during fear conditioning assay (Sekeress et al., 2012). This evidence thus suggests a region-specific role for CRTTC1 in the regulation of long-term memory. Work from Uchida and colleagues have also shown that mouse hippocampal CRTTC1 is capable of inducing chromatin changes upon associative learning and the loss of CRTTC1 impacts that process. However, the overexpression of nuclear CRTTC1 reverses this phenotypic deficit, suggesting that CRTTC1 nuclear import is crucial for sustaining target gene transcription and enhancing memory strength (Uchida et al., 2017).

In *Drosophila*, there is only a single member of CRTTC, which regulates a form of long-term appetitive memory in the mushroom body when flies were subjected to food deprivation (Hirano et al., 2013). Fasting negatively regulates the insulin signalling pathway in which under normal conditions, would result in SIK2-induced phosphorylation of CRTTC, thereby inactivating CRTTC (Hirano et al., 2013). However, constitutively active CRTTC simulates nuclear CRTTC, which is shown to be necessary for the formation of long-term memory and the extension of memory extinction (Hirano et al., 2016). Progression from memory formation to memory maintenance involves histone acetylation and the shift in the transcriptional complex of CREB/CBP to CRTTC/CBP (Hirano et al., 2016). Following a CREB-dependent mechanism, it is expected that any disruption to this CREB/CRTTC1 association will potentially impair CREB-dependent transcription, synaptic plasticity and long-term memory.

Transcriptionally, activity-dependent nuclear entry of CRTTC1 in hippocampal and cortical neurons can selectively upregulate a subset of CREB-target genes, among which includes immediate early genes (IEG) *cFos*, *Zif268/Egr1*, *Arc*, *Nr4a1* and *Nr4a2*, *Bdnf*, and the brain-specific growth factor *Fgf1*. Many of these genes play crucial roles in synaptic plasticity as well as memory formation (Breuillaud et al., 2012; Ch'ng et al., 2015; Ch'ng et al., 2012; Espana et al., 2010; Fukuchi et al., 2015; Nonaka, Kim, Fukushima, et al., 2014; Parra-Damas et al., 2014; Parra-Damas, Chen, et al., 2017; Uchida et al., 2017; Zhou et al., 2006). CRTTC1-mediated gene transcription is also linked to dendritic morphology – the overexpression of CRTTC1 in cultured rat cortical neurons showed an increase in basal as well as activity-induced dendritic length;

conversely, the dominant negative form of CRTC1 significantly reduced these dendritic growths (Finsterwald, Fiumelli, Cardinaux, & Martin, 2010). However, to date, there is no direct investigation on the role of CRTC1 knockout on transcription and its impact in neuronal function.

1.2.3 CRTC1 function in neurodegenerative diseases

Dysregulation of CREB function has been reported in several neurodegenerative diseases in humans and rodent models (Chaturvedi et al., 2012; Giralt et al., 2012; Jeong et al., 2012; Mendioroz et al., 2016; Parra-Damas et al., 2014; Saura & Valero, 2011; Won et al., 2016). Alzheimer's disease (AD), the leading cause of dementia is characterised by the abnormal accumulation of plaques and tangles in memory circuits. Presence of these β -amyloid peptides corresponded well with impaired CRTC1 function, thus suggests a causal link between the progression of AD, and disruption of CRTC1-CREB mediated transcription (Parra-Damas et al., 2014; Wilson et al., 2017). Impairment to the Ca^{2+} /calcineurin-mediated CRTC1 dephosphorylation led to reduced nuclear CRTC1 thereby affecting the occupancy of CREB-target gene promoters which resulted in the downregulation of neuroplasticity (Espana et al., 2010; Parra-Damas et al., 2014). On the other hand, overexpression of CRTC1 reverses memory impairments in AD, providing further evidence that increased CRTC1 activity is valuable to combat against cognitive declination in AD (Parra-Damas et al., 2014; Parra-Damas, Chen, et al., 2017).

In mouse models of Huntington's disease (HD), CRTC1-mediated transcription is compromised by defects in the huntingtin (*Htt*) gene. CRTC1-driven neuroprotective transcriptional response is aborted as well. The failure to promote CRTC1-mediated transcription led to motor deficits, which can be ameliorated through Sirt1 deacetylase by activating CRTC1 (Jeong et al., 2012). CRTC1 overexpression, on the other hand, increases transcription of peroxisome proliferator-activated receptor γ coactivator (PGC-1 α), which is important for mitochondrial biogenesis in HD pathogenesis (Chaturvedi et al., 2012). Finally, work by Won and colleagues showed that neuronal p21-activated kinase 4 (PAK4) mediates neuroprotection in rat models of Parkinson's disease (PD) by phosphorylating CRTC1 at S215 to induce CREB-target genes. Their results signify the importance of phosphorylated CRTC1 at S215 for PAK4

neuroprotection against motor dysfunction of dopaminergic neurons at the substantia nigra pars compacta caused by 6-hydroxydopamine (6-OHDA) and α -synuclein (Won et al., 2016). Collectively, CRTTC1 and CREB represent as attractive targets for novel therapies aimed to modulate synaptic plasticity, which may help to alleviate cognitive impairments.

1.3 Research Objectives

At the molecular level, overexpression of CRTTC1 or the inhibition of CREB activity via a dominant negative form of CRTTC1 corresponds to either the strengthening or the disrupting of memory. Yet the molecular mechanism of how CRTTC1 impair memories are not well understood. Many gaps still exist for CRTTC1 function in hippocampal neurons. For example, we still do not know how the loss of CRTTC1 leads to CREB dysregulation. While CRTTC1 selectively impacts transcription of activity-dependent CREB target genes in cultured hippocampal neurons (Ch'ng et al., 2012), we are yet clear of how CRTTC1 drives expression of IEGs during long-term plasticity. Transcriptome studies in the context of studying long-term plasticity via RNA-seq in cultured hippocampal neurons have never been done. Thus, it would be interesting to profile the nuclear transcriptome of stimulated hippocampal neurons that lack CRTTC1 expression and compare that with wild-type (WT) hippocampal neurons for the identification of changes in gene expression that are attributed to CRTTC1. Apart from CREB, it is possible that CRTTC1 may bind to other bZIP transcription factors. CRTTC1 nuclear translocation has been associated with epilepsy (Dubey & Porter, 2016; Nishimura, Casanova, & Swann, 2017) and in a related study, mice deficient in PAR bZIP transcription factors were found to be highly seizure-prone (Gachon et al., 2004). Perhaps, there is a possible link between CRTTC1 and PAR bZIP transcription factor family during epileptogenesis.

Currently, there exists a germline CRTTC1 knockout mouse line generated from the gene trap approach (Breuillaud et al., 2012). These CRTTC1 deficient mice showed no signs of brain developmental deficits (Altarejos et al., 2008); however, they underscore the importance of CRTTC1 function in the nervous system (Meylan, Halfon, Magistretti, & Cardinaux, 2016). CRTTC1-null mice developed several metabolic deficits such as

infertility, hyperphagicity and obesity (Altarejos et al., 2008) as well as neurobehaviour disorders, including impulsive aggression, social withdrawal, and reduced sexual motivation (Breuillaud et al., 2012). These animals suffer from glucose and metabolic imbalance, and since energy homeostasis can impact memory performance, they cannot be used to study memory effects with high predictability and accuracy.

Presently, CRTTC1 gene knockout specifically in the hippocampus, has never been generated or studied in the context of learning and memory. Therefore, the overall goal for this project is to rigorously examine how the absence of CRTTC1 affects neuronal form and function at the cellular level. To achieve this, we will pursue the following aims:

- 1) Aim 1: Establish a viral-mediated CRISPR/cas9 gene knockout paradigm which allows *in vitro* and *in vivo* study of CRTTC1 protein function in the hippocampus
- 2) Aim 2: Assess the impact of CRTTC1 knockout on morphology in cultured hippocampal neuron as well as animal behaviour
- 3) Aim 3: Analyse the role of CRTTC1 in the regulation of gene expression during LTP and LTD in cultured hippocampal neurons

To address the first aim, we sought to knock out expression of CRTTC1 in hippocampal neurons using genome editing technology, in particular, CRISPR/Cas9. We will deliver CRISPR/Cas9 editing constructs via lentiviral transduction in dissociated hippocampal neurons as well as stereotaxic injection of these lentiviruses into the hippocampus of wild-type adult mice and verify the efficiency of the knockout using well-characterised antibodies against CRTTC1. We have decided to adopt the use of viral delivery approach for several benefits. Firstly, previous studies have shown that virally delivered CRISPR/Cas9 constructs can successfully knock out gene expression and disrupt brain function (Incontro, Asensio, Edwards, & Nicoll, 2014; Straub, Granger, Saulnier, & Sabatini, 2014; Swiech et al., 2015). Secondly, CRISPR-embedded lentiviruses can also be made to couple to epigenetic modifiers modified for altering gene expression without inducing DSBs. Moreover, directed evolution of SpCas9 to yield high fidelity forms have remarkably improved both fidelity and efficiency of gene editing while dramatically reducing off-target effects (Chavez et al., 2015; Kleinstiver et al., 2016).

CRISPR-driven knockout of target genes in neurons is efficient, though not complete and one of our largest caveat in the study will be off-target effects. To eliminate concerns for off-target effects, we have taken steps to address each of these issues by generating at least two independent gRNAs to target CRTTC1 to which we will rigorously test. It is possible that CRTTC1 knockout in hippocampal neurons can lead to upregulation of other CRTTC family members to compensate for the loss of CRTTC1 activity. However, according to Koo and colleagues, it appears that CRTTC2 is not able to compensate for loss of CRTTC1 function in *Crttc1*^{-/-} mice, indicating that gene targets and functions of each CRTTC gene may not coincide (Koo et al., 2005), in particular, CRTTC3, as its expression in the brain is almost non-existent. Finally, we recognise that some cognitive functions may not be regulated exclusively by the hippocampus and one benefit of using this lentiviral CRISPR to target specific genes for knockout is the flexibility of targeting other specific regions of the brain such as the amygdala.

Once we have established that CRTTC1 can be effectively knocked out in hippocampal neurons, our next aim is to understand the role of CRTTC1 in the hippocampus during long-term plasticity. Thus far, we have not come across any study that has examined the effects of knocking out CRTTC1 in hippocampal neurons. Therefore, it is of interest for us to know if hippocampal neurons lacking CRTTC1 expression exhibit any morphological deficits from WT hippocampal neurons. More specifically, we will examine if dendritic arborisation as well as the composition of pre- and postsynaptic structural markers are compromised. We have a good reason to examine spine morphology as our preliminary data using siRNA-mediated knockdown of CRTTC1 in dissociated hippocampal cultures indicate an altered synapse distribution as well as protein composition of the postsynaptic density (data not shown). With a knockout in mature hippocampal neurons via CRISPR/Cas9, we expect to see a more robust mutant phenotype.

While collective data involving the manipulation of CRTTC1 expression levels in the brain indicate that CRTTC1 is vital in the regulation of long-term plasticity, the actual impact of CRTTC1 function during memory formation and how it regulates gene expression crucial for long-term plasticity remains unclear. For example, no one has yet to explore changes in structural plasticity in the absence of CRTTC1. Therefore, we are also curious to ask whether deficits observed in CRTTC1 knockout hippocampal neurons

are activity-dependent or constitutively present. Dominant negative inhibition of CRTTC1 causes L-LTP failure, drawing the inference that LTP requires CRTTC1 (Zhou et al., 2006). However, it is possible that the dominant negative form of CRTTC1 may interfere with CREB's function; another reason why knocking out CRTTC1 will help delineate its function during long-term plasticity.

One of our immediate goals here is to ask if CRTTC1 contributes to long-term synaptic plasticity. Using our CRISPR viruses, we can eliminate CRTTC1 expression in a specific subpopulation of mature hippocampal neurons in dissociated cultures and examine whether CRTTC1 expression influences different forms of synaptic plasticity. Coupling this with whole-cell patch clamp, we will be able to monitor intrinsic electrophysiological properties of pyramidal neurons, including basal synaptic transmission and excitability. We are also interested in monitoring changes in other transcription-dependent events such as spontaneous neurotransmitter release by measuring miniature neurotransmission (Nelson, Kavalali, & Monteggia, 2008; Wiegert, Hofmann, Bading, & Bengtson, 2009).

Previous publication showed that theta burst stimuli at the Schaffer collateral pathway and tetrodotoxin (TTX) withdrawal causes robust nuclear accumulation of CRTTC1 (Ch'ng et al., 2012) but it is not known if physiological L-LTP and long-term memory stimulation paradigms are capable of triggering CRTTC1 dephosphorylation and nuclear translocation. As such, it is of interest for us to know how activity-dependent nuclear translocation of CRTTC1 in hippocampal neurons can trigger changes in expression of immediate early genes (IEGs) during long-term plasticity through a candidate approach. It will also be useful for us to profile the nuclear transcriptome of basal or stimulated hippocampal neurons that lack CRTTC1 expression and compare them to WT hippocampal neurons via whole-cell sequencing with RNA-Seq. To study long-term plasticity, we will be looking into inducing two forms of activity-dependent synaptic paradigms for studying learning and memory: long-term potentiation (LTP) as well as long-term depression (LTD). Calcineurin-mediated dephosphorylation of CRTTC1 is equally important in driving LTD, and with preliminary data showing that CRTTC1 responds to DHPG-mediated activation of mGluR receptors as well as chemical LTD stimulation. This result hints at a possible link between activation of calcineurin during LTD with translocation of CRTTC1 to the nucleus to trigger CREB transcription. By

subjecting hippocampal neurons to well-defined induction protocols, we will then be able to attribute these changes to loss of CRTCL1 during long-term plasticity.

2 MATERIALS AND METHODS

2.1 Animals

C57BL/6J mice were fed *ad libitum* and maintained in a temperature-controlled colony room of 20-25 °C with relative humidity between 70-80%. They have also been synchronised to a 12:12 light-dark cycle (lights on from 07:00 h to 19:00 h). Experiments were conducted during the light phase. All animal procedures were performed under the policies approved by Nanyang Technological University Institutional Animal Care and Use Committee (IACUC #E0019).

2.2 Dissociated primary hippocampal cultures

Unless otherwise stated, all experiments in this study employed the use of mature hippocampal neurons (DIV17 – 21) dissected from new-born (P0 – P3) C57BL/6J pups as previously described (Ch'ng et al., 2012). Briefly, hippocampi were dissected under a stereomicroscope (Stereo 508, Zeiss), placed in ice-cold 1x Hank's Balanced Salt Solution (HBSS, Invitrogen; supplemented with sodium pyruvate and HEPES) and dissociated with 2.5% Trypsin (containing 2.5M calcium chloride and DNase I). Next, neurons were cultured in Neurobasal-A media (Invitrogen); B27 supplement (Gibco); 125 µM GlutaMAX (Gmax; Gibco); 25 µM monosodium glutamate (MSG; Sigma); 25 µM β-mercaptoethanol (Sigma) and seeded on 0.1 mg/mL Poly-D-lysine-coated (Sigma) 12 mm glass coverslips (Marienfield Superior). Cells were maintained in a humidified incubator at 37°C supplied with 5% CO₂. Hippocampal neurons were fed once a week by removing a third of the old media and replaced with new media.

2.3 Mammalian cell cultures

HEK293T cells were maintained in Dulbecco's Modified Eagle Media (DMEM; Nacalai Tesque) supplied with 10% fetal bovine serum (FBS; Hyclone) and 1% Penicillin/Streptomycin (Invitrogen) in a 37°C humidified incubator with 5% CO₂.

2.4 Plasmid Transfection

Cells were seeded in 6-well plates coated with 0.1 mg/mL Poly-D-Lysine (Sigma) at a density of 5×10^5 per well. Cells were transiently transfected with plasmid DNA using DNAfectin (ABM) with protocols recommended by the manufacturer. Briefly, HEK293T cells were transfected at about 70 – 80 % confluency. Reconstituted EGFP fluorescence was observed at 24 h and 48 h after transfection.

2.5 Plasmid Expression Constructs

The human codon-optimised SpCas9 chimeric gRNA expression vector, pX330, was a gift from Feng Zhang (Addgene plasmid #42230) while the pCAG-EGxxFP was a gift from Masahito Ikawa (Addgene plasmid #50716); commercial plasmid includes dendra2 (Evrogen). To generate the desired gRNA plasmids, a pair of targeting oligos of 20 base pairs long were phosphorylated, annealed and inserted into BbsI site of pX330 using standard restriction-ligation cloning. The lentiCRISPR v2 expression vector was a gift kindly shared by Feng Zhang (Addgene plasmid #52961). A single 20 base-pair oligo targeting CRTTC1 was phosphorylated, annealed and inserted into the said lentiviral vector using standard restriction-ligation cloning (Le Cong et al., 2013; Mashiko et al., 2013; Sanjana et al., 2014). FUGW lentiviral plasmid was a kind deposition made by David Baltimore (Addgene plasmid #14883; Lois, Hong, Pease, Brown, & Baltimore, 2002).

2.6 T7 Endonuclease I (T7E1) assay

NIH3T3 cells were transfected with CRTTC1-dendra2 and either negative pX330control or CRTTC1^{A1}/ CRTTC1^{B4}. After 48 h of post-transfection, 200 ng of genomic NIH3T3 DNA was harvested for PCR using Q5 high fidelity DNA polymerase and the following primers were designed:

(CRTTC1^{A1}[F]:GTTTCGCCTTTTGCAGAGTC, [R]: GGGGCTTCCTTCTGAGAACT; CRTTC1^{B4}[F]: GCTCTGCCTTGATCCTCTTG, [R]: GATATCTGTCGCCCCATGCTT).

Next, PCR products were subjected to T7E1 digestion according to the manufacturer's instructions. Thereafter, digested DNA were analysed via gel electrophoresis (Vouillot, Thlie, & Pollet, 2015).

2.7 Third generation lentiviral production

HEK293T cells were seeded at 6×10^6 onto 4×10 cm Poly-D-Lysine-coated (0.1 mg/mL) dishes in D10 (DMEM supplied with 10% fetal bovine serum and 1% Pen-Strep) in 5% CO₂ at 37°C. At about 90% confluency, cells were transfected with DNAfectin (ABM) according to manufacturer's instructions for at least 6 h before replacing with DMEM supplemented with 1% FBS and 1% Pen-Strep. Approximately at 48 h post-transfection, the supernatant from each dish was pooled together and centrifuged at 2000 rpm for 3 min at RT. The clarified supernatant is then kept at 4 °C. Each dish is then replaced with DMEM supplemented with 1% FBS, 1% Pen-Strep and 2 mM Sodium Butyrate (Sigma) and kept in a humidified CO₂ incubator. A second harvest is repeated within 24 h from the first harvest. Supernatant from both harvests were combined and filtered through a 0.45 µm filter unit (Merck). The filtrates were then transferred into disposable polyallomer tubes (Beckman) and send for ultracentrifugation using SW28 rotor at 23, 000 rpm for 90 min in 4°C. After spinning, the supernatant is discarded, and another layer of supernatant is added back to the same tube. A second spin is repeated, and the supernatant is drained completely. The virus is then resuspended in 90 µL of ice-cold 1x PBS (Thermo Fisher Scientific). A sterile parafilm is sealed over the tube and left inside the 4°C overnight. Virus pellet was gently mixed, dissolved and stored in small aliquots in -80°C (Cooper et al., 2011).

2.8 Lentivirus Transduction

Lentiviral particles (CRTTC1^{Scr} and CRTTC1^{Al}) were commercially packaged and purified from Vector Builder. All lentiviral transduction of hippocampal neurons was performed on day 7 after plating (DIV, days *in vitro*) at a reduced volume for 24 h before replacing with pre-warmed conditioned medium. The integrated constructs were allowed to express for at least 10 days before experiments.

2.9 Pharmacological Treatments

The following pharmacological agents were used: (-)-Bicuculline methiodide (Bic, 40 µM; Tocris), tetrodotoxin (TTX, 1 µM; Tocris), (RS)-3,5-Dihydroxyphenylglycine (DHPG, 50 µM; Tocris), Cyclosporin A (CsA, 5 µM; Sigma), BAPTA-AM (25 µM; Tocris), Cycloheximide (CHX, 60 µM; Tocris), N-methyl-D-aspartate (NMDA, 20 µM; Sigma). Cultured hippocampal neurons were incubated with various pharmacological

agents in conditioned neuronal media and maintained in a 37°C incubator with 5% CO₂ for an appropriate amount of time before cells were fixed for immunocytochemistry (Ch'ng et al., 2012).

2.10 Chemical LTP/LTD induction

Briefly, mature hippocampal neurons were equilibrated in a pre-warmed bath solution: NaCl 140 mM, CaCl₂ 1.3 mM, KCl 5.0 mM, HEPES 25 mM, glucose 33 mM, TTX 0.0005 mM, strychnine 0.001 mM, bicuculline methiodide 0.02 mM and pH and osmolarity adjusted to 7.4 and 325 – 335 mosmol⁻¹ respectively (Lu et al., 2001) for 10 minutes prior to application with 200 µM glycine for 3 minutes to induce LTP. The stimulation solution was then removed and recovered in a pre-warmed bath solution without glycine for the next 30 minutes. For the induction of LTD, hippocampal neurons were incubated in conditioned media supplemented with 50 µM DHPG for 5 minutes (Snyder et al., 2001) and following stimulation, cells were returned to conditioned media for the next 55 minutes.

2.11 Immunocytochemistry

Cells were fixed at room temperature with 3.2% PFA for 10 minutes, permeabilised with 0.1% Triton-X 100 (Sigma) for 5 minutes and blocked in 10% goat serum (Invitrogen) at room temperature for 30 minutes. Cells were then incubated in primary antibodies either at room temperature for 4 h or overnight at 4°C. Secondary antibodies and Hoechst nuclear dye (2 µg/mL, Invitrogen) were incubated at room temperature for 1 h. All antibodies were diluted in 10% goat serum, and coverslips were mounted with aqua-poly/mount (Polysciences).

2.12 GluA1 surface labelling

Cells were fixed in 4% PFA with 4% sucrose for 5 minutes, blocked in 10% goat serum for 1 h and incubated with GluA1 primary antibody overnight at 4°C. Next, cells were permeabilised in blocking buffer containing 0.1% Triton-X 100 for 20 minutes at room temperature and incubated with other primary antibodies at 4 °C overnight. Subsequent corresponding secondary antibodies were incubated for 1 h at room temperature (Snyder et al., 2001).

2.13 Internalised GluA1 labelling

The protocol as described by Snyder et al., (2001): briefly, surface AMPARs were labelled in live cells (GluA1 antibody; 5 µg/mL) and incubated at 37°C for 30 minutes. After live labelling, cells were treated with (R,S)-DHPG (50 µM in medium) for 5 minutes, warmed in a 37°C incubator and recovered in conditioned media for either 10 or 55 minutes. Cells were then chilled briefly in 4°C 1x Tris-Buffered Saline (TBS) to stop endocytosis, and exposed to 0.5 M NaCl/0.2 M acetic acid with pH adjusted to 3.5 for 4 minutes on ice to remove extracellular-bound GluA1. Next, cells were fixed in 4% PFA with 4% sucrose, permeabilised and blocked in 1x TBS containing 0.1% Triton-X 100, 4% goat serum and 2% BSA (Sigma). Subsequently, cells were incubated with secondary antibodies for 1 h at room temperature alongside Hoechst dye.

2.14 Stereotaxic injections

For all stereotaxic surgical procedures, animals were kept under deep anaesthesia (Ketamine 100 mg/mL, Xylazine 20 mg/mL) at a dose of 85 mg of Ketamine and 10 mg Xylazine per kg of animal body mass as previously described (Chew et al., 2015). Anaesthetised animals were mounted onto a stereotaxic frame (Kopf Instruments). Buprenorphine (0.1mL/10g) and lidocaine (0.1mL/20g) were both administered subcutaneously before surgery for pain relief and to alleviate any discomfort on animals according to per kg of animal body mass. A small incision was made along the midline of the shaven head to expose the skull. Small holes were drilled into the skull according to the following coordinates: 2 mm posterior to bregma, ± 1.5 mm lateral from the midline and 2.3 mm ventral from the skull surface. Lentivirus was injected at a volume of 1.5 µL per site on each hemisphere with a flow rate of 150 nL/min.

2.15 Perfusion and sectioning

Two weeks after lentivirus injection, animals were anaesthetised with pentobarbital and transcardially perfused with ice-cold sterile 0.9% saline followed by 4% paraformaldehyde diluted in 1x PBS. Mice brains were extracted and post-fixed for the next 1 – 2 h before being transferred to 30% sucrose solution for 48 h at 4°C. Fixed mice brains were quick frozen in a cryomold containing OCT compound, and sliced on a cryostat (Leica) into 40 µm-thick coronal sections. Slices were stored in Tissue Collecting Solution (TCS) containing 25% Glycerol, 30% Ethylene Glycol and 45%

DEPC-treated PBS at -20°C and collected for every 5th interval in an anterior to posterior manner for downstream immunohistochemistry.

2.16 Immunohistochemistry

Sections retrieved from TCS were washed and blocked in 10% goat serum and 0.2% Triton-X 100 for 1 h at room temperature, followed by primary antibody incubation for 2 overnights in blocking buffer at 4°C. Next, sections were rinsed, blocked for another 10 minutes and incubated with secondary antibodies for the next 4 – 6 h at room temperature. Stained sections were mounted on glass slides and stored away from light.

2.17 Electrophysiological acquisition[#]

Whole-cell patch clamp recordings were made from cultured hippocampal neurons, as described in (Song & Augustine, 2016). Action potential was measured under the current clamp, and postsynaptic miniature currents were measured under the voltage clamp. 4 – 5 MΩ of patch pipettes were used for recording. The extracellular solution contained (in mM): 150 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 20 glucose and 10 HEPES-NaOH, pH 7.3 in all of the recordings. For the recording of spontaneous miniature excitatory postsynaptic current (mEPSC), patch pipettes were filled with intracellular solution containing (in mM): 135 K-gluconate, 3 Na₂ATP, 0.2 EGTA, 0.3 Na₃GTP, 10 and 10 HEPES, pH 7.3. The extracellular solution contained (in mM): 150 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 20 glucose and 10 HEPES-NaOH, pH 7.3. Action potential recording was performed gap-free mode in the same internal and external solution with mEPSC without any pharmacological treatment and any current injection. mEPSCs were pharmacologically isolated by adding GABA receptor blockers, bicuculline (20 μM) and sodium channel blocker, TTX (1 μM) to the extracellular bath solution. For mIPSC, intracellular solution contained (in mM): 140 CsCl, 4 NaCl, 0.5 CaCl₂, 5 EGTA, 2 MgATP, 0.4 Na₃GTP and 10 HEPES-KOH (pH 7.4, adjusted with CsOH). mIPSCs were pharmacologically isolated by adding AMPA and NMDA receptor blockers CNQX (20 μM) and APV (50 μM), and sodium channel blocker, TTX (1 μM) to the extracellular bath solution. Hippocampal neurons were voltage clamped at -70 mV with a Multiclamp 700B (Molecular Devices) for recording spontaneous postsynaptic currents. All experiments were performed at room temperature (~ 25°C). Electrophysiological data were sampled and filtered at 10 kHz. Spontaneous synaptic

events were analysed using the MiniAnalysis program (Synaptosoft). For mEPSC and mIPSC analyses, detection parameters were set at 5 pA amplitude and <3 ms rise time and events were screened and verified by visual examination before the inclusion in the final data analysis.

[#]In collaboration with Professor George Augustine, Dr Song Sang Ho performed and analysed data for all electrophysiological experiments.

2.18 Fluorescent Microscopy

Thermo Fisher EVOS FL Cell Imaging System

Objectives: 10x

Contrast: Epifluorescence and transmitted light

2.19 Confocal Microscopy

Fluorescent images were captured with the following instrument:

Zeiss Scanning Confocal Microscope LSM 800

Objectives: Plan Apochromat 63X 1.40 Oil DIC

Temperature: 25 °C

Solid state lasers: 405, 488 and 561 nm

Data acquisition: Zen 2011

2.20 Immunoblotting and Quantification of Western Blots

Cell lysates were harvested in RIPA buffer (150 mM NaCl; 1% Triton-X 100; 0.5% sodium deoxycholate; 0.1% SDS; 50 mM Tris pH 8.0) supplemented with protease inhibitors (Nacalai's Tesque) and centrifuged at 13,200 rpm for 5 min at 4°C. Protein concentration was determined with Pierce BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instruction. Approximately about 20 uL of extracted proteins were loaded and electrophoresed on a 10 – 12% bisacrylamide mini gel (Biorad) and ran on standard PAGE buffers at 120V. Proteins were subsequently transferred onto an Immobilon-P membrane (Millipore) at either 100V for 90 min at room temperature or 30 V overnight at 4°C. The membrane was blocked in 5% skim milk for 1 h at room temperature, rinsed in 1% Tween/PBS (PBS-T), and probed with primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibody for 1 h at room temperature. All protein bands were detected with Luminata Crescendo Western HRP Substrate (Millipore), and western blot images were captured on Image

Lab (Bio-Rad) under non-saturating conditions. For quantification, identical regions were selected for each sample to obtain pixel intensities and normalised against the respective GAPDH or β III-tubulin (TUJ1) loading control.

2.21 RNA extraction and cDNA conversion

Total RNA from cultured hippocampal neurons were extracted with PicoPure RNA Isolation kit (Applied Biosystems) according to the manufacturer's instructions. The RNA concentration was measured from the Nanodrop instrument (Thermo Scientific). Reverse transcription of total RNA (1 μ g; RevertAid First Strand cDNA Synthesis kit, Thermo Fisher Scientific) was performed in a 20 μ L of a mix containing oligo(dT)₁₈ primers, dNTP mix (10 mM), RiboLock RNase Inhibitor (20 units), RevertAid M-MuLV reverse transcriptase (200 units), using the following program: 42 °C for 60 minutes and heat inactivation at 70 °C for 5 minutes.

2.22 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) reactions were conducted in triplicates using 1:10 dilution of 1 μ g cDNA in a mixture containing custom-designed primers (IDT, 200 nM; see Appendix – Table 1 for list of primers used) and SYBR Green PCR Master Mix (Applied Biosystems). All reactions were tested in the presence of negative controls (no template control, no reverse transcriptase control and no amplification control) and ran in a StepOnePlus instrument (Applied Biosystems). Primers were designed using Primer3 online software tool (Kõressaar et al., 2018; Koressaar & Remm, 2007; Untergasser et al., 2012) for CREB target genes that have not been previously published (Ch'ng et al., 2012). Each respective melting curve was generated experimentally (see Appendix – Figure S5). Baselines were defined starting from cycle 3 and ending with cycle 13. Target gene expression levels (Ct-values) were normalised to the mean of housekeeping control, hypoxanthine guanine phosphoribosyl transferase-1 (*Hprt1*), a nonactivity-dependent gene. Amplification data were acquired and exported for analysis with Δ Ct. Fold change of each target gene was determined by calculating for $\Delta\Delta$ Ct.

2.23 Image Analysis

All data were collected over three independent experiments unless otherwise specified. Raw confocal images were taken using a 63x 1.4NA oil objective. For the quantification of nuclear-to-cytoplasmic ratio, a subtraction was applied for both whole cell (MAP2-positive) and nuclear masks to obtain a separate mask for cytoplasm using ImageJ. The average intensity determined by each mask from the respective compartment was used for individual hippocampal neurons. For the quantification of PSD95 puncta, each dendritic branch image was linearised on NIH ImageJ software (Abràmoff, Magalhães, & Ram, 2004) with a width setting of 120 pixels. Based on the MAP2 staining, the proximal dendritic distance was measured from the base of the cell body and extended along the length of the dendrite whereas distal dendritic distance was measured from the terminal ends of the dendrite and extended upwards along the dendrite. Since dendrites were of different lengths at image acquisition, only dendrites that fall below 100 µm were considered as proximal, and those that were less than 100 µm from the terminal ends of the dendrite were regarded as distal. The pixel intensity of PSD95-positive puncta was adjusted accordingly using the threshold function. A constant threshold is applied to the same experimental set. The size of the puncta was selected at >10-pixel units with a circularity of 0.5 – 1.0. The number of puncta identified was used as an approximate indication of the number of synapses. For the morphometric measurement of GluA1, GAD67 and Gephyrin terminals, only immuno-reactive puncta defined as distinct points along proximal and/or distal dendrites with intensity twice the background staining were manually counted and included in analyses. All data points were plotted on GraphPad Prism and error bars presented as mean $\pm$ SEM, and unless specified otherwise, all statistical significance was determined by Student's t-test or One-Way ANOVA coupled with either Tukey's post-hoc analysis (parametric) or Dunn's post-hoc analysis (non-parametric). A probability value of $\alpha = 0.05$ was used to indicate statistical significance (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.0001$).

3.1 Generation of CRISPR/Cas9-mediated CRTTC1 knockout in hippocampal neurons

3.1.1 Selection of CRISPR-Cas9 gRNAs targeting *Crttc1* genomic locus

The first step to generating a CRISPR knockout is the selection and identification of the Cas9 target sites in the *Crttc1* gene. To begin, we designed a short 20-nucleotide guide RNA (gRNA) complementary to the site of the target genome. Each gRNA seed site is followed by a PAM motif (NGG) that is necessary for target binding.

To knock out the *Crttc1* genomic locus, we screened candidate gRNAs using a CRISPR design tool (<http://crispr.mit.edu>) which is an online database that scans for all possible CRISPR guides and their off-target matches throughout the genome. The job output ranks according to the on-target score in the order from 0 to 100%, that is, the higher the on-target score, the better the affinity of the on-target activity of each guide. The online tool computes 100% minus a weighted sum of off-target hit-scores in the target genome by evaluating the total number of mismatches, mismatch position (mismatches falling close to the PAM site have a higher disruption rate to gRNA cleavage activity) as well as distance between mismatches (close mismatches create a more considerable steric hindrance to the gRNA-DNA interaction).

We created a list of five criteria to optimise for gRNA selection:

1. Target the 5' of the protein-coding region that is not associated with alternative splicing. Exons near the N-terminus are ideal because frameshift mutations will increase the likelihood of a non-functional protein product.
2. Overall low off-target scores (an aggregate score higher than 85% is regarded as a potential candidate target) to offset the risk of editing additional genomic sites.
3. Manually screen gRNA selections for possible off-targets effects in the genome that potentially encode crucial cellular functions.

4. Target sequences are not homologous to other motifs to ensure highest gRNA specificity.
5. Manually verify to ensure no secondary or complementary folding from gRNA.

Our aim here was to use the above criteria as a guideline to select potential gRNAs with minimal off-target effects. Eventually, we narrowed down to four target sites: CRTCL1^{A1}, CRTCL1^{A3}, CRTCL1^{B2} and CRTCL1^{B4}, corresponding to exons coding for the N-terminal region of *Crtcl* (Figure 3-1).

3.1.2 Construction and validation of CRISPR/Cas9 plasmids targeting *Crtcl* genomic locus with pX330

Based on our selection, we next asked which of these targets are most effective in cleavage efficiency. We then proceeded to validate our chosen gRNAs for targeting *Crtcl* in HEK293T cells. These gRNA target sequences were cloned into pX330 vector containing a human codon-optimised Cas9 derived from the bacteria *Streptococcus pyogenes* (Figure S1; see <https://www.addgene.org/crispr/zhang/#spcas9>). For examining the effectiveness of the selected gRNAs in cleaving *Crtcl*, we used the Split-GFP reconstitution assay. First, the gRNAs are directionally cloned into plasmid vector pX330. Next, partial coding regions of *Crtcl*, which contains all the target sites are inserted between EGFP fragments of the pCAG-EGxxFP plasmid. The “Split-GFP” CAG-EGxxFP plasmid contains two partial EGFP fragments, with significant overlap in homology (482 base pairs) driven under ubiquitous CAG promoter (Figure 3-2).

Since the coding region of the GFP is not in frame due to the presence of *Crtcl* insert, no GFP is produced in cells transfected with the plasmid. If the selected gRNA binds to *Crtcl* partial fragment sandwiched by the split GFP sequence, then DSBs are induced, and the plasmid will be linearised. The linearised plasmid cannot express GFP unless recombination occurs in the overlapping GFP fragment, allowing reconstitution of GFP. Subsequent homologous recombination of the EGFP regions of homology allows reconstitution of the GFP enabling expression of full-length GFP protein. In short, only successful gRNA-directed Cas9 cleavage of the target sequence will result in GFP-labelled cells. We hope to employ this reconstitution system to map the efficiency of recombination with different gRNA targets.

Partial *Crtc1* genome (from 1 – 810 base pairs of the coding region that contained all the gRNA target sites) was inserted between the EGFP fragments and used as a reporter plasmid. To validate which gRNA sequence works, we co-transfected pX330 and pCAG-EGxxFP-CRTC1 plasmids into HEK293T cells along with pCAG-mCherry as a label for transfected cells, and the reconstituted EGFP fluorescence was observed at two intervals: 24 h (not shown) and 48 h after transfection. At each given time point, we observed no EGFP fluorescence in our negative control samples, in which the pX330 plasmid had no gRNA sequence inserted. In contrast, when pX330 contained appropriate gRNA targeting CRTC1, HEK293T cells exhibited EGFP signal.

We then quantified the number of GFP-positive cells (expressed over the number of transfected cells in percentage) induced by pX330 constructs and compared using one-way ANOVA. We found that all gRNAs had more than 70% of the transfected cells with reconstituted EGFP by 48 h post-transfection, but there was no distinct difference between each gRNA (Figure 3-3), suggesting that all our gRNAs effectively induce DSBs in targeted sites of reporter plasmids in HEK293T cells.

Figure 3-1: Construction of CRISPR/Cas9 plasmids for targeting *Crtc1* in HEK293T

(A) Genomic mouse *Crtc1* sequence selected as gRNA target site (green) with adjacent PAM motifs (red).

(B) Schematic representation of the genomic locus on chromosome 8 for *Crtc1* as well as the location of each gRNA (black arrowheads).

Figure 3-2: Schematic illustration for EGFP reconstitution assay

(A) pX330-expressing gRNA sequences and pCAG-EGxxFP reporter plasmid containing 5' and 3' EGFP in two coding fragments that share a significant 482 base pairs overlap under ubiquitous CAG promoter. Partial genomic fragment of *Crtc1* (~800 bp) was placed between EGFP fragments of the reporter plasmid.

(B) The two expression plasmids were co-transfected into HEK293T cells. When *Crtc1* target sequence was cleaved by gRNA guided Cas9 endonuclease, homology-dependent repair took place and reconstituted the EGFP expression cassette. The efficiency of the DSB-mediated homologous recombination was validated by observing EGFP fluorescence 24 and 48 h after transfection.

Figure 3-3: Verifying CRISPR/Cas9-mediated CRTC1 knockout through reconstituted EGFP fluorescence

- (A) Representative confocal micrographs of pX330 and pCAG-EGxxFP co-transfection in HEK293T after 48 h; mCherry is used as a marker of transfection.
- (B) After staining with mCherry (red) and with Hoechst nuclear dye (blue), the number of EGFP-positive cells was expressed as a percentage over the number of mCherry-positive cells captured in a single field. No significant difference was detected between gRNAs in their cleavage efficiency. Data were collected over three independent experiments where n=15 scores the number of random confocal images analysed for each group from the same coverslip. Scale bars, 10 μ m.

3.1.3 Knocking out CRTC1-dendra2 protein expression in HEK293T cells

All of our four constructs showed very similar abilities in cleaving CRTC1 using the EGFP reconstitution assay, but we eventually narrowed down to two candidate choices, namely, CRTC1^{A1} and CRTC1^{B4} based on their slightly higher reconstitution readout at 48 h post-transfection relative to the other candidates. The percent GFP reconstitution for CRTC1^{B4} is comparable to that of CRTC1^{A1}. Then, we asked whether CRTC1^{A1} and CRTC1^{B4} can mediate efficient knockout of full-length CRTC1 by overexpressing the fusion of CRTC1 to photo-switchable fluorescent protein dendra2 (green) in cultured HEK293T cells. We co-transfected CRTC1 gRNAs (CRTC1^{A1} or CRTC1^{B4}) and CRTC1-dendra2 plasmids into HEK293T cells and observed for a change in the number of GFP-positive cells at both 24 h and 48 h intervals after transfection.

Co-transfection of the negative pX330 vector (with no gRNA target sequence) did not result in reduced CRTC1-dendra2 expression. In contrast, cells co-transfected with either CRTC1^{A1} or CRTC1^{B4} showed a dramatic loss of dendra2 fluorescence (Figure 3-5A). We also quantified for the number of GFP-positive cells as a percentage over total cell number. At the end of the 48 h post-transfection, our results revealed that there is no significant difference in the GFP fluorescence output between CRTC1^{A1} and CRTC1^{B4}. This data suggests that the corresponding gRNAs (A1 and B4) that target *Crtc1* are equally capable of mediating knockout of CRTC1 *in vitro*.

We collected lysates after 48 h post-transfection in which the proteins were separated by SDS-PAGE and probed with an antibody against CRTC1 to determine relative amounts of CRTC1 expression. The same blot was also probed with an antibody against the constitutively-expressed housekeeping gene, GAPDH, which serves as a loading control. As shown in Figure 3-5B, delivery of CRISPR/Cas9 constructs containing CRTC1^{A1} and CRTC1^{B4} produced a significant reduction in CRTC1 protein expression in comparison to the negative pX330 (no gRNA).

As a screen for Cas9-induced mutations, we used T7 endonuclease I assay for detecting DNA heteroduplexes resulting from non-homologous recombination after gRNA/Cas9-mediated cleavage. This assay is uniquely different from the plasmid-based systems as it allows us to assess gRNA cleavage on the genome. In this method, edited cells were

used as a direct template for PCR amplification with specific primers that target the region of editing. The products after rounds of amplification were then denatured and reannealed back to form heteroduplex mismatches where they will then be recognised and cleaved by T7E1. We chose to utilise T7E1 assay over Surveyor because of its higher sensitivity (Vouillot et al., 2015). We designed two sets of primers corresponding to CRTCl respectively (see materials and methods). NIH3T3 cells were transfected with negative pX330 (without any targeting gRNA) or CRTCl targeting gRNA (CRTCl^{A1} or CRTCl^{B4}). Genomic DNA (gDNA) was extracted from these transfected cells and used as a template for PCR amplification flanking the CRISPR/Cas9-mediated cleavage site of about 800 bp using Q5 high fidelity DNA polymerase and cleavage at heteroduplex mismatch sites were analysed on agarose gel electrophoresis. As can be seen in Figure 3-5C, Cas9 endonuclease protein when combined with CRTCl^{A1} provides effective gene editing (cleaved fragment size of 500 bp). Surprisingly, CRTCl^{B4} did not show a positive result for the T7 assay. It is likely that both CRTCl^{A1} and CRTCl^{B4} cleave the DNA since they showed positive results in reconstitution assay and also knocked out CRTCl expression in transient transfections. We proceeded to generate both CRTCl^{A1} and CRTCl^{B4} lentiviruses, but most of the experiments described in the subsequent sections will focus on the deployment of CRTCl^{A1} unless otherwise stated.

Figure 3-4: Schematic illustration for CRTC1-dendra2 fluorescence assay

A

B

- (A) Two plasmids were being used: single vector pX330 containing either CRTC1^{A1} or CRTC1^{B4} and Cas9; full-length coding sequence of mouse *Crtc1* fused to the dendra2 photoconvertible protein in an expression vector driven by a CMV vector.
- (B) The two expression plasmids were co-transfected into HEK293T cells. When the *Crtc1* target sequence was cleaved by gRNA guided Cas9 endonuclease, it will result in lower levels of CRTC1-dendra2. The efficiency of the DSB-mediated non-homologous end joining was assessed by observing dendra2 fluorescence 24 and 48 h after transfection.

Figure 3-5: CRISPR/Cas9 mediates transient knock out of CRTC1-dendra2 expression

Figure 3-5

- (A) The coding sequence of mouse *Crtc1* was fused to dendra2 photoconvertible protein in an expression vector driven by a CMV vector. Representative confocal micrographs showing co-transfection of CRTC1 gRNAs (CRTC1^{A1} and CRTC1^{B4}) and CRTC1-dendra2 in HEK293T cells and left to express for 48 h before being fixed and visualised. Quantification of the total number of GFP-positive (green) cells (upper panel) and percentage of GFP-positive cells over total cells (lower panel) was analysed at 48 h after transfection (**** $p < 0.0001$ relative to negative pX330; n.s., not significant). Data were collected over three independent experiments. Numbers within each column represent total micrographs analysed per group. Scale bars, 400 μ m.
- (B) Immunoblot analysis of HEK293T cells at 48 h post-transfection showing CRTC1 protein expression when co-transfected with gRNAs CRTC1^{A1} and CRTC1^{B4} in HEK293T cells. Antibodies against GAPDH were used as loading controls. Data were collected from two independent experiments.
- (C) The gRNA-Cas9 construct was transfected in NIH3T3 cells, and CRISPR/Cas9-induced mutations were tested by mismatch T7E1 assay. Negative pX330 did not express any targeting gRNA and was used as control. The asterisk shows heteroduplex DNA as a result of gRNA/Cas9-mediated cleavage to DNA strands.

Figure 3-6: Schematic illustration of lentiviral constructs

The schematic diagram shows CRISPR lentiviral constructs with gRNA (CRTC1^{A1} and CRTC1^{Scr}) expression driven by U6 promoter and Cas9-FLAG expression driven by elongation factor 1-alpha promoter.

3.1.4 Knocking out CRTCl expression in cultured hippocampal neurons

To examine how the absence of CRTCl affects neuronal form and function at the cellular level, we needed to knock out expression of CRTCl in hippocampal neurons. We showed in the previous section that CRTCl^{A1} efficiently knocks out transient expression of CRTCl in HEK293T cells (Figures 3-3 and 3-5). As a control for CRTCl knockout, we also designed a scrambled gRNA control (CRTCl^{Scr}) whose sequence does not match any human nor rodent genome (the sequence is taken from OriGene; 5' GCACTACCAGAGCTAACTCA 3').

We cloned gRNAs CRTCl^{Scr/A1} into a single lentiviral vector construct (Figure 3-6), and we will test to see if CRTCl^{A1} knocks out the expression of CRTCl in dissociated hippocampal neurons (DIV 17 – 21). Our CRISPR constructs harbouring CRTCl^{Scr} and CRTCl^{A1} gRNA sequences were commercially purified and packaged by VectorBuilder as high-titre lentiviral particles of about 2×10^9 transforming units/mL, whereas CRTCl^{B4} was self-packaged.

We first established basic parameters for optimal transduction of cultured hippocampal neurons with two considerations: 1) the age of neurons to produce efficient knockouts and 2) the time it takes to detect knockout. It has been reported that transduction occurs much more effectively in younger primary neurons (Levin, Diekmann, & Fischer, 2016). We first transduced dissociated hippocampal neurons at 4 DIV and measured CRTCl expression levels after 14 DPT (days post-transduction). Surprisingly, transduction at 4 DIV showed that CRTCl^{A1} nuclear-to-cytoplasmic ratio remains relatively comparable to CRTCl^{Scr}, which suggests that CRTCl knockout was not efficient (Figure 3-7A).

To test both efficiency of lentiviral transduction as well as the duration after the transduction to achieve a complete CRTCl knockout in hippocampal neurons, we transduced them at 7 DIV and then measured for their CRTCl nuclear-to-cytoplasmic ratio at different post-transduction time-points: 7, 10 and 14 DPT. We observed that the earliest time-point at 7 DPT showed an increase in CRTCl levels between CRTCl^{Scr} and CRTCl^{A1} (Figure 3-7B), indicating that the knockout is incomplete. However, hippocampal neurons at 10 and 14 DPT showed an equal reduction in CRTCl expression levels as no difference was detected between these time-points (Figure 3-

7C), suggesting that transduction can be terminated earliest at 10 DPT. We then proceeded to immunostain for Cas9 expression with a well-characterised antibody against the FLAG epitope tag. To determine if a cell is Cas9-positive, we normalised the Cas9 pixel intensity against the highest background staining in wildtype (WT) non-transduced hippocampal neurons. Our experimental validation established that viral transduction was 100% efficient as almost all cells tested were Cas9-positive (Figure 3-8A).

Because CRTC1 is sensitive to neural activity and accumulates in the nucleus (Ch'ng et al., 2012), we treated dissociated hippocampal neurons with bicuculline (40 μ M, 30 minutes) and measured both nuclear CRTC1 expression as well as its nuclear-to-cytoplasmic ratio. As anticipated, we found that on average 90 – 95% of all Cas9-positive hippocampal neurons exhibited a dramatic reduction of nuclear CRTC1 expression (Figures 3-8C and D) and did not respond to bicuculline induction which drives nuclear translocation of CRTC1, indicating that in a large proportion of hippocampal neurons, CRTC1 expression is abolished. To ensure that both somatic and nuclear CRTC1 were reduced, we also quantified CRTC1 signals in entire neuronal cell bodies and showed a robust reduction (Figure 3-8B). To summarise, we found that transducing hippocampal neurons at DIV7 and performing experiments at DIV17 (after 10 days) gives us the most complete and efficient knockout of CRTC1 expression.

Figure 3-7: CRTC1 knockout in cultured hippocampal neurons is most effective when transduced at DIV7 and allowed to go for 14 days (DIV 21)

Figure 3-7

- (A) Representative confocal micrographs showing hippocampal neurons transduced at 4 DIV and after 14 DPT was stimulated with bicuculline (40 μ M, 30 min) before immunostaining with MAP2 (cyan), Cas9 (red), CRTCl (green) and Hoechst nuclear dye (blue). Corresponding CRTCl nuclear-to-cytoplasmic ratio was quantified (n.s., not significant relative to CRTCl^{Scr}). Scale bar, 10 μ m. Data were collected over three independent experiments, and numbers within column represent n of cells analysed per group.
- (B) Representative confocal micrographs showing hippocampal neurons transduced at 7 DIV and at various time-points of at 7, 10 and 14 DPT (days post-transduction) were stimulated with bicuculline (40 μ M, 30 min) before immunostaining with MAP2 (cyan), Cas9 (red), CRTCl (green) and Hoechst nuclear dye (blue). Corresponding CRTCl nuclear-to-cytoplasmic ratio was quantified (****p < 0.0001 relative to CRTCl^{Scr}). Scale bar, 10 μ m. Data were collected over three independent experiments, and numbers within column represent n of cells analysed per group.
- (C) Reduction in CRTCl nuclear-to-cytoplasmic ratio normalised to averaged CRTCl^{Scr} at three time-points: 7, 10 and 14 DPT, expressed in percentage.
- (D) Overview of CRTCl nuclear-to-cytoplasmic ratio across all three time-points (****p < 0.0001 relative to CRTCl^{Scr}). Scale bar, 10 μ m. Data were collected over three independent experiments, and numbers within column represent n of cells analysed per group.

Figure 3-8: High efficiency of CRISPR/cas9 CRTC1 knockout in cultured hippocampal neurons

Figure 3-8

- (A) Hippocampal neurons were cultured for 7 DIV before being transduced with CRTCl^{Δ1} or CRTCl^{Scr}. After 10 days transduction, cells were treated with bicuculline (Bic, 40 uM, 30 min) in order to visualise nuclear CRTCl. The threshold for scatter plot of Cas9 pixel intensity is set at the highest data point scored for WT. Scale bar, 20 μm. Scale bar, 10 μm.
- (B) Hippocampal neurons were immunostained with antibodies corresponding to MAP2 (cyan), Cas9 (red), CRTCl (green) and Hoechst nuclear dye (blue). Data showed a reduction in CRTCl of the whole soma in CRTCl^{Δ1} neurons (****p < 0.0001).
- (C) Mean nuclear intensity of CRTCl was quantified (left) and data for CRTCl^{Δ1} was further normalised against the average nuclear readout of CRTCl^{Scr} (right; ****p < 0.0001).
- (D) The nuclear-to-cytoplasmic ratio of CRTCl was quantified (left) and data for CRTCl^{Δ1} was further normalised against the average N:C readout of CRTCl^{Scr} (right; ****p < 0.0001). Data were collected over three independent experiments. Numbers within the column represent n of cells per group.

3.2 Assessing consequences of knocking out CRTTC1 in dissociated hippocampal neurons

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3.2.1 Loss of CRTTC1 does not affect total CREB and its neuronal activity-dependent CREB phosphorylation

We have demonstrated in the last section that CRISPR-mediated knockout of CRTTC1 in hippocampal neurons achieved between 90 – 95% reduction in CRTTC1-mediated nuclear response in Cas9-positive of cultured hippocampal neurons. Since CRTTC1 function is directly tied to CREB activity, we needed to ensure that loss of CRTTC1 does not directly impact CREB levels and phosphorylation patterns post-stimulation.

Hippocampal cultures were subjected to virus transduction at 7 DIV (determined from the previous chapter), and 10 DPT neurons were stimulated with bicuculline stimulation to trigger neuronal activity. Unless otherwise stated, all subsequent viral transduction parameters will be as stated in materials and methods (section 2.4). Hippocampal neurons were fixed and immunolabelled with CREB to determine total CREB levels. For analysis, we selected only pyramidal neurons and omitted GAD67-positive inhibitory neurons (note that the GAD67 boutons that ring around the pyramidal neuron soma comes from GAD67 positive inhibitory neurons and does not denote the intracellular presence of GAD67). Our results revealed that CRTTC1^{A1} neurons showed no significant difference when compared to CRTTC1^{Scr} in total CREB levels with or without stimulation (Figure 3-9), thereby indicating that total CREB levels remained unaltered despite the loss of CRTTC1.

CREB is activated in an activity-dependent manner and binds to CRE elements in the promoter for transcription critical in establishing and consolidating long-term memories. Activation of CREB is associated with phosphorylation at multiple residues in the kinase-inducible domain. Similar to evaluating for total CREB levels, we measured phosphorylation of CREB at serine 133 (pCREB S133) as it is widely used as a proxy for transcriptional activation (Ch'ng et al., 2012). We showed that basal pCREB levels in hippocampal neurons were low, but upon bicuculline induction, pCREB S133 levels become robustly elevated. Moreover, bicuculline stimulation induced equivalent CREB

phosphorylation at S133 in CRTCl^{Scr} or CRTCl^{A1} neurons. As expected, knocking out CRTCl does not affect pCREB S133, suggesting that CREB phosphorylation is independent of CRTCl association (Figure 3-10).

Figure 3-9: Loss of CRTC1 does not alter CREB expression

- (A) Representative confocal micrographs showing no change in CREB expression of hippocampal neurons at 17 DIV: compare immunostaining of basal unstimulated and bicuculline-stimulated (40 μ M, 30 min).
- (B) Quantitative of total CREB in cultured hippocampal neurons remained the same with or without bicuculline treatment across WT, CRTC1^{Scr} and CRTC1^{A1} (n.s., not significant) neurons. Data were collected over three independent experiments. Numbers within the column represent n of cells per group. Scale bar, 10 μ m.
(Note that the GAD67 boutons that ring around the pyramidal neuron comes from GAD67 positive inhibitory neurons and not from the selected neurons)
- (C) Western blot and corresponding quantitative analysis showing no change in CREB expression levels at basal and that stimulated with bicuculline at 17 DIV (n.s., not significant).

Figure 3-10: CRTC1 knockout does not alter phosphorylation of CREB (S133) during activity-induction

- (A) Representative confocal micrographs of CREB phosphorylation at S133 in cultured hippocampal neurons transduced at 7 DIV with CRTC1^{A1} and CRTC1^{Scr} virus and stimulated with bicuculline at 17 DIV. Neurons were immunostained for pCREB at basal unstimulated (left), and bicuculline stimulated (right).
- (B) Quantitative of pCREB in cultured hippocampal neurons showed increased nuclear staining upon bicuculline treatment with no difference between CRTC1^{Scr} and CRTC1^{A1} (n.s., not significant). Data were collected over three independent experiments, and numbers within the column represent n of cells per group. Scale bar, 10 μ m.
- (C) Western blot and corresponding quantitative analysis showing no change in pCREB expression levels of cultured hippocampal neurons when stimulated with bicuculline stimulation at 17 DIV (n.s., not significant).

3.2.2 Loss of CRTTC1 induces AP bursting in hippocampal neurons

Studies have demonstrated that CREB plays a crucial role not only in synaptic plasticity but also in its ability to exert control on neurons to fire action potential (AP) in response to an input signal (Benito & Barco, 2010; Han et al., 2006; Lopez de Armentia et al., 2007). It was shown that the expression of constitutively-active CREB showed enhanced intrinsic excitability, while dominant negative CREB reduced intrinsic excitability. According to Porte and colleagues, they found that the longer the duration of constitutive CREB activation, the better an animal performs in overall tasks (Porte, Buhot, & Mons, 2008).

Because CRTTC1 enhances CREB activity, we hypothesised that the loss of CRTTC1 might affect neuronal excitability, which could in turn, compromise overall network activity. Since CREB enhances excitability, one hypothesis would be that loss of CRTTC1 downregulates CREB activity, which in turns reduces overall neuronal excitability. To examine whether CRTTC1 contributes to neuronal excitability, we randomly selected single hippocampal neurons in culture after viral transduction and performed whole-cell patch clamp recordings[#] between 1 to 3 hours at basal unstimulated condition. Contrary to our initial hypothesis, we found an overall increase in the proportion of cells at the basal level, displaying an increased firing of action potentials in hippocampal neurons transduced with CRTTC1^{A1}. We recorded 11 out of 15 neurons (73.3%) showing increase AP bursting as compared to 3 out of 16 CRTTC1^{Ser} neurons (18.8%) (Figure 3-11D). Our basal culture conditions indicate very low network activity as most hippocampal neurons appear quiescent with low bursting activity. Apart from fewer cells firing, the frequency of firing is low in CRTTC1^{Ser} neurons, often appearing as single spikes while in CRTTC1^{A1} neurons, AP firing occurs in bursts or a cluster of spikes (Figures 3-11B and E). These findings suggest that loss of CRTTC1 in hippocampal neurons enhances action potential firing in neurons under basal, non-stimulated conditions.

This form of AP bursting in CRTTC1^{A1} neurons is reminiscent of firing patterns observed when neurons are exposed to bicuculline to block GABA_A receptors to reduce inhibition (Wiegert et al., 2009). To determine if excitability in CRTTC1^{A1} neurons can be further affected by blocking inhibitory synaptic transmission, we used bicuculline, to block

inhibitory synaptic transmission in dissociated hippocampal cultures for 15 minutes before washing out bicuculline. Hippocampal neurons were recorded 60 minutes after bicuculline washout (Figure 3-11A). GABAergic inhibition will cause neurons to synchronously fire AP in bursts (characterised by clusters of spikes with relatively short spike-free intervals). A brief exposure of bicuculline in neurons followed by washout triggers a synchronous and sustained AP bursting that can last for more than 24 hours. This form of plasticity is NMDA receptor-dependent and is also shown to be transcription-dependent as the application of actinomycin D blocks sustained AP firing (Arnold et al., 2005; Wiegert et al., 2009).

In general, we found that bicuculline enhances overall AP firing in CRTTC1^{Scr} neurons such that the number of cells firing AP now closely matches that of CRTTC1^{A1} neurons at basal state (11 out of 17 CRTTC1^{Scr} neurons (64.7%) and in 10 out of 15 CRTTC1^{A1} neurons (66.7%) (Figures 3-11C and D). Bicuculline treatment and washout also appeared to impact the overall frequency of AP firing for both CRTTC1^{A1} and CRTTC1^{Scr} neurons, but group data analyses suggest that the increase was not statistically significant (Figures 3-11E-F). This data was surprising because bicuculline-induced sustained bursting after removal of bicuculline was previously reported (Wiegert et al., 2009). Moreover, a clear trend was present between control neurons and neurons exposed to bicuculline treatment regardless of the type of viral transduction (Figure 3-11E). Sample traces indicate neurons exposed to bicuculline and measure 60-120 min after washout to have a more complex and sustained bursting patterns that were not present in the control group (Figure 3-11C). Based on the trends, an increase in sample size measurements between groups or a change in the window of treatment and the start of recordings may yield a more significant result (Figures 3-11C and E). Nevertheless, our results clearly show regardless of bicuculline, the frequency of AP firing in CRTTC1^{A1} neurons is enhanced over and CRTTC1^{Scr}. Finally, measurements of resting membrane potential showed no difference between CRTTC1^{A1} and CRTTC1^{Scr} neurons either under basal or stimulated conditions, suggesting that loss of CRTTC1 does not affect intrinsic excitability of neurons nor alter the threshold of activation (Figure 3-11F).

To verify if AP bursting stems from a synaptic effect, we used kynurenic acid (KynA, 200 μ M) to block all glutamatergic receptors. The blockade of glutamatergic receptors eliminated AP bursting across all groups regardless of the type of viral transduction (CRTC1^{A1} or CRTC1^{Scr}) or stimulation (with or without BIC induction), thereby confirming that enhanced AP bursting seen in the knockout requires glutamatergic receptor activation and is likely synaptic in origin. Again, there were no observable differences in the respective resting membrane potentials during KynA treatment and recording (Figure 3-11I). Together, our data informs us that loss of CRTC1 in neurons increases AP firing which could most likely be mediated by an increase in glutamatergic driven synaptic activation.

Figure 3-11: Loss of CRTTC1 leads to increased AP firing

Figure 3-11

#Dr Song Sang Ho performed and analysed data for all electrophysiological experiments.

- (A) Schematic showing the stimulation protocol used to induce action potential bursting (refer to Methods). Bicuculline (40 μ M) or vehicle was applied over 15 minutes. AP bursting activity was recorded 1 hour after washout up to 3 hours.
- (B) Representative traces of AP bursting recorded from primary hippocampal neurons at 17 – 20 DIV with vehicle treatment.
- (C) Representative traces of AP bursting recorded from primary hippocampal neurons at 17 – 20 DIV stimulated with bicuculline.
- (D) Histograms show the number of cells firing action potential expressed in percentage. Numbers per group are indicated on top of each graph column.
- (E) Analysis of action potential frequency measured from cultured neurons (* $p < 0.05$).
- (F) Measurement of resting membrane potential of cultured neurons across all treatment groups.
- (G) Separate quantification showing number of neurons treated with kynurenic acid (KynA) firing an action potential, expressed in percentage. Numbers per group are indicated on top of each graph column.
- (H) Neuronal frequency during action potential firing when treated with KynA.
- (I) Comparison of resting membrane potential in neurons treated without KynA and those treated with KynA.

3.2.3 Loss of CRTC1 leads to reduced mIPSC frequency

We hypothesised that enhanced action potential firing in CRTC1^{A1} neurons could be due to loss of inhibition resulting in enhanced neuronal excitability. To analyse if inhibitory connections are impacted, we compared miniature inhibitory postsynaptic currents (mIPSCs) between CRTC1^{Scr} and CRTC1^{A1} neurons to assess potential changes in synaptic transmission.

From our recordings, we observed a reduction in mIPSC frequency but not amplitude in the absence of CRTC1 (Figure 3-12B). The observation that only frequency but not the amplitude of mIPSC are altered suggests the possibility that the deficit stems from the presynaptic release of GABA (Gao, Cheng, & Ziskind-Conhaim, 2017; Zhou, Chen, & Roper, 2009) which is congruent with our observation that CRTC1^{A1} neurons increase AP bursting in dissociated cultures. The lack of difference in amplitude may also reflect the fact that there is no change in response to single quantal release of GABA-packaged vesicles at functional synapses of postsynaptic inhibitory receptors. We also saw no changes in either the rise time or decay time of the mIPSC which means that there are no changes in cable properties (cell shape, capacitance and membrane resistance) that could alter the kinetics of mIPSCs.

Figure 3-12: Loss of CRTC1 reduces frequency but not amplitude of mIPSC

Figure 3-12

#Dr Song Sang Ho performed and analysed data for all electrophysiological experiments.

- (A) Representative traces of WT, CRTCl^{Scr} and CRTCl^{A1} neurons showing mIPSCs recorded in the presence of APV (50 μ M), CNQX (20 μ M) and TTX (1 μ M) from cultured hippocampal neurons between 17 – 20 DIV.
- (B) Reduction of mIPSC frequency in CRTCl^{A1} neurons (*p < 0.01).
- (C) mIPSC amplitude was not significantly different in scrambled control and CRTCl^{A1} neurons.
- (D) Rise time and (E) decay time constant of mIPSCs were not affected in CRTCl^{A1} compared to CRTCl^{Scr} neurons.
- (F) Cumulative probability histogram showing the distribution of inter-event interval of mIPSC events.
Number of neurons measured per group are indicated within each column.

3.2.4 Increase AP firing in CRTTC1^{A1} neurons due to loss of GABAergic terminals in cultured neurons

We have established that CRISPR-Cas9 targeting CRTTC1 effectively reduced CRTTC1 expression between 10 to 14 DPT (Figure 3-7). However, the loss of CRTTC1 could affect the viability of excitatory neurons. We thus quantified cultured neurons to show that knocking out CRTTC1 expression did not affect the overall viability of GAD67-positive inhibitory neurons, which accounts for < 15% of total neuronal population (Figure 3-13). Since GABAergic inhibition using bicuculline resulted in CRTTC1^{Scr} neurons firing AP bursts similar in numbers to CRTTC1^{A1} transduced neurons, we hypothesised that defects in inhibition could result in increased AP firing in neurons lacking CRTTC1. Moreover, mIPSC measurements also indicate defects in miniature inhibitory currents (Figure 3-12). We set out to test the idea that CRTTC1 may contribute to the regulation of inhibitory inputs received by excitatory neurons. We investigated if the loss of CRTTC1 impacts the number of inhibitory terminals (presynaptic GABA-producing enzyme GAD67 and postsynaptic scaffolding protein Gephyrin) forming onto a pyramidal neuron. Therefore, we hypothesised that CRTTC1^{A1} neurons have fewer inhibitory terminals.

For our analyses, we considered only inhibitory terminals forming on excitatory pyramidal neurons (GAD67-negative) as well as the peripheral dendrites (<50 μ m) surrounding the soma (perisomatic). Additionally, we quantified Gephyrin-positive puncta on proximal dendrites (defined as dendrites <100 μ m extended from the soma) and distal dendrites (defined by the last 100 μ m from the terminal ends of the dendritic branch). We observed that CRTTC1^{A1} neurons but not CRTTC1^{Scr} neurons had a significant reduction in the number of perisomatic GAD67-positive terminals (Figure 3-14) at basal unstimulated state. Moreover, CRTTC1^{A1} neurons also showed a decrease in Gephyrin-positive puncta in both proximal and distal dendrites (Figure 3-15). The presence of neuronal activity induced by bicuculline (40 μ M, 30 min) did not further alter distribution or density of either GAD67-positive terminals or Gephyrin-positive puncta and showed similar result when compared to those measured during basal state (Figures S3 – S4). These data suggest that CRTTC1 regulates the number of pre- and postsynaptic inhibitory specialisations in an activity-independent manner.

Figure 3-13: Loss of CRTC1 does not affect viability of GAD67-positive inhibitory neurons in cultured hippocampal neurons

Figure 3-14: Loss of CRTC1 reduces perisomatic GAD67 inhibitory terminals

- (A) Representative confocal micrographs showing perisomatic GAD67-positive terminals of excitatory hippocampal neurons. Cultured hippocampal neurons were transduced at 7 DIV and at 17 DIV, these neurons were immunostained for MAP2 (cyan), GAD67 (red) and Hoechst nuclear dye (blue). Scale bar, 5 μ m.
- (B) Quantification showing the total and average number of GAD67-positive terminals analysed per condition. Sample size indicates the total number of perisomatic excitatory neurons in a given field across three independent experiments.
- (C) Bar graph showing a significant reduction in the average number of GAD67-positive terminals in CRTC1^{A1} neurons (**** $p < 0.0001$; n.s. not significant).

Figure 3-15: Loss of CRTC1 reduces Gephyrin puncta in proximal dendrites

- (A) Representative confocal micrographs showing Gephyrin-positive expression on proximal dendrites of excitatory hippocampal neurons transduced at 7 DIV and immunostained with MAP2 (cyan) and Gephyrin (red) at 17 DIV. Scale bar, 5 μm .
- (B) Quantification showing the total and average number of positively-stained Gephyrin terminals analysed per condition. Sample size indicates the total number of proximal dendrites in a given field across three independent experiments.
- (C) Bar graph showing a significant reduction in the average number of Gephyrin-positive terminals in CRTC1^{A1} neurons (**** $p < 0.0001$; n.s. not significant).

3.2.5 CRISPR-mediated knockout of CRTTC1 in hippocampal neurons is specific

We expect CRTTC1^{A1} uniquely targets CRTTC1 genomic region during genome editing, however, CRISPR/cas9 targeting could have non-specific targets and the mutant phenotypes we observed could be attributed to mutations in other regions of the genome that is not associated with CRTTC1. Previously, we described and characterised another CRTTC1^{B4}, which also specifically targeted CRTTC1 in the reconstitution assay and knocked out transient expression of CRTTC1 in HEK293T cells. We, therefore, generated another knockout virus (CRTTC1^{B4}) encoding a different target cassette of CRTTC1 (Figure 3-1B) to try and replicate the deficits we observed from CRTTC1^{A1}. We reasoned that it was unlikely the deficits attributed to the loss of CRTTC1 in neurons would be due to non-specific CRISPR/cas9 knockouts generated from two independent gRNAs.

Similar to CRTTC1^{A1} transduced neurons, to assess the efficiency of transduction and knockout efficiency, we transduced cultured hippocampal neurons at 7 DIV, and after 10 DPT, we treated neurons to bicuculline (40 μ M, 30 mins) before immunostaining with specific antibodies to Cas9 and CRTTC1. As a measure of transduction efficiency, we normalised Cas9 pixel intensity against the average background staining in wildtype (WT) non-transduced neurons. Our experimental validation established that viral transduction for CRTTC1^{B4} were about 70% efficient as 16 out of 24 cells were Cas9-positive (Figure 3-17A).

Our results showed that neurons subjected to CRTTC1^{B4} also behaved similarly when compared to CRTTC1^{Scr} neurons under electrophysiological measurements. In Figure 3-16A, we see the same AP firing pattern as with CRTTC1^{A1} (Figure 3-11) as well as a significant reduction in AP frequency in CRTTC1^{B4} neurons (Figure 3-16C).

We found that CRTTC1^{B4} fared as well as CRTTC1^{A1} in terms of knockout efficiency, with an average of 92 – 96% drop in nuclear CRTTC1 accumulation (Figures 3-17C and D respectively). This indicates that majority of the 70% of CRTTC1^{B4} Cas9-positive cells had a complete loss of CRTTC1 expression be it through the measurement of its nuclear intensity or its nuclear-to-cytoplasmic ratio.

Additionally, we also saw a significant reduction of inhibitory terminals in CRTC1^{B4} neurons at basal (Figures 3-18 and 3-19). The results here are therefore indicative that CRTC1^{B4} is able to recapitulate results to that of CRTC1^{A1} neurons.

Figure 3-16: CRTC1^{B4}-mediated loss of CRTC1 leads to increased AP firing

Figure 3-16

#Dr Song Sang Ho performed and analysed data for all electrophysiological experiments.

- (A) Representative traces of AP bursting recorded from primary hippocampal neurons (CRTCl^{Scr} and CRTCl^{B4} respectively) at 17 – 20 DIV with vehicle treatment.
- (B) Histograms show the number of cells firing AP expressed in percentage. Numbers per group are indicated in the table below (n = 13 and n = 16 for CRTCl^{Scr} and CRTCl^{B4} respectively).
- (C) Reduction in the analysis of AP frequency measured from cultured neurons (*p < 0.05).
- (D) Measurement of resting membrane potential of cultured neurons across all treatment groups.
- (E) Averaged single AP trace in CRTCl^{Scr} hippocampal neurons.
- (F) Averaged single AP trace in CRTCl^{B4} hippocampal neurons.
- (G) No change in the AP amplitude in CRTCl^{Scr} and CRTCl^{B4} neurons.
- (H) The decay time constant of APs were not affected in CRTCl^{B4} compared to CRTCl^{Scr} neurons.
- (I) Rise time constant of APs were not affected in CRTCl^{B4} compared to CRTCl^{Scr} neurons.
- (J) No difference detected in the afterhyperpolarisation (AHP) area between CRTCl^{Scr} and CRTCl^{B4} neurons.
- (K) Half-width comparisons between CRTCl^{Scr} and CRTCl^{B4}.
- (L) Quantification merged with Figure 3-11 showing the number of neurons treated with kynurenic acid (KynA) firing an action potential, expressed in percentage.
- (M) Neuronal frequency during action potential firing when treated with KynA.
- (N) Comparison of resting membrane potential in neurons treated without KynA and those treated with KynA.

Figure 3-17: Knockout efficiency and specificity of CRTC1^{B4} is comparable to CRTC1^{A1}

Figure 3-17

- (A) Hippocampal neurons were cultured for 7 DIV before being transduced with CRTCl^{Scr}, CRTCl^{A1} and CRTCl^{B4} viruses. After 10 days transduction, cells were treated with bicuculline (Bic, 40 μ M, 30 min) to visualise nuclear CRTCl. Neurons were immunostained with antibodies corresponding to MAP2 (cyan), Cas9 (red), CRTCl (green) and Hoechst nuclear dye (blue). Scale bar, 10 μ m.
- (B) Threshold for scatter plot of Cas9 pixel intensity is set at the average pixel intensity scored for WT. Scale bar, 20 μ m.
- (C) Mean nuclear intensity of CRTCl were quantified (left; ***p and ****p < 0.0001). Data on right compares between CRTCl^{A1} and CRTCl^{B4} was further normalised against the average nuclear readout of CRTCl^{Scr}.
- (D) Nuclear-to-cytoplasmic ratio of CRTCl were quantified (left; ****p < 0.0001). Data on right compares between CRTCl^{A1} and CRTCl^{B4} was further normalised against the average N:C readout of CRTCl^{Scr}.
- Data were collected over three independent experiments. Numbers (n) within column represent cells analysed per group.

Figure 3-18: CRTC1^{B4}-induced loss of CRTC1 reduces basal GAD67 inhibitory terminals

- (A) Representative confocal micrographs CRTC1 expression in primary hippocampal neurons. Neurons were transduced at 7 DIV and at 17 DIV were immunostained for antibodies against MAP2 (cyan), GAD67 (red), and Hoechst nuclear dye (blue). Scale bar, 5 μ m.
- (B) Quantification showing the total and average number of perisomatic GAD67-positive terminals per condition. Sample size indicates the number of neurons analysed over three independent experiments.
- (C) Bar graph showing a significant reduction in the average number of perisomatic GAD67-positive terminals in CRTC1^{A1} and CRTC1^{B4} (**** $p < 0.0001$ relative to CRTC1^{Scr}). There is however, no difference between CRTC1^{A1} and CRTC1^{B4} (n.s., not significant).

Figure 3-19: CRTC1^{B4}-induced loss of CRTC1 reduces basal proximal Gephyrin puncta

Figure 3-19

- (A) Representative confocal micrographs showing Gephyrin expression on proximal dendrites of cultured neurons transduced at 7 DIV and immunostained with MAP2 (cyan) and Gephyrin (red) at 17 DIV. Scale bar, 10 μ m.
- (B) Quantification showing the total and average number of Gephyrin-positive puncta analysed per condition. Sample size indicates the total number of proximal dendrites in a given field across three independent experiments.
- (C) Bar graph showing a reduction in the average number of Gephyrin-positive puncta in CRTTC1^{A1} and CRTTC1^{B4} neurons (**p < 0.001 relative to CRTTC1^{Scr}).
- (D) Representative confocal micrographs showing Gephyrin expression on distal dendrites of cultured neurons transduced at 7 DIV and immunostained with MAP2 (cyan) and Gephyrin (red) at 17 DIV. Quantification showing reduction in the average number of Gephyrin-positive puncta in CRTTC1^{A1} and CRTTC1^{B4} neurons (*p < 0.01 relative to CRTTC1^{Scr}). Scale bar, 10 μ m. Data were collected over three independent experiments.

3.2.6 Loss of CRTC1 alters number of GAD67-positive GABAergic terminals during formation and maturation

To determine the significance of knocking out CRTC1 at the synaptic level, we wanted to ascertain the GABAergic synaptogenesis profile across different stages of our *in vitro* hippocampal cultures. Based on a previous publication, it was shown that GABAergic synapses of embryonic rats form between 7 DIV to 14 DIV *in vitro*, after which proliferation remained constant from 14 DIV to 21 DIV (Swanwick, Murthy, Mtchedlishvili, Sieghart, & Kapur, 2006).

To examine if our cultured mouse neurons share a similar profile for inhibitory terminal development, we defined two distinct phases of GABAergic development: 1) synaptic formation where we expect growth of GABAergic terminals (between 7-14 DIV) and 2) synaptic maintenance where these GABAergic synapses mature and stabilise (14-24 DIV). To this end, we measured total GAD67 puncta as a marker for GABAergic synapses in neurons across the different developmental stages. According to our results, there were barely any GAD67-positive immunoreactive puncta at 7 DIV, but at 14 DIV, there was about 15-fold increase in the number of GAD67-positive terminals. However, between 17 DIV and 24 DIV, there was no change in the number of these GABAergic synapses. This data supports results published by Swanwick and co-workers, indicating that inhibitory synaptogenesis occurs from 7 DIV to 14 DIV, after which these synapses mature and remain unaltered *in vitro* (Figure 3-20).

Following the experimental setup above, we wanted to identify if knocking out CRTC1 will affect either inhibitory synaptic formation, maintenance, or both. We addressed the aim by transducing neurons at 7 DIV, where we have shown that at this time point, nascent GAD67 synapses first emerge. After about 10 DPT, we then proceeded to immunostain with specific antibodies against GAD67. In Figure 3-14, we showed that CRTC1^{Δ1} neurons had a dramatic reduction of about 3-fold in the number of GAD67-positive terminals during synaptic formation (7 – 17 DIV), which suggests that loss of CRTC1 can negatively affect the proper formation of GABAergic terminals.

Similarly, in Figure 3-21, we showed that a reduction in GAD67-positive puncta (2-fold) is seen in CRTC1^{Al} neurons when compared to CRTC1^{Scr} neurons, suggesting that knocking out CRTC1 also affected the maintenance of inhibitory terminals once the neurons reach maturity. We note that the loss of inhibition is more dramatic if CRTC1 expression is lost during synapse formation than maintenance, which indicates that perhaps CRTC1 plays a critical role in GABAergic proliferation during *in vitro* development.

Figure 3-20: Synaptogenesis for GAD67-positive GABAergic terminals occurs between 7 – 14 DIV in cultured hippocampal neurons

- (A) Representative confocal micrographs showing neuronal GABA synaptogenesis across different stages of growth at basal conditions. At each time point, neurons were immunostained with antibodies specific for MAP2 (cyan), GAD67 (red) and Hoechst nuclear dye (blue). Scale bar, 5 μ m.
- (B) Quantification showing the total and average number of GAD67-positive terminals analysed per time point. Sample size indicates the total number of perisomatic excitatory neurons in a given field across three independent experiments.
- (C) Bar graph showing the average number of GAD67-positive terminals during growth (between 7 to 14 DIV) and the stabilisation of these terminals from 14 DIV onwards.

Figure 3-21: Loss of CRTC1 affects perisomatic GAD67 inhibitory terminal formation and maintenance

B

Perisomatic	WT	CRTC1 ^{Scr}	CRTC1 ^{A1}
Total number of GAD67 ⁺ terminals	637	554	267
Sample size	n=26	n=30	n=30
Avg. number of GAD67 ⁺ terminals	24.50 ± 1.75	22.97 ± 1.60	10.43 ± 0.84

- (A) Representative confocal micrographs showing CRTC1 expression in primary hippocampal neurons. Cultures were transduced with lentiviruses at 14 DIV and at 24 DIV were immunostained for MAP2 (cyan), GAD67 (red), and Hoechst nuclear dye (blue). The number of perisomatic GAD67 GABAergic puncta is significantly reduced by CRTC1^{A1} (**** $p < 0.0001$). Scale bar, 5 μ m.
- (B) Quantification showing the total and average number of GAD67-positive terminals analysed per condition. Sample size indicates the total number of perisomatic excitatory neurons in a given field across three independent experiments.
- (C) The average number of GAD67-positive terminals was plotted as a bar graph showing a significant reduction in CRTC1^{A1} neurons (**** $p < 0.0001$; n.s. not significant).

3.2.7 Increase AP firing due to changes in GABAergic receptor signalling at inhibitory terminals

We hypothesise that another way that AP firing can be enhanced in CRTC1^{A1} neurons is due to reduction or changes in chloride channel composition at inhibitory terminals which disrupts the transport of chloride ions at GABAergic terminals. This is a plausible explanation as CRTC1 acting as transcription coactivator can influence CREB transcription and expression of different chloride channels at inhibitory terminals. We decided to test this hypothesis by studying the gene expression of several inhibitory receptor subunits of GABAergic receptors in cultured neurons. We first had to determine which chloride channels contain CRE elements and are potentially CREB target genes. Looking at the literature, we noted that genes encoding the GABA_A receptor subunit alpha-1 (*Gabra1*) (Hu et al., 2008), GABA_B receptor subunit-1b (*Gabbr1b*) (Steiger, 2004) and glycine receptor beta and alpha subunit (*Glrbl* and *Glr1* respectively) (Lynch, 2004; Malosio, Marquze-Pouey, Kuhse, & Betz, 1991; Young-Pearse, Ivic, Kriegstein, & Cepko, 2006) were reported to be CREB target genes. A check on the CREB online database indicates that these four genes do indeed have CRE elements (<http://natural.salk.edu/CREB/>; Table S2).

As shown in Figure 3-22, CRTC1^{A1} neurons had significantly reduced *Gabra1*, *Gabbr1b* and *Glrbl* mRNA expression as compared to CRTC1^{Scr} neurons via candidate qPCR analysis. In contrast, GABA_B receptor subunit 1a (*Gabbr1a*) was not affected by the loss of CRTC1. This was in line with reports that indicated the GABA_B 1a subunit is an ATF4 (CREB2) target gene and does not respond to CREB activation despite having CRE elements. Interestingly, *Glr1* increased upon knocking out CRTC1. We postulate that knocking out CRTC1 may have compensatory effects for reduced GABAergic transmission, and one way of achieving a homeostatic balance between excitation and inhibition could be through increasing *Glr1* mRNA which encodes glycine receptors. Altogether, these results suggest that loss of CRTC1 affects basal transcription of selective GABA receptors (GABA_A and GABA_B) and chloride channels.

3.2.8 Increase AP firing due to disruption in *Npas4* mRNA resulting in excitatory-inhibitory imbalance

One other possibility for the loss of CRTC1 affecting inhibition is the reduction of *Npas4*-mediated transcription. As described in the introduction, *Npas4* is a major activity-dependent transcription factor that is regulated by CREB during activity (Lin et al., 2008; Maya-Vetencourt, 2013). One of the critical function for *Npas4* is that it regulates the expression of downstream genes which determine the balance between excitatory and inhibitory neural circuits (Lin et al., 2008; Sun & Lin, 2016). We postulate that the loss of *Npas4* mRNA due to the loss of CRTC1-mediated transcription could lead to the imbalance in inhibitory synapse formation. We then asked whether the loss of CRTC1 had any effect on *Npas4* transcription. From our real-time qPCR analysis, we also found reduced mRNA levels of *Npas4* in CRTC1^{A1} neurons at the basal state (Figure 3-22F). This suggests that the reduction in *Npas4* transcription could indeed further disrupt the excitatory-inhibitory balance in neuronal circuits. Indeed in *Npas4*-RNAi, the number of GABAergic terminals were significantly reduced, whereas excitatory synapses were not affected (Lin et al., 2008).

Figure 3-22: Loss of CRTC1 affects basal transcription of *Npas4*, GABA_A and GABA_B receptor subunits

Quantification showing real-time RT-PCR analysis of gene expression levels of GABAergic receptor subunits and putative *Npas4* in cultured hippocampal neurons (17 DIV) at basal condition. Basal levels of (A) *Gabra1*, (C) *Gabbr1b*, (E) *Glr1* and (F) *Npas4* mRNAs were differentially decreased in CRTC1^{A1}. On the contrary, (B) *Gabbr1a* mRNA levels were not significantly different between CRTC1^{Scr} and CRTC1^{A1}, and (D) *Glr1* mRNA levels were increased in CRTC1^{A1} neurons. Gene expression levels were normalised to *Hprt1* housekeeping gene (*p < 0.01; **p < 0.001; n.s., not significant). Data were collected over three independent experiments.

3.2.9 Loss of CRTC1 alters activity-dependent structural plasticity of excitatory postsynaptic protein expression but not mEPSC

Next, we wanted to examine the consequences of CRTC1 deletion on the composition, density and distribution of excitatory synaptic terminals. To determine if the loss of CRTC1 affects excitatory synaptic activity, we once again transduced hippocampal neurons with lentiviral constructs CRTC1^{Scr} and CRTC1^{A1} as previously described. We asked if at basal levels, excitatory synaptic proteins were altered, and miniature excitatory postsynaptic currents were affected (mEPSC). For this, we transduced dissociated hippocampal neurons at 7 DIV as previously described and looked at neurons 10 DPT (at DIV17). Under basal, non-stimulated conditions, we imaged and quantified excitatory postsynaptic scaffold (PSD95) protein distribution and density along the neuronal dendrites. Since PSD95 scaffolding proteins are clustered and enriched in the postsynaptic compartment, we measured PSD95 puncta in dendrites as a proxy for postsynaptic spines. We first separated quantifications of the proximal length of the dendrite (defined as distance less than 100 μ m extending from the soma) and the distal length of the dendrite (defined as the last 100 μ m from the terminal ends of the dendritic branch). Our measurements showed no noticeable changes in either the density or distribution of PSD95-positive puncta in either the proximal or distal dendrites of pyramidal excitatory neurons transduced with CRTC1^{A1} or CRTC1^{Scr} (Figure 3-24). We next measured miniature EPSC in these neurons, and no deficits were detected in either the frequency or amplitude of the mEPSC (Figure 3-23). This indicated that at least in basal, non-stimulated conditions, loss of CRTC1 did not disrupt the localisation of excitatory PSD95 proteins that may be involved in synapse formation, maintenance or in the spontaneous release of neurotransmitters.

Since CRTC1 nuclear translocation is activity-dependent, it is plausible that major deficits in CRTC1 knockout hippocampal neurons is not detectable under basal state and deficits in transcription and structural plasticity at synapses can only be observed upon induction of neuronal activity. With that in mind, we treated neurons with bicuculline for two specific purposes: 1) to drive CRTC1 translocation in cultured neurons and 2) and to increase activity-dependent spine density which was previously reported in hippocampal neurons (Papa & Segal, 1996). In this paper, Papa and

colleagues showed a 21.5% increase in spine density with the application of bicuculline. In Figure 3-25, we observed diminished levels of the average number of PSD95-positive puncta as well as density (puncta per 10 μm of dendrite) of PSD95-positive puncta in CRTC1^{Al} compared to CRTC1^{Scr} neurons in both proximal and distal dendrites. Indeed, loss of CRTC1 completely abolished an activity-dependent increase of PSD95 positive puncta on dendrites upon bicuculline treatment.

Moreover, western blot analysis showed a decrease in activity-dependent postsynaptic scaffolding protein, PSD95. However, the expression of pre-synaptic scaffold protein Synaptophysin remains unaltered (Figure 3-26). Altogether, our results suggest that loss of CRTC1 did not affect PSD95 protein localisation under basal conditions, but disrupted its increase in spine density following an activity-dependent stimulation.

Figure 3-23: Loss of CRTCl does not affect mEPSC

- (A) Representative trace of mEPSC recordings from WT, CRTCl^{Scr} and CRTCl^{A1} neurons in the presence of bicuculline (20 μ M) and TTX (1 μ M).
- (B) Frequency and (C) amplitude of mEPSCs measured in WT, CRTCl^{Scr} and CRTCl^{A1} neurons.
- (D) Rise and (E) decay time constant of mEPSCs were not affected in scrambled compared to CRTCl^{A1} neurons. The number of neurons analysed per group is indicated within each graph column.

Figure 3-24: Loss of CRTC1 does not affect number of PSD95-positive puncta in basal hippocampal neurons

Figure 3-24

- (A) Representative confocal micrographs showing no difference in number and density of proximal PSD95-positive puncta of CRTC1^{Al} neurons in comparison to CRTC1^{Scr} neurons. Cultures were transduced at 7 DIV and immunostained for MAP2 (cyan) and PSD95 (red) (n.s., not significant relative to CRTC1^{Scr}). Scale bar, 5 μ m.
- (B) Quantification showing the total number of PSD95-positive puncta analysed per condition. Sample size indicates the total number of proximal dendrites in a given field across three independent experiments.
- (C) Bar graph showing no change in the average number of PSD95-positive puncta across all three conditions (n.s. not significant).
- (D) Quantification showing the density of PSD95-positive puncta analysed per condition.
- (E) Bar graph showing no change in the proximal density of PSD95-positive puncta per 10 μ m across all three conditions (n.s. not significant).
- (F) Representative confocal micrographs showing no difference in number and density of distal PSD95-positive puncta of CRTC1^{Al} in comparison to CRTC1^{Scr}. Cultures were transduced at 7 DIV and immunostained for MAP2 (cyan) and PSD95 (red) (n.s., not significant relative to CRTC1^{Scr}). Scale bar, 5 μ m.
- (G) Quantification showing the total number of PSD95-positive puncta analysed per condition. Sample size indicates the total number of distal dendrites in a given field across three independent experiments.
- (H) Bar graph showing no change in the average number of PSD95-positive puncta across all three conditions (n.s. not significant).
- (I) Quantification showing the density of PSD95-positive puncta analysed per condition. Sample size indicates the total number of distal dendrites in a given field across three independent experiments.
- (J) Bar graph showing no change in distal density PSD95-positive puncta per 10 μ m as a bar across all three conditions (n.s. not significant).

3-25: Loss of CRTC1 abolishes activity-dependent increase of PSD95-positive puncta in both proximal and distal dendrites

Figure 3-25

- (A) Representative confocal micrographs of cultured hippocampal neurons transduced at 7 DIV and at 17 DIV, stimulated with bicuculline (40 μ M, 30 min) prior to immunostaining with antibodies for MAP2 (cyan), CRTC1 (green) and Hoechst nuclear dye (blue). Scale bar, 10 μ m. Quantification showing a reduction of CRTC1 protein expression in CRTC1^{A1} neurons, but not CRTC1^{Scr} (**p < 0.001; ****p < 0.0001; n.s. not significant). The number of neurons analysed per group over three independent experiments is indicated within each graph column.
- (B) Representative confocal micrographs of cultured hippocampal neurons transduced at 7 DIV and at 17 DIV, stimulated with bicuculline (40 μ M, 30 min) prior to immunostaining with antibodies for MAP2 (cyan), and PSD95 (red). Scale bar, 10 μ m.
- (C) Quantification showing the average number of PSD95-positive puncta analysed per condition. Sample size refers to the number of proximal dendrites analysed per group over three independent experiments.
- (D) Bar graph showing a significant reduction in the average number of glutamatergic postsynaptic PSD95-positive puncta on proximal dendrites in CRTC1^{A1} neurons (****p < 0.0001).
- (E) Quantification showing the density of PSD95-positive puncta analysed per condition. Sample size refers to the number of proximal dendrites analysed per group over three independent experiments.
- (F) Bar graph showing a significant reduction in the density of glutamatergic postsynaptic PSD95-positive puncta on proximal dendrites in CRTC1^{A1} neurons (****p < 0.0001).
- (G) Representative confocal micrographs of cultured hippocampal neurons transduced at 7 DIV and at 17 DIV, stimulated with bicuculline (40 μ M, 30 min) prior to immunostaining with antibodies for MAP2 (cyan), and PSD95 (red). Scale bar, 10 μ m.
- (H) Quantification showing the average number of glutamatergic postsynaptic PSD95 puncta on distal dendrites in CRTC1^{A1} neurons. Sample size refers to the number of dendrites analysed per group over three independent experiments.
- (I) Bar graph showing a significant reduction in the average number of glutamatergic postsynaptic PSD95 puncta on distal dendrites in CRTC1^{A1} neurons (****p < 0.0001).
- (J) Quantification showing the density of glutamatergic postsynaptic PSD95 puncta on distal dendrites in CRTC1^{A1} neurons. Sample size refers to the number of dendrites analysed per group over three independent experiments.
- (K) Bar graph showing a significant reduction in the density of glutamatergic postsynaptic PSD95 puncta on distal dendrites in CRTC1^{A1} neurons (****p < 0.0001).

Figure 3-26: Loss of CRTC1 reduces expression of postsynaptic density proteins

Bicuculline stimulation

Representative western blot showing total protein lysates of Synaptophysin, PSD95 and CRTC1 from transduced cultures stimulated with bicuculline (40 μ M, 30 min) at 17 DIV. Band intensities were quantified against TuJ1 and normalised against CRTC1^{Scr} (**p < 0.001; *p < 0.01; n.s., not significant).

3.3 CRTTC1 – a central player in transcription-dependent synaptic plasticity during LTP and LTD

3.3.1 Glycine-induced LTP in neurons leads to nuclear translocation of CRTTC1

Theta pulse stimulation at the Schaffer collateral pathway leads to a robust nuclear accumulation of CRTTC1 while simultaneously depleting the synaptic pool of the said protein (Ch'ng et al., 2012). However, antidromic stimulation at the axons of CA1 neurons did not alter CRTTC1 localisation, thereby indicating that synaptic activity, but not depolarisation alone, was required for nuclear import (Ch'ng et al., 2012). However, no data yet exists to show CRTTC1 translocation during stimuli that trigger long-term potentiation. While non-physiological synaptic activity such as bicuculline treatment to block GABAergic receptors or TTX withdrawal can drive CRTTC1 nuclear translocation, the type of physiological pattern of activity that drives translocation in neurons has not been explored. Previously, Ch'ng and colleagues showed that CREB target genes in hippocampal cultures can be activated using a TTX withdrawal protocol – briefly, neurons were silenced with TTX (sodium channel blocker), and thereafter a quick washout of TTX then results in an AP firing and the induction of several IEGs (Ch'ng et al., 2015; Saha et al., 2011). Instead of TTX withdrawal, we ask if a chemical potentiation protocol which has been shown to trigger transcription-dependent synaptic strengthening can also trigger CRTTC1 translocation. We wanted a chemical LTP induction protocol that fulfilled the following criteria: (1) has a long-lasting synaptic effect and (2) one that triggers transcription. These are relevant considerations since CRTTC1 co-activates CREB in an activity-dependent manner, and the result of which is the activation of a multitude of other downstream targets.

Glycine was demonstrated to be an effective chemical method of inducing LTP in organotypic hippocampal slices (Musleh, Bi, Tocco, Yaghoubi, & Baudry, 1997) and in dissociated hippocampal cultures (Fortin et al., 2010; Lu et al., 2001). The induction of LTP resulted in increased recruitment of AMPARs at active synapses (Hayashi et al., 2000; Shi et al., 1999) as new receptors were being rapidly redistributed to the postsynaptic membrane surface. The selective blocking of synaptic NMDARs with MK-801 prevented the production of a reliable LTP, demonstrating the prerequisite for

activating synaptic NMDARs in the initiation of this process (Carroll et al., 1999; Lu et al., 2001). To this end, we utilised glycine-induced LTP (GI-LTP) protocol established by Lu and colleagues as it was shown to demonstrate stable potentiation in cultured neurons when postsynaptic NMDAR were activated in the presence of glycine as measured by an increase in mEPSCs (Lu et al., 2001). To examine if GI-LTP also resulted in changes in surface labelling of GluA1 subunit of AMPAR in our neuronal cultures, we used a polyclonal antibody targeted against the extracellular domain of GluA1 subunit under non-permeant conditions. An increase in GluA1 labelling on the surface of spines has been widely used as a proxy for synaptic activity and plasticity (Malenka, 2013). We determined the effect of GI-LTP induction on AMPARs expressed on the surface of hippocampal neurons by treating cells with glycine for 3 minutes, followed by a 30 minutes recovery in a glycine-free bath solution. We found that glycine application stimulated a 2-fold increase in surface GluA1 puncta on dendrites (Figure 3-27A). The proportion of cell surface receptors were determined using ImageJ (refer to materials and methods, section 2.23).

To investigate whether CRTC1 respond to the glycine-induced activation of NMDAR-dependent synaptic potentiation in cultured neurons (17 – 21 DIV), we treated neurons to either mock (no glycine) or LTP (200 μ M glycine) treatment and terminated the experiment with an immediate PFA fixation. As shown in Figure 3-27B, CRTC1 was excluded from the nucleus in mock-treated neurons, whereas a 10-minute glycine treatment showed CRTC1 accumulation in the nucleus. Even though the original protocol called for a recovery period after glycine stimulation to observe for changes in structural plasticity (Lu et al., 2001), for this particular set of experiment, we had to treat neurons with glycine for 10 minutes without a recovery period to better observe for CRTC1 accumulation in the nucleus. CRTC1 faithfully tracks neuronal activity and will rapidly exit the nucleus once the stimulus is removed (Ch'ng et al., 2012). If we had allowed the neurons to recover after stimulation, CRTC1 would have exited the nucleus.

Figure 3-27: Gl-LTP induces synaptic surface AMPA receptors

- (A) Representative confocal micrographs of WT hippocampal neurons at 21 DIV either mock control or LTP-treated. After 30 minutes of recovery, neurons were immunostained with MAP2 (cyan) and GluA1 (green). Scale bars, 10 μm .
- (B) Quantification showing the total number of surface GluA1 puncta in mock and LTP-treated neurons. Sample size indicates the number of dendrites analysed over three independent experiments.
- (C) Bar graph showing a significant increase in surface GluA1 puncta (**** $p < 0.0001$ relative to mock).
- (D) Representative confocal micrographs comparing CRTC1 expression levels across WT, CRTC1^{Scr} and CRTC1^{A1} hippocampal neurons with 10 minutes of LTP stimulation. Neurons were immunostained with MAP2 (cyan), GAD67 (red), CRTC1 (green), Hoechst nuclear dye (blue). Scale bars, 10 μm . Quantification showing a significant increase in the nuclear-to-cytoplasmic ratio in CRTC1^{A1} neurons (**** $p < 0.0001$ relative to CRTC1^{Scr}). The number of neurons quantified is indicated within each graph column with data collected over three independent experiments.

3.3.2 LTP induction evokes phosphorylation of CREB and its target genes

The induction of LTP at excitatory synapses in the hippocampal neurons is thought to be a likely cellular process to underlie learning and memory which involves CREB (Lynch, 2004). We have already established that GI-LTP enhances AMPA receptor insertion on the plasma membrane and triggers CRTC1 nuclear accumulation (Figure 3-27). We now examine if the glycine-induced nuclear import of CRTC1 can also activate CREB. Following the original Lu et al. (2001) protocol where neurons are subjected to a brief GI-LTP treatment in the presence of glycine followed by 15 – 20 minutes recovery in a bath solution, neurons showed robust phosphorylation of CREB at S133 (Figure 3-28). The effect of CREB phosphorylation peaked at 5 minutes after GI-LTP onset and sustained up to 10 minutes post-GI-LTP treatment. However, pCREB S133 levels returned to basal levels by 30 min after glycine withdrawal, indicating that the temporal activation of CREB activation requires constitutive stimulation. The phosphorylation of CREB occurs in parallel with the entry of CRTC1 during stimulation.

Next, we seek to identify specific CREB-mediated IEGs that may be triggered by nuclear translocation of CRTC1 upon GI-LTP treatment. Using a candidate-based approach, we selected well-known activity-dependent IEGs that are relevant to the maintenance of long-term plasticity. We used cultured hippocampal neurons treated with mock (no glycine) or LTP (200 μ M glycine) treatment followed by recovery for 30 minutes. We then harvested RNA from these neurons and subjected them to quantitative PCR. LTP-stimulated neurons responded via upregulation of specific CREB-target genes, including *Arc*, *cFos*, *Egr4*, *Cyr61* and *Zif268* compared to neurons receiving mock treatment. Most of these activity-dependent genes increase between 2-4 fold in comparison to mock (Figures 3-29: A-F). As we saw in Figure 3-27, CRTC1 responds to GI-LTP treatment through nuclear accumulation, but its mRNA levels remain unaltered even after GI-LTP (Figure 3-29) which suggests that *Crtc1* transcript levels are not regulated by activity.

To address the function of CRTC1 in activity-dependent transcription during LTP, we knocked out CRTC1 expression in hippocampal neurons as previously described. After 10 DPT, we then stimulated neurons with GI-LTP to induce CREB target genes in hippocampal neurons, in which cultures were treated with 200 μ M glycine for 3 minutes,

followed by glycine withdrawal, and replacement of bath solution containing no glycine for the next subsequent 30 minutes. Our qPCR results of CRTC1^{Scr} and CRTC1^{A1} neurons showed substantial downregulation for all five of our tested CREB specific target genes which have been induced by the application of glycine: *Arc*, *cFos*, *Cyr61*, *Egr4* and *Zif268* compared to CRTC1^{Scr} neurons (Figures 3-29: G-L). Our data are consistent with the previous report that CRTC1 knockdown inhibited the expression levels of activity-dependent transcripts (Ch'ng et al., 2012). Collectively, our results indicate that GI-LTP can drive CRTC1 nuclear translocation, CREB phosphorylation and that loss of CRTC1 during GI-LTP resulted in deficits in transcription-dependent CREB-target genes.

Figure 3-28: LTP induction leads to CREB phosphorylation at S133

A

B

- (A) Representative confocal micrographs of mock control and LTP-stimulated neurons coupled with different recovery intervals at 5, 10, 30 and 60 min recovery accordingly. Neurons were immunostained with pCREB S133 (red), MAP2 (cyan) and Hoechst nuclear dye (blue).
- (B) Nuclear signals of phosphorylated CREB at S133 was quantified (* $p < 0.01$; **** $p < 0.0001$ relative to mock; n.s., not significant). Scale bars, 10 μ m. The number of neurons quantified is indicated within each graph column with data collected over three independent experiments.

Figure 3-29: GI-LTP induces activation of CREB gene targets and loss of CRTC1 impairs transcription of CREB target genes

- (A-F) RT-qPCR data represent changes in total mRNA expression of immediate early genes of hippocampal cultures treated with 200 μ M glycine for 3 minutes following a 30 minutes recovery. Analysis of quantitative real-time PCR revealed a significant increase in expression of candidate genes (* $p < 0.01$; ** $p < 0.001$; n.s., not significant).
- (G-L) RT-qPCR data of immediate early genes treated with 200 μ M glycine for 3 minutes following a 30 minutes recovery in dissociated hippocampal neurons. Comparison between *CRTC1^{Scr}* and *CRTC1^{A1}* neurons revealed a significant decrease in expression of candidate genes (* $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$).

3.3.3 Induction of DHPG mediated mGluR-LTD results in endocytosis of cell surface GluA1 receptors

While we know from previous publication that CRTC1 undergoes nuclear translocation in response to high-frequency stimulation in acute hippocampal slices (Zhou et al., 2006), much less is known about CRTC1 localisation and its contribution during LTD. We show that CRTC1 responds to glycine-induced LTP (Figure 3-27B). We are also interested in studying LTD because as with LTP, several forms of chemical and electrical stimulation of LTD require transcription (Kemp, Tischmeyer, & Manahan-Vaughan, 2013; Sohyun, Ginty, & Linden, 1999; Wang & Zhuo, 2012). The importance of CRTC1 stimulus-induced gene transcription during LTD has never been directly explored even though calcineurin's role in the maintenance of late-phase LTD is well established, and so is CRTC1's dependence on calcineurin to mediate dephosphorylation for its nuclear localisation (Mulkey, Endo, Shenolikar, & Malenka, 1994).

There are several forms of LTD in neuronal culture systems (Fitzjohn et al., 2001; Goda & Stevens, 1996; Henson, Tucker, Zhao, & Dudek, 2017). NMDA-mediated LTD has been performed on slices to achieve synaptic depression (Lee, Kameyama, Huganir, & Bear, 1998) but it is not well established in cultured hippocampal neurons. While the application of NMDA to cultured neurons can trigger CRTC1 nuclear translocation, CREB is not activated because neurons trigger extrasynaptic NMDA NR2B-subunit mediated excitotoxicity program (Ch'ng et al., 2015). As such, NMDA-LTD in neuronal cultures is not feasible. Hence, we turn our focus on another well-established form of synaptic LTD: DHPG-induced mGluR-LTD.

It was published that mGluR-LTD require protein synthesis (Naie & Manahan-Vaughan, 2005) and mGluR-LTD signalling cascade converge on common pathways (MAPK and ERK) that involve transcriptional regulation (Gallagher, 2004; Gladding, Fitzjohn, & Molnar, 2009; Wang & Zhuo, 2012; Wang, Fibuch, & Mao, 2007). Initial studies show that transcriptional inhibitor actinomycin D did not block mGluR-LTD in CA1 hippocampal slices (Huber et al., 2000). However, critical consensus indicates that the initial study focused mainly on the early phases of LTD (< 90 minutes) instead of the late phase (2 – 3 hours) of LTD when transcriptional requirements became critical.

Subsequent publication from the Levenson group replicated and extended Huber's initial LTD study (Huber et al., 2000) and showed that transcription inhibitor (DRB) can indeed block DHPG-LTD if recordings were extended to the late phase > 90 minutes (O'Riordan, 2006). Several groups argued that early phase LTD relies more on translation, whereas the late phase LTD requires transcription of new genes (Wang & Zhuo, 2012). To further support LTD's role in mediating gene transcription, recent publication indicates that hippocampal LTD likely triggers gene transcription in which behavioural conditions that led to LTD in behaving rodents also trigger immediate somatic early gene expression in the hippocampus (Hoang, Aliane, & Manahan-Vaughan, 2018). This study supports an earlier report on the necessity of learning-induced LTD on activation of cFos expression (Kemp et al., 2013). But what is striking about both studies is that the relative number of soma that expresses IEGs after LTD-related events are very low, which could confound specific detection of transcription-dependent plasticity in an *in vivo* preparation. However, this also suggests that *in vitro* approaches that aim for less potent LTD responses may be the most appropriate route to take when addressing transcription issues in the slice preparation.

Both NMDA-dependent LTD (NMDA-LTD) and mGluR-LTD lead to reduced surface expression of AMPARs (Huber et al., 2000). In our experiments, we stimulated cultured hippocampal neurons with 50 μ M of (R, S)-DHPG for 5 minutes to induce mGluR-LTD (Hernandez & Abel, 2008) and replaced with conditioned media for the next 55 minutes. We then used an acid-strip staining protocol to label GluA1 subunit on live neurons under non-permeabilising conditions to monitor changes in surface AMPA receptors concentrations (Snyder et al., 2001). Additionally, we included a control group (basal permeabilised) where neurons were permeabilised immediately with Triton X-100 and subsequently immunostained with GluA1 antibody – neurons will stain both surface and internalised GluA1. Our aim here is to ask whether DHPG treatment is effective in inducing mGluR-LTD in our mouse hippocampal neurons by looking at endocytosis of surface GluA1 from the membrane.

Neurons at 60 minutes after the onset of receiving DHPG treatment showed a close to 2-fold increase in the number of internalised GluA1 puncta due to endocytosis compared to WT neurons with no treatment (Figure 3-30). In parallel to increased internalisation

of AMPARs, surface AMPARs were also shown to significantly reduced at synapses upon DHPG treatment (Snyder et al., 2001). Note that internalisation of GluA1-positive vesicles occurs as early as 15 minutes and persists up to 60 minutes post-treatment with DHPG (Snyder et al., 2001).

Figure 3-30: mGluR-LTD stimulates internalisation of GluA1

- (A) Representative confocal micrographs of basal WT neurons permeabilised with Triton X-100 prior to staining with GluA1 stains both internalised GluA1 as well as surface GluA1. Basal neurons that were non-permeabilised were first immunostained for GluA1 receptor internalisation prior to permeabilisation with Triton X-100. DHPG-treated neurons at 50 μ M for 5 min, and following 55 min of recovery were immunostained for GluA1. Scale bars, 10 μ m. Sample size indicates the number of dendrites analysed over three independent experiments.
- (B) Quantification showing an increased in the average number of GluA1 puncta after 15 and 60 minutes onset of DHPG treatment (* $p < 0.01$; *** $p < 0.0001$ relative to basal; n.s., not significant).

3.3.4 CRTC1 translocates to the nucleus of cultured hippocampal neurons following induction of mGluR-LTD

To examine if CRTC1 mediates mGluR-LTD dependent transcription, we first asked whether CRTC1 responded to DHPG-mediated mGluR-LTD in cultured hippocampal neurons. To induce mGluR-LTD, we stimulated cultured neurons with (R, S)-DHPG (50 μ M) for 5 minutes and immediately fixed the cells for immunostaining. Additionally, we have also included controls where WT neurons were incubated with tetrodotoxin (TTX; 1 μ M, 1 h), a sodium channel blocker which silences neurons, and with GABA_A receptor antagonist bicuculline (Bic; 40 μ M, 30 min), which drives excitatory synaptic transmission in cultures. As shown in Figure 3-31, CRTC1 significantly accumulates in the nucleus following incubation with bicuculline and DHPG.

To further monitor the persistence of DHPG-induced CRTC1 nuclear accumulation, we incubated neurons with DHPG for 5 minutes, followed by a subsequent recovery of either 10 or 55 minutes in conditioned media without DHPG. At 15 minutes after DHPG onset, moderate levels of CRTC1 remained in the nucleus, comparable to that of the bicuculline-treated neurons. However, at 60 minutes after DHPG treatment onset, CRTC1 nuclear signal returned to basal levels. Interestingly, the nuclear persistence of CRTC1 during DHPG induction is longer as compared to bicuculline-mediated AP bursting (Ch'ng et al. 2012).

Figure 3-31: mGluR-LTD induces CRTC1 translocation

Bicuculline (Bic, 40 μ M, 30 min) and TTX (1 μ M, 1 h) were added to hippocampal cultures in conditioned media. CRTC1 nuclear-to-cytoplasmic ratio was quantified. Comparison of CRTC1 nuclear-to-cytoplasmic ratio in WT neurons between basal and DHPG-treated (50 μ M, 5 min) neurons showed increased in CRTC1 nuclear accumulation (**** $p < 0.0001$ relative to basal; n.s., not significant relative to basal). Scale bars, 10 μ m. The number of neurons quantified are indicated within each graph column with data collected over three independent experiments.

3.3.5 Calcium influx and calcineurin activity is required for DHPG-induced CRTC1 nuclear translocation

Because the kinetics the CRTC1 translocation has never been reported or studied during LTD, we wanted to ask whether the nuclear translocation with DHPG requires the same signalling cascade as NMDA-receptor mediated CRTC1 translocation. First, we examined whether nuclear entry of CRTC1 is dependent on calcineurin since activity-dependent translocation of CRTC1 with conventional synaptic NMDAR activation is dependent on calcineurin activation (Ch'ng et al). We treated cultured hippocampal neurons with mGluR-LTD protocol in the presence or absence of cyclosporine A (CsA; 5 μ M, 30 min) to block calcineurin function. Our results showed that CsA completely blocked the nuclear enrichment of CRTC1 (Figure 3-32A). Next, we asked if intracellular calcium is a required component for CRTC1 nuclear translocation during mGluR-LTD-induced synaptic activation in hippocampal neurons. To do this, we again treated neurons with DHPG (50 μ M, 5 min) to induce mGluR-LTD in the presence or absence of membrane permeable AM-BAPTA. BAPTA is a commonly used calcium chelator and should remove all traces of intracellular calcium. As shown in Figure 3-32B, the presence of BAPTA-AM also abolished the nuclear accumulation of CRTC1 induced either by bicuculline or DHPG. These experiments indicate that calcium is required for CRTC1 nuclear import likely through the activation of calcineurin to dephosphorylate CRTC1.

Figure 3-32: mGluR-LTD is sensitive to both calcineurin activity and calcium

(A) WT hippocampal neurons were incubated with DHPG (50 μ M, 5 min) in the absence or presence of pre-treatment with CsA (5 μ M, 30 min). Neurons were then fixed and immunostained with antibodies against MAP2 (cyan), CRTC1 (green) and Hoechst dye (blue), and the nuclear-to-cytoplasmic ratio was quantified (** $p < 0.001$ relative to basal; **** $p < 0.0001$ relative to DHPG-stimulated neurons; n.s. not significant).

(B) WT hippocampal neurons were treated with either DHPG (50 μ M, 5 min) or bicuculline (Bic; 40 μ M, 30 min) in the presence or absence of pre-treatment with BAPTA (25 μ M, 30 min). The nuclear-to-cytoplasmic ratio of CRTC1 was quantified (**** $p < 0.0001$ relative to either Bic or DHPG-treatment). Scale bars, 10 μ m. The number of neurons quantified are indicated within each graph column with data collected over three independent experiments.

3.3.6 mGluR-LTD induces CREB activation and is independent of protein synthesis

While CREB phosphorylation was demonstrated in cultured striatal neurons upon DHPG treatment (Snyder et al., 2001), a similar response has never been observed in cultured hippocampal neurons. As such, we seek to test whether DHPG-induced mGluR-LTD alters CREB phosphorylation status in cultured hippocampal neurons. We found few pCREB-expressing cells at basal conditions (without treatment) in comparison to cells receiving 5 minutes of DHPG treatment (Figure 3-33A). We then went on to determine the persistence of CREB phosphorylation by applying a brief pulse of DHPG in neurons for 5 minutes, followed by a washout of DHPG and allowing the neurons to recover in conditioned media for a further 10 and 55 minutes prior to fixing and immunostaining the cells. We observed robust CREB immunoreactivity immediately after DHPG onset (0 minute recovery) (Figure 3-33B). However, 60 minutes after the onset of DHPG treatment, pCREB levels returned to that of basal condition. Since DHPG mediated mGluR-LTD is known to engage protein synthesis machinery (Mao & Wang, 2002). Treatment with cycloheximide (CHX) inhibited not only translation but also reduced mGluR-stimulated GluA1 receptor endocytosis (Huber et al., 2000). We asked if CRTC1 nuclear entry during DHPG-mediated mGluR-LTD requires protein synthesis. Treatment of neurons with cycloheximide (CHX; 60 μ M, 30 minutes) did not inhibit CRTC1 nuclear translocation (Figure 3-34). Our findings corroborated well with a previous study showing that protein synthesis inhibitor emetine did not prevent bicuculline-induced CRTC1 nuclear translocation (Snyder et al., 2001). Thus, we show that mGluR-LTD triggers CREB S133 phosphorylation in parallel with CRTC1 nuclear translocation, which is independent of protein translation but requires calcium influx and calcineurin activation.

Figure 3-33: mGluR-LTD triggers CREB phosphorylation at S133 in dissociated hippocampal neurons

- (A) Representative confocal micrographs of hippocampal neurons at basal (no treatment) and LTD-stimulated. Neurons were stained with antibodies specific for pCREB S133 (green; $**p < 0.001$ relative to basal).
- (B) Neurons were immunostained with antibodies for GAD67 (red), pCREB S133 (green), MAP2 (cyan) and Hoechst nuclear dye (blue). Quantification showed robust pCREB expression upon LTD stimulation but decays gradually over time ($*p < 0.01$; $****p < 0.0001$ compared to basal). Scale bars, 10 μm . The number of neurons quantified are indicated within each graph column with data collected over three independent experiments.

Figure 3-34: CRTC1 nuclear entry during mGluR-LTD is independent of protein synthesis

Hippocampal neurons were treated with DHPG (50 μ M, 5 min) in the presence or absence of pre-treatment with CHX (60 μ M, 30 min). Neurons were then fixed and immunostained with antibodies against MAP2 (cyan), CRTC1 (green) and Hoechst dye (blue), and the nuclear-to-cytoplasmic ratio was quantified (** $p < 0.001$ relative to DHPG-stimulated neurons; n.s. not significant relative to CHX-treated neurons). Scale bars, 10 μ m. The number of neurons quantified are indicated within each graph column with data collected over three independent experiments.

3.3.7 mGluR-LTD induces CRTC1-dependent CREB target gene expression

Similar to assessing CREB-target genes during LTP induction, we wanted to ask if the absence of CRTC1 can affect specific CREB-mediated gene transcription during mGluR-LTD induction. So far, we have shown that the induction of mGluR-LTD triggers the CRTC1/CREB signalling pathway. To better understand the function of CRTC1 expression during mGluR-LTD, we assessed six candidate CREB-mediated target genes by quantifying mRNA levels in cultured hippocampal neurons.

After applying DHPG (50 μ M, 5 min) to induce mGluR-LTD, we recovered cultures in conditioned media for the next subsequent 55 minutes. We then harvested these cultures as it matches with our AMPA receptor internalisation data, and at this time point, synaptic depression occurs (Snyder et al., 2001). One criterion for choosing the CREB-dependent gene targets was because reports previously implicated these genes in DHPG-mediated mGluR-LTD (Lindecke et al., 2006). As an example, ATF4 is a CREB2 transcriptional repressor; it is a transcription factor known to be regulated by mGluR-LTD (Guan et al., 2003).

Our candidate qPCR data showed between 1- to 2- fold upregulation of activity-induced transcript levels of CREB-target genes: *Arc*, *Atf4*, *cFos*, *JunB*, *Nr4a1* and *Zif268* (Figures 3-35: B to G). These results indicate rapid and robust activation of CREB-mediated transcription by mGluR-LTD in neurons. Interestingly, we observed overlapping candidate genes in both LTP and LTD, namely *Arc*, *cFos* and *Zif268*, providing evidence that CREB-mediate a pool of common target genes during long-term plasticity. The data here suggests that transcription is critical for mGluR-LTD in cultured neurons. Following the loss of CRTC1, these upregulated genes were significantly reduced in comparison to CRTC1^{Scr} neurons (Figures 3-35: I to N). Together with our findings from LTP, the downregulation of mRNAs upon knocking out CRTC1 indicate that CRTC1 not only influences glycine-mediated LTP mRNA synthesis but also impacts mGluR-LTD-mediated transcription.

In summary, sections 3.3.3 to 3.3.7 investigated the role of CRTC1 in the regulation of gene expression during mGluR-LTD in the hippocampal neurons. The results demonstrate that:

1. Induction of DHPG-mediated mGluR-LTD results in the endocytosis of cell surface GluA1 receptors as reported previously (Figure 3-30; Snyder et al., 2001).
2. Next, we saw that following the application of DHPG, CRTC1 translocates to the nucleus of cultured hippocampal neurons (Figure 3-31).
3. Calcium influx and calcineurin activity are required for DHPG-induced CRTC1 nuclear translocation (Figure 3-32).
4. mGluR-LTD induces CREB activation (Figure 3-33) and is independent of protein synthesis (Figure 3-34).
5. mGluR-LTD induces CRTC1-dependent CREB-target gene expression (Figure 3-35).

Figure 3-35: CRTC1 is important for the expression of CREB-mediated target genes during mGluR-LTD

- (A-G) mRNA expression of CREB-dependent transcripts after induction of chemical LTD by 50 μ M DHPG for 5 min. Data represent fold changes of these transcripts 60 min after treatment onset with reference to expression in WT hippocampal neurons (* p < 0.01; n.s., not significant).
- (H-N) Comparison of mRNA expression levels between CRTC1^{Scr} and CRTC1^{A1} neurons after DHPG treatment showed a significant decrease in candidate genes (* p < 0.01; ** p < 0.001; *** p < 0.0001).

3.3.8 *In vivo* CRTTC1^{A1} lentivirus delivery to cells in the dentate gyrus (DG)

Given that our CRTTC1^{A1} lentivirus was capable of knocking out CRTTC1 efficiently *in vitro*, we then asked if the same knockout efficiency can be achieved at the hippocampus, specifically at the dentate gyrus via *in vivo* stereotaxic injections. To study the impact of CRTTC1 expression during learning and memory, we aimed to knock out CRTTC1 expression in the hippocampus of wild-type mice, and thereafter assess their cognitive performances through multiple behavioural assays. As CRTTC1 germline knockout mouse was reported to exhibit anxiety and depressive-like behaviours (Breuillaud et al., 2012), and the presence of multiple abnormalities and behavioural deficits cannot be directly attributed to the loss of CRTTC1 function in the brain. However, direct knockout of CRTTC1 in hippocampal neurons to assess memory assays has never been tested.

To begin, we first had to ascertain that our lentivirus injection is uniformly expressed throughout our intended target site: the DG. As such, we performed a pilot study on the delivery efficiency of a high-titre GFP-expressing lentivirus, FUGW. We injected the virus into wild-type C57BL6/J male mice at 6 – 8 weeks old with approximately 1.5 μ L of virus on each cerebral hemisphere. A total of 5 mice were used for this pilot injection.

As seen in Figure 3-36A, representative confocal micrographs were organised in an anterior to posterior fashion with focus only on the DG site. In most images, GFP expression was detected in cells within the granular (GrDG) layer as well as the polymorph (PoDG) layer on both hemispheres of the brain as early as 7 days post-injection (DPI). We observed visible mossy fibre projections from GFP-expressing cells extending towards the CA3 region. Figure 3-36B shows a closeup of brain section at - 2.00 mm away from bregma and individual insets of the GrDG and PoDG layers.

Next, we determined if the viral spread for CRTTC1^{Scr} and CRTTC1^{A1} is comparable to that of the FUGW lentivirus by performing bilateral stereotaxic injection of the virus. Unlike FUGW, the global expression of the virus in the DG indicated by Cas9 staining was limited only to the top of the PoDG layer (Figures 3-10Bi & ii) in many of the injected mice. Furthermore, Cas9 expression in both hemispheres is inconsistent, and the coverage in the DG was low. Unlike the control FUGW virus which had GFP expression in a much larger area of the injection site, the CRTTC1 injections were

inconsistent even between hemispheres. In all, we tested many variables in our injections, including different coordinate site for stereotaxic injections in the dorsal DG, different viral volumes and injection apparatus as well as using multiple injection sites (data not shown). The inconsistent and insufficient spread of CRTTC1^{Scr/A1} virus in the DG renders the study of cognitive tasks challenging. In particular, we wanted to use the knockout animal in fear conditional tests, Morris Water Maze as well as object recognition task to assess spatial reference and working memories respectively in these rodents.

We are also further limited by the availability of suitable antibodies to specifically immunostain our sections. At the moment, we are using the rabbit polyclonal Cas9 antibody for all our *in vivo* sections; the mouse monoclonal FLAG, as well as Cas9 antibodies, were ineffective in our hands. We are thus unable to establish transduction and knockout efficiencies *in vivo* as a result of having both Cas9 and CRTTC1 antibodies being raised in the same host species. There are, however, CRTTC1 antibodies raised in a different host species in the market but those antibodies have not been validated to work as well as the rabbit CRTTC1.

Figure 3-36: Lentivirus infection in DG of injected hippocampus

- (A) Representative confocal micrographs showing five different animals showing the extent of GFP lentiviral spread in DG from anterior to posterior at 14 days post-injection. Scale bar, 100 μ m.
- (B) Confocal micrograph from (A) of animal #4 at -2.0 mm from bregma -2.0 mm. Insets in red boxes are the zoomed-in high magnification of lentivirus-infected single cells demonstrating virus penetration in the granular and polymorph layers, respectively. Scale bar, 20 μ m for inset.

Figure 3-37: Sparse and inconsistent spread of CRTC1^{Scr} in DG

A

B

C

Figure 3-37

- (A) Images showing a single animal receiving CRTCL^{Scr} injection in both sides of the brain (right hemisphere). The white insets show the zoomed-in high magnification extent of Cas9 spread in DG at -2.3 mm from bregma at 14 days post-injection. Scale bar, 100 μ m; 20 μ m for inset.
- (B) Images showing a single animal receiving CRTCL^{Scr} injection in both sides of the brain (left hemisphere). The white insets show the zoomed-in high magnification extent of Cas9 spread in DG at -2.3 mm from bregma at 14 days post-injection. Scale bar, 200 μ m; 10 μ m for inset.
- (C) Images of a single animal showing successful CRTCL^{Scr} spread of Cas9 in DG from anterior to posterior at 14 days post-injection. Scale bar, 100 μ m.

Figure 3-38: Sparse and inconsistent spread of CRTC1^{A1} in DG

Representative confocal images showing a single animal receiving CRTC1^{A1} injection in both sides of the hemispheres at 14 days post-injection. Refer to materials and methods (section 2.14) for injection coordinates and volume of virus injected. Scale bar, 100 μ m.

4 DISCUSSION

4.1 Developing a method for conditional deletion of CRTTC1 expression in cultured hippocampal neurons

The cAMP-response element binding protein (CREB)-regulated transcription coactivator, CRTTC1, is identified as a potent modulator of cAMP response element (CRE)-driven gene transcription in multiple tissues (Altarejos & Montminy, 2011). Numerous studies have established that CRTTC1 plays important roles in long-term plasticity (Saura & Cardinaux, 2017). There are also experimental evidence to indicate that dysfunctional CRTTC1 leads to neuropathological conditions (Zhan-Cheng et al., 2015). Moreover, we are aware of the existence of a germline CRTTC1 knockout mouse (Breuillaud et al., 2012). However, since CRTTC1 expression is absent early during development, this mouse is infertile, hyperphagic and obese (Altarejos et al., 2008). As CREB function is crucial during growth and development of the brain, the genetic abnormalities and metabolic deficits can easily influence cognitive function, and thus the knockout mouse is not suitable for studying long-term plasticity in adult mice. Therefore, to address this issue, we sought to characterise the function of CRTTC1 by knocking out protein expression in the hippocampus using genome editing constructs and studying whether loss of the coactivator in adult mice leads to defects at the cellular level.

The work described in this thesis provides the first study on CRTTC1 knockout by co-expressing endonuclease Cas9 and a gRNA specific to the CRTTC1 gene to be targeted. To study the function of CRTTC1, we established the CRISPR/Cas9 gene editing system to knock out the expression of CRTTC1. The gRNAs were designed and optimised to target mouse genomic *Crttc1*. Since the success of *Crttc1* deletion hinges on a highly efficient CRISPR/Cas9 activity, a series of validation assays were pursued to identify the most efficient gRNA in targeting the *Crttc1* genomic locus. One principal concern that this system brings about is that of off-target effects, which is not unusual for a method that relies on a 20-bp recognition motif and its ability to withstand mismatches. Jinek and colleagues reported that mismatches in the first 7 bases of the gRNA are well tolerated in terms of cleavage efficiency *in vitro* using the plasmid-based system but

additional studies showed that mismatches in the 8th – 12th base pairs from the 3' end of the target sequence leads to reduced or inactive target cleavage (Cong et al., 2013; Jinek et al., 2013; Jinek et al., 2012; Semenova et al., 2011; Wiedenheft et al., 2011).

In our efforts to address this issue, we generated four independent gRNAs to target *Crtc1* to which we have rigorously tested (Figures 3-3 and 3-5), and eventually narrowed down to a single candidate gRNA. Thus far, our results with gRNA CRTC1^{A1} has provided reliably consistent results; hence, we proceeded to use this gRNA for all our subsequent studies. While CRTC1 protein expression levels were reduced in Figure 3-5, a complete loss of CRTC1 is not expected primarily due to the efficiency of co-transfection of two expression plasmids. As such, it is likely that a population of cells expresses the dendra2 fusion protein but lacks the pX330 CRISPR/cas9 plasmid. The number of targeted cells largely depends on the transfection efficiency, and many other variable factors including cell type, cell cycle progression, gene expression activity, promoter activity, among many others (Cong et al., 2013; Jinek et al., 2013; Jinek et al., 2012; Semenova et al., 2011; Wiedenheft et al., 2011).

Collectively, our *in vitro* overexpression assays reveal that the CRISPR/Cas9 system is capable of inducing target-specific cleavage of *Crtc1*. We had set up the reconstitution assay and others to determine cutting efficiency of gRNA, but it turns out the *in vitro* assays worked very well for most if not all constructs tested. Therefore, it is pertinent that we test our gRNA CRTC1^{A1} in hippocampal neurons and to examine if it cuts the genome. To understand the importance of CRTC1 in specific neuronal functions, it is imperative that we can optimally manipulate CRTC1 protein levels in neurons. Thus, it is necessary that we adopt a carrier that can consistently introduce genetic material in these neuronal models both *in vitro* and *in vivo*. Viral vectors have emerged as a much better tool than conventional transfection methods of delivering foreign gene (Sandbichler, Aschberger, & Pelster, 2013).

More importantly, we needed a viral vector that is capable of transducing non-dividing cells since neurons are post-mitotic. Being post-mitotic, these neurons do not undergo cell division, and so it is unlikely that HDR can occur efficiently in neurons; as such, NHEJ is the dominant mechanism of DSB repair (Iyama & Wilson, 2013). Moreover, several published works have shown that CRISPR can be successfully elicited in post-

mitotic neurons. For example, a complete loss of NMDA-mediated plasticity from hippocampal pyramidal neurons using CRISPR/Cas9 knockout of *Grin1* (gene encoding for GluN1 subunit of NMDA receptor) exhibited electrophysiological patterns and behavioural defects (Straub et al., 2014). In a separate study by Incontro and colleagues, they showed that CRISPR-targeted *Gria2* (AMPA receptor subunit 2) had close to 100% mutation efficiency in their Cas9-positive neurons, of which 90% of their mutations were indels with frameshifts (Incontro et al., 2014). These findings, thus, suggest that mutagenesis in post-mitotic neurons using CRISPR are highly efficient, perhaps even more so than in dividing cells (Lee, Sundberg, Sigafos, & Clark, 2016).

Although both lentivirus and adeno-associated virus (AAV) are capable of transducing dividing and non-dividing cells, we wanted to construct an all-in-one vector system that allows expression of both Cas9 and the gRNA simultaneously in a transduced cell. This is critical for *in vivo* as single neuron whether for molecular or imaging studies incorporates the use of a single viral vector to deliver both gRNA and cas9 ensures that neurons expressing cas9 will have a corresponding gRNA to target CRTC1. Since AAV has a smaller packaging limit of about 4.5 kb, a split virus system had to be employed to express the gRNA and cas9 in separate viral vectors. As such, we decided to go with a single lentiviral expression vector to knock out CRTC1 expression. Clearly, our *in vitro* results have reliably established transduction efficiency for our CRISPR lentiviruses, especially in cultured hippocampal neurons, to be close to 100% as every randomly selected cell expresses Cas9 (Figure 3-8A).

However, every neuron with Cas9 expression does not indicate CRTC1 has been knocked out in these neurons. In our study, CRISPR-driven knockout of CRTC1 in dissociated hippocampal neurons is highly efficient although the strength of our CRTC1 knockout using CRTC1^{A1} is not 100% complete with about 90 – 95% reduction in CRTC1 protein expression (Figures 3-8C & D). As discussed previously, Cas9 creates DSBs through the combined activity of its duo-functional endonuclease domains – RuvC and HNH (Gasiunas, Barrangou, Horvath, & Siksnys, 2012; Jiang & Doudna, 2017; Jinek et al., 2014). Hence, it is possible that Cas9 cleavage may be slightly more efficient on one allele but not on the other, resulting in a partial deletion of CRTC1 which resembles that of a knock-down. To circumvent this, Cas9 can be modified such that one of two domains can be kept inactive, allowing a DNA nick rather than a DSB,

or otherwise also known as Cas9 nickase. In this way, two nickases can be introduced to target on opposite alleles to generate double nicks. The use of a dual nickase, however, does not further increase gRNA cleavage efficiency (i.e. having more alleles being knocked out) but rather aids in enhancing its specificity and reduces the possibility of off-target effects since two cuts are required.

We next wanted to determine the duration of lentiviral transduction required to achieve maximal knockout of CRTTC1 expression. This is critical as we want to ensure that the majority of the neurons have lost CRTTC1 expression without affecting neuronal viability. In our case, we showed that maximal knockout required at least 10 days post-transduction. This timing, however, is dependent on the age of cultured neurons with which transduction occurs. As previously discussed, in our hands, transduction at 4 DIV showed no apparent knockout even though the knockout analysis was conducted 14 days post-transduction. We postulate that perhaps during early culture time points (3 – 4 DIV) when synaptic connections have yet to mature, possible cell death and cellular debris that are left behind after plating may interfere with knockout efficiency. That said, we believe that transduction in our experiments should happen on DIV 7 and beyond because we are looking into the effects of CRTTC1 loss in mature synaptic structures and plasticity. Therefore, there is no single best timing for transduction and time required for efficient protein depletion as it has to be empirically determined for each target protein for every experiment.

Our Westerns show that CRTTC1^{A1} reduced the overall amount of protein expression (Figure 3-23). However, from our immunocytochemistry results, overall neurons expression of CRTTC1 is reduced either at the whole soma level (Figure 3-8B) or when neurons are stimulated with bicuculline to accumulate in the nucleus (Figures 3-8C & D). The percentage of CRTTC1^{A1} neurons that responded to bicuculline stimulation and subsequently accumulates in the nucleus is low (5 – 10%). One explanation for this disparity is that the lentiviruses mainly transduce neurons in culture and not astrocytes. We know that CRTTC1 is also expressed in astrocytes (data not shown; LWL). As such, in Westerns, we may detect astrocytic CRTTC1 that have not been knocked out by lentiviral transduction.

We initially expected that commercially-purified and concentrated high titre lentiviral particles would allow sufficient viral transduction of dorsal hippocampal neurons in the DG. We manipulated the injection parameters (needle length, injection coordinates, viral volume, multiple injection sites, etc.) without much success. We also performed a series of stereotaxic injection in P24 – P32 mice and found limited Cas9 immunoreactivity in the DG and inconsistent Cas9 staining pattern on both hemispheres, an indication that transduction efficiency is not robust. According to Kumar and colleagues, there exists a direct relationship between packaging size and titre (Karra & Dahm, 2010). Although our CRISPR lentiviruses were guaranteed with 10^9 titre from a commercial vendor, it has never been tested for any *in vivo* applications. Given the limited packaging of the lentivirus machinery, it is not surprising why we would expect much drastically less efficient transduction during our attempted microinjections.

While SpCas9 is the most commonly used endonuclease in genome editing, it might not fit best for every application. Given the advances in CRISPR genome engineering, there are Cas9 orthologs that may prove to be more useful than SpCas9 depending on the requirement of the application to be used. For example, an all-in-one AAV system is possible – by substituting SpCas9 with *Staphylococcus aureus* Cas9 (SaCas9) as it is 1 kb shorter than SpCas9, therefore enabling the cDNA to be efficiently packaged into AAV (Friedland et al., 2015; Nishimasu et al., 2015). Similar to SpCas9, the SaCas9 is also equally capable of modifying target genes both *in vitro* as well as *in vivo*. Moreover, AAV is ideal for gene delivery *in vivo* due to its mild toxicity (Kumar, Keller, Makalou, & Sutton, 2001).

Overall, there is still potential to use the virus for further *in vivo* studies if the system could be further optimised or tweaked. For instance, we could repackage the cas9 virus using a split AAV vector that expresses the gRNA and cas9 in separate AAV particles. While this eliminates the benefits for a single virus transduction, it could help solve the weak transduction coverage encountered with our lentiviruses. To locate neurons that are transduced with the virus, we could also add a fluorescent fusion protein such as tdTomato after the P2A peptide sequence to serve as a visual marker of transduction. Finally, while the virus-mediated gene transduction in DG may not be sufficient for behavioural analysis, but with the current setup, we can still use the virus to study the effects of knocking out CRTCL1 expression at the single cell level such as examining

spine morphology and dynamics upon behavioural paradigms. Additionally, CRTC1 knockout effects can be possibly tested with *in situ* RNA hybridisation techniques.

4.2 CRTC1 expression is required for proper synaptic composition and density

There are reports of full-length or dominant negative overexpression forms of CRTC1 modulating certain forms of long-term plasticity and memory consolidation at the hippocampus (Lau & Suh, 2017). However, a CRTC1 loss of function study and its impact on long-term plasticity at the cellular and molecular level has never been done. Our electrophysiological data show an apparent effect that basal CRTC1^{Al} neurons displayed increased AP frequency in comparison to CRTC1^{Scr} neurons (Figure 3-11E) and the addition of bicuculline resulted in an even higher rate of firing pattern (Figure 3-11C). Hence, the effect on AP bursting frequency is probably due to enhanced synaptic excitation rather than depolarisation since AP properties are identical across the other groups (Figure 3-11F). To ascertain that the effect we see is of a synaptic origin, we incubated neurons with KynA, a broad glutamatergic receptor antagonist, and we no longer see the bursting phenotype (Figure 3-11H). The data thus indicate that enhanced AP bursting is due to enhance glutamatergic synaptic activity and not due changes in intrinsic excitability of the neurons (Figure 3-11I).

There are several possibilities that neurons exhibit increase AP firing due to loss of CRTC1. Our first hypothesis is that the increased neuronal excitability could be due to disinhibition of excitatory neurons. We then used single cell patch clamp techniques to measure mIPSC, and indeed, we saw a near three-fold reduction in the frequency, but not in the amplitude (Figures 3-12B and C). Following the conventional school of thought that mIPSC frequency is dependent upon vesicular release, whereas the mIPSC amplitude corresponded to the activation of the postsynaptic receptor (Pinheiro & Mulle, 2008), we therefore believe that the change we saw in frequency could reflect a possible reduction in either the probability of presynaptic vesicular release at the synapse or the number of functional inhibitory synapses. As for amplitude, we think that there is probably no change in the conductance or activation of postsynaptic GABA_A receptors. In parallel to patch clamp experiments, we used presynaptic marker GAD67 as well as postsynaptic marker Gephyrin as indicators for inhibitory input in our dissociated hippocampal neurons. In our study, we report that neurons lacking CRTC1 exhibited

reduced inhibitory boutons in pre- and postsynaptic terminals that synapsed on excitatory neurons (Figures 3-13 and 3-14).

Loss of CRTC1 impacting inhibitory connections could be a result of synaptogenesis during neuronal development or maintenance of inhibitory terminals after maturation. To differentiate between the two scenarios, we needed to knock out expression of CRTC1 either early during neuronal development or after the neurons have matured and synaptogenesis is largely completed. Indeed, we show that at 7 DIV, inhibitory terminals still developing and by 14 DIV, no new inhibitory synapses were detected (Figure 3-20). This matches what was previously reported (Swanwick et al., 2006). We then knocked out CRTC1 by transducing neurons either at 7 DIV (development) or at 14 DIV (maintenance). Our results reveal that loss of CRTC1 at both phases affected total inhibitory synapses (Figures 3-14 and 3-21) even though the effect at development is more severe than maintenance. We postulate that perhaps synaptogenesis developmental stage is more important functionally for inhibitory development. However, we do think that CRTC1 expression is also equally vital for continued maintenance especially, in response to activity. CRTC's role in inhibitory synaptogenesis has never been described and this perhaps could be one of the many emerging roles of CRTC1 to be looked into. In Figure 3-15, we show that the number of inhibitory neurons remains the same even in CRTC1^{Δ1} neurons, ruling out the possibility that loss of CRTC1 may affect the viability of inhibitory neurons. Therefore, the enhanced AP firing is not due to CRTC1-mediated loss of inhibitory neurons.

Since CRTC1 is a transcriptional coactivator, one other possibility is that transcription of different GABAergic receptors and/or chloride channels are affected. Considering that maintaining proper synaptic connections are critical for the downstream transcription-dependent gene expression, our results show that the loss of CRTC1 led to a reduction in the transcript levels of some of these GABAergic receptor subunits as well as chloride channels (Figure 3-22). These are CREB-induced target genes that may potentially regulate excitatory-inhibitory balance, one of which includes *Npas4*. *Npas4* is an activity-dependent transcript that is induced by CREB during AP firing; its expression is predominantly found in excitatory neurons (Sun & Lin, 2016) but could also be detected in inhibitory neurons (Shepard, Heslin, Hagerdorn, & Coutellier, 2019;

Spiegel et al., 2014). While *Npas4* was first thought to modulate only GABAergic synaptic connections, there are increasingly more evidence to suggest that *Npas4* plays a broader role in regulating activity-dependent synaptic connections (Lin et al., 2008; Spiegel et al., 2014).

A reduction of *Npas4* expression in cultured hippocampal pyramidal neurons via RNAi led to a loss of inhibitory terminals that synapses on these neurons, whereas the overexpression of which resulted in the overall increase of these terminals (Lin et al., 2008). In organotypic hippocampal slices, similar trends were also observed (Bloodgood et al., 2013; Lin et al., 2008). Moreover, it appears that reduced *Npas4* had a larger effect on postsynaptic GABA_A- γ 2 puncta in comparison to presynaptic GAD65 puncta (Lin et al., 2008), perhaps suggesting that the effect of *Npas4* on presynaptic puncta is a secondary effect resulting from its effect on postsynaptic puncta.

Apart from excitatory neurons, *Npas4* can be robustly induced by neuronal activity in GABAergic neurons (Lin et al., 2008; Spiegel et al., 2014). The deletion of *Npas4* from somatostatin (SST)-expressing GABAergic neurons *in vitro* and *in vivo* led to the reduced number of excitatory terminals forming synapses on these neurons but not GABAergic synapses (Spiegel et al., 2014). Results gathered from both Lin and Spiegel, thereby confirm that *Npas4* works to regulate excitatory-inhibitory homeostasis in the neural network through the recruitment of the corresponding synapses in response to activity as well as the neuronal cell type.

Based on work provided by these extensive studies, we believe that disruption to this balance could potentially contribute to disinhibition or that the composition of specific GABAergic receptors or chloride channels are altered such that the inhibition is lowered and firing is enhanced. Given that very little is known about the upstream transcriptional regulators of *Npas4* expression, loss of *Npas4* mRNA due to loss of CRTTC-mediated transcription could very well indeed lead to an imbalance in inhibitory synapse formation. So far, only one study has provided evidence that serum response factor (SRF) could be a potential upstream regulator of *Npas4* as the deletion of *Srf* gene resulted in a corresponding decrease in *Npas4* expression (Kuzniewska, Nader, Dabrowski, Kaczmarek, & Kalita, 2016). Collectively, the data hints at a potential role for CRTTC1

in the regulation of GABAergic connections received by excitatory neurons by modulating *Npas4* mRNA levels.

We next asked if excitatory terminals were similarly affected by the loss of CRTC1. We first measured for changes in glutamatergic synaptic boutons based on PSD95 markers in the postsynaptic compartment. In addition, we also recorded mEPSC from cultured neurons transduced with either CRTC1^{Al} or CRTC1^{Scr}. At basal unstimulated state, there was no change in PSD95-positive terminals (Figure 3-23); we did not observe much of an effect in mEPSC measurements (Figure 3-23) and CREB is not activated at quiescent state as well (Figure 3-10). However, upon activity induction with either bicuculline (Figures 3-10) or LTP (Figure 3-28), we saw a dramatic difference. Since CRTC1 is a transcriptional coactivator and many of its effects are likely activity-dependent, we would expect that activity is required to drive in the protein to trigger transcription required for long-term plasticity. In the absence of any activity, it is likely we may not see any robust deficits in mEPSCs. Indeed, increased in activity induced by bicuculline drives CRTC1 activation, which then leads to changes in spine density on excitatory neurons. However, this activity-induced increase in the number of spines does not occur in CRTC1^{Al} neurons when compared to CRTC1^{Scr} (Figure 3-25). One possibility is that CRTC1 could either alter genes required for spine formation or impair existing spines. Most of the data points evidence towards the direction that deficits in the structure are as a result of activity-dependent changes. At this juncture, it remains unknown to us why the increased in AP firing, and synaptic activity at basal state was insufficient to induce spine growth that is dependent on CRTC1.

4.3 CRTC1 and its contribution to transcription-dependent plasticity

Synaptic activity triggers CRTC1 nuclear translocation (Ch'ng et al., 2012; Herbst & Martin, 2017), but the type or pattern of activity that permits this translocation remains virtually unexplored. In this part of our study, we focus on long-term plasticity by inducing two primary forms of cellular mechanisms underlying learning and memory: long-term potentiation (LTP) as well as long-term depression (LTD). For us to establish the effectiveness of each paradigm, we asked specifically if our chosen synaptic stimulations can initiate nuclear translocation of CRTC1 in cultured hippocampal neurons. While knockdown studies of CRTC1 (Ch'ng et al., 2012; Finsterwald et al.,

2010; Mamoru Fukuchi et al., 2014) has shown its importance during L-LTP (Nonaka, Kim, Fukushima, et al., 2014), no study has ever selectively knocked out CRTTC1 in the hippocampus. To our knowledge, it is also not known if CRTTC1 is equally important for LTD. However, in this thesis, we provide the first evidence to demonstrate that CRTTC1 responds to both mechanistically distinct forms of synaptic plasticity in the hippocampus – LTP and LTD – in an activity-dependent manner (Figures 3-27B and 3-31). We postulate that CRTTC1 may serve as a potent transducer of different synaptic signals for regulating genes during transcription-dependent plasticity. It is therefore of great interest that we determine how a single protein is capable of impacting transcription depending on different cell types and type of stimuli applied.

Chemical forms of LTP in cultured neurons are well established and can trigger the spontaneous release of glutamate from the presynaptic site, mimicking that of a physiological environment. Lu et al. showed that the application of glycine in this protocol elicits larger and higher frequency mEPSCs and even after the removal of glycine (for up to an hour), the amplitude and frequency of mEPSCs is potentiated. Moreover, glycine-treated neurons also showed an increase in AMPA receptor at synapses (Lu et al., 2001). And this is significant because the relative abundance of AMPA receptors can be modulated by neuronal activity through gene transcription and translation (Evans, Gurung, Wilkinson, Stephens, & Henley, 2017; Gan, Salussolia, & Wollmuth, 2015). We account for LTP in cultured neurons by first measuring for recruitment of native AMPA receptors assembled on the neuronal surface plasma membrane. Consistent with findings from Lu et al., our confocal micrographs show less cell surface AMPA receptors in the mock control cultures compared to treatment of cultures with glycine (200 μ M for 3 min). These analyses show an increased in the number of AMPA receptors along dendrites (Figure 3-30A), suggesting that glycine-induced LTP is associated with an increase in the number of cell surface AMPA receptors. Glycine applications have also been replicated in organotypic slices exhibiting GluA1 immunoreactivity similar to that in cultured neurons (Li et al., 2009; Sekeres et al., 2012). We study the function of CRTTC1 in the transcriptional response to cLTP in cultured neurons by using an established protocol (Lu et al., 2001). Our results reveal that knocking out CRTTC1 in cultured neurons inhibited the expression of all five of our tested CREB target genes – *Arc*, *cFos*, *Cyr61*, *Egr4* as well as *Zif268*

(Figure 3-29), indicating that CRTC1 is critical for the induction of these downstream transcripts.

As with LTP, we are motivated to study CRTC1 effects on the postsynaptic group I metabotropic receptors (mGluRs)-mediated LTD for several reasons: first, like LTP, mGluR-LTD requires transcription (Musleh et al., 1997). It is pertinent for us as CRTC1 is a transcriptional regulator with its subcellular localisation tightly associated with its ability to respond rapidly to synaptic stimulation, allowing it to traffic from dendrites and synapses to the nucleus. Second, calcineurin is involved in the maintenance of late-phase LTD and also responsible for the dephosphorylation of CRTC1. To examine the activation of mGluR-mediated LTD paradigm, we adopted an acid-strip immunocytochemical staining protocol from Snyder et al. (2001) and show that activation of mGluR via DHPG application leads to increased endocytosis of surface AMPA receptors (Figure 3-30). This finding is aligned with other published reports that indicate LTD results in loss of functional surface AMPA receptors due to enhanced receptor internalisation (Kemp et al., 2013; Sohyun et al., 1999; Wang & Zhuo, 2012). Similar to LTP, we explored the impact of CRTC1's influence on gene expression during mGluR-LTD. We chose a few unique targets that were previously identified, specifically, *Nr4a1* and *JunB* as these were among the 7000 genes screened to have the greatest induction upon LTD stimulation (Lindecke et al., 2006). Another characteristic feature of LTD is that it is dependent on ATF4 (CREB2), a known repressor of LTP (Alberini, 2009; Bartsch, Casadio, Karl, Serodio, & Kandel, 1998; Guan et al., 2002; Kandel, 2012), and therefore we have chosen *Atf4* as one of our putative target candidate gene to be tested. Here, we show that CREB-dependent IEGs are reduced upon knocking out CRTC1 in cultured neurons (Figure 3-35), signifying that CRTC1 is also required for mGluR-induced CREB target genes.

While it has been shown that activity can induce CRTC1-mediated activation of CREB target gene transcription (Ch'ng et al., 2012), we show for the first time that both chemical LTP and DHPG-mGluR LTD (henceforth labelled as LTD) can also alter transcription of a subset of IEGs. And interestingly, despite LTP and LTD being “opposites”, here we provide evidence via qualitative real-time PCR that some candidate genes that are upregulated by mGluR-LTD are also upregulated in LTP – *Arc*, *cFos* and *Zif268*. From these data, we further speculate that IEGs common to both LTP

and mGluR-LTD could share the same mechanistic pathway, whereas IEGs that are unique to either LTP or LTD must have distinct molecular signature, suggesting that they are differentially regulated regardless of its dependence or independence on the presence of CRTC1.

Critically, all of the targets we chose contains CRE elements in the promoter region and are known to be CREB target genes (Ch'ng et al., 2012; Zhang et al., 2005). In the absence of CRTC1, there is a general decrease in transcription for both LTP and LTD IEGs. *Crtc1* mRNA, however, is unaltered upon induction of LTP and LTD because CRTC1 nuclear accumulation is not transcription-dependent.

Although mGluR-LTD has always been thought to require protein synthesis (Manahan-Vaughan, Kulla, & Frey, 2000; Naie & Manahan-Vaughan, 2005), there are several indications that point LTD to converge on common pathways (MAPK and ERK) that may also involve transcriptional regulation. The inconsistent findings could be accounted by the fact that most studies were largely focused on the early phase of LTD (< 90 minutes) instead of the late phase (2 – 3 hours of LTD). Publication from the Levenson group extending Huber's LTD study (Huber et al., 2000) showed that transcription inhibitor (DRB) can indeed block DHPG-LTD if recordings were extended to the late phase > 90 minutes (O'Riordan, 2006). Furthermore, there exist other published records of using anisomycin D to successfully block late-phase LTD *in vitro* (Kauderer & Kandel, 2000; Palmer, Irving, Seabrook, Jane, & Collingridge, 1997). As such, it may be possible that the early phase LTD is translation-dependent and the late phase LTD is transcription-dependent (Wang & Zhuo, 2012).

4.4 Functional significance of CRTC1 at basal levels

Basally, CRTC1 expression and activity is low and only upon glutamatergic synaptic activity will it dissociate from its anchoring protein 14-3-3 ϵ and enter the nucleus to enhance CREB transcription. In our culture system, CREB activity is also relatively low under basal conditions in neurons and CRTC1 serves to enhance CREB-driven transcription only in the presence of stimulus such as bicuculline by inhibiting GABA_A receptors (Conkright et al., 2003). Furthermore, we have shown that the presence or

absence of CRTCL does not alter CREB phosphorylation at S133 during induction (Figure 3-10). It is therefore somewhat surprising to see that neurons transduced with CRTCL^{A1} exhibit elevated synaptically-driven AP firing yet did not show elevated CREB phosphorylation at S133. This suggests that CREB-transcription is not activated even though neurons show enhanced bursting and AP firing. Under physiological conditions, nuclear CRTCL directly informs the cell of the presence of synaptic activity. The addition of bicuculline triggers nuclear translocation of CRTCL, which then goes on to activate CREB activity. However, in the absence of CRTCL, neurons have bursting AP firing, which is reflective of what happens in bicuculline-treated neurons. Yet, CREB is not active. One possibility here is that increase in AP does not trigger CREB activation, but rather CREB activation requires either a more robust and sustained activity such as in the presence of bicuculline or a more physiological stimulus such as with cLTP (Figures 3-10 and 3-28). Another possibility is that since the sustained firing is happening throughout the lifetime of neurons after the knockout occurs, these neurons have homeostatic mechanisms to dampen the effects of CREB transcription which would otherwise be toxic to them if CREB is always turned on.

5 Conclusion and future work

The use of CRISPR/Cas9 as a model system to study CRTTC1 function opens up many windows of opportunities. However, there are still many questions that remain unanswered, and there will be much work required before we have a comprehensive understanding of the significance of CRTTC1 in the encoding of long-term memories. With CRISPR/Cas9 off-target effects to be constantly an issue, it is important to show that we can rescue CRTTC1 loss of function deficits in neurons. Furthermore, the CRTTC1 knockout construct we are using express cas9 driven by a ubiquitous EF1 promoter. All neurons and astrocytes theoretically express cas9 (though lentiviral transduction generally favours neurons rather than glia). It will be important to ask whether observed deficits in CRTTC1^{Δ1} transduced cells stem from deficits in excitatory neurons, inhibitory neurons or even glia.

A good place to start might be to design CRISPR-resistant CRTTC1 overexpression construct where the gRNA target site is altered to prevent gRNA binding, but yet retains the primary amino acid sequence. We would then overexpress these CRISPR-resistant CRTTC1 proteins driven by specific promoters (CaMK2 α for excitatory neurons, GAD67 synthetic construct for inhibitory neurons and GFAP for glia). We would use a lentiviral vector that also encodes a GFP-IRES to target and identify the transduced neurons. We can then rescue the phenotype and at the same time, identify which specific cell population is responsible for the deficits attributed to the loss of CRTTC1.

While candidate based qPCR approach is limited in scope, our results obtained from stimulating neurons with LTP and LTD paradigms paves the way to proceed with a larger study – to understand how CRTTC1 expression of IEGs during long-term plasticity by analysing the neuronal transcriptome. There are several compelling reasons for this study: First, CRTTC1 is known to enhance transcription in non-neuronal cells, and since CREB is the master switch for regulating IEG expression during long-term memories, studying CRTTC1 impact on transcription will provide valuable insight into understanding how CREB and other bZIP transcription factors regulate activity-dependent gene expression. Second, it is yet unknown to us how CREB differentially select target genes for transcription in neurons. In the human genome, there are 4000

CRE elements that bind CREB *in vivo* (Zhang et al., 2005). However, depending on cell type and stimuli, as few as hundred CREB target genes are induced during stimulation. How then does CREB respond to specific stimuli and bind to distinct CRE elements to modulate transcription?

Additionally, there are other activity-dependent transcription factors, apart from CREB, that contribute toward long-term plasticity. Some of these also belong to the bZIP family of transcription factors and are also potential binding partners with CRTTC1 (Alberini, 2010; Alberini, 2009; Canettieri et al., 2009). The identification of additional nuclear proteins that binds CRTTC1 in an activity-dependent manner will take us one step closer to assembling a comprehensive profile of the transcriptional machinery during the activation of transcription-dependent plasticity. This profile will help, therefore, to pinpoint how extensive CRTTC1's role in regulating global gene expression during transcription-dependent plasticity. Transcriptome studies have reported that nuclear RNA-sequencing can faithfully recapitulate transcription patterns observed via whole-cell sequencing and shows less variability due to lower RNA degradation (Grindberg et al., 2013; Lacar et al., 2016). By profiling the nuclear transcriptome of basal or stimulated CRTTC1^{Al} neurons and comparing them to CRTTC1^{Scr}, we want to identify changes in gene expression attributed to CRTTC1 during long-term plasticity. Moreover, we can then categorise and sort these activity-dependent transcripts based on rapid or delayed IEG expression and examine if a specific subset of CREB target genes are altered and whether they are CRTTC1-dependent. It stands to reason that since CRTTC1 rapidly enters the nucleus in an activity-dependent manner, perhaps only rapid but not delayed IEGs depend on CRTTC1. It would be interesting to see if CRTTC1 is responsible for the temporal expression of CREB target IEGs. Analysing upstream of the transcripts will also enable us to isolate promoters that may lack CRE elements and aid in our identification of other possible candidates that may be co-activated by CRTTC1.

Finally, it is also worth generating a Cre-dependent Cas9 knockout mouse, as illustrated in Appendix Figure S6. The idea here is to flank a STOP sequence with two loxP sites so that Cas9 expression can be driven by neuron-specific promoters (Nathanson, 2009) (e.g. α -CaMKII for excitatory glutamatergic neurons or decarboxylase for inhibitory GABAergic neurons) in a Cre-dependent manner at Rosa26 locus. Thus, transcription

will be blocked unless Cre recombinase is present. As previously stated, the existence of CRTC1 germline knockout mouse has multiple abnormalities (Bourtchuladze et al., 1994) and the behavioural deficits cannot be directly attributed to the loss of CRTC1 function in the brain. With this mouse model, we would be able to achieve spatial and temporal control of CRISPR/Cas9-mediated genome editing. A single AAV vector integrating gRNA CRTC1 as well as having a Cre recombinase expression cassette can be delivered into Cre-dependent Cas9 mouse. Therefore, Cre recombinase activity on transduced cells leads to the excision of the STOP sequence and subsequent Cas9 expression. Cas9 nuclease activity directed by gRNA introduces DSB at the target site of interest. On the contrary, genomic DNA of non-transduced cells remains intact since Cas9 expression and activity relies on Cre recombinase and gRNA(s).

In particular, we would be interested in assessing several forms of hippocampal-dependent cognitive tasks: using Morris Water Maze to test both spatial reference and working memories (either the standard maze or the delayed placement tasks). Furthermore, we would also be interested in measuring non-spatial hippocampal-dependent function through contextual fear conditioning. An unpublished preliminary analysis using cFos-GFP mice showed an enhanced nuclear CRTC1 accumulation in CA1 neurons at the hippocampus relative to caged control mice (not shown). Interestingly, these cFos-GFP neurons partially overlap with CRTC1 expression, indicating the different population of neurons may differentially express CREB target genes.

6 References

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7 APPENDIX

Figure S1: Expression map of pX330

- (A) Circular graphic map of a human codon-optimized SpCas9 and chimeric guide RNA expression plasmid, pX330.
- (B) Partial linearised map of pX330 vector showing where restriction enzyme digestion of BbsI can allow a pair of annealed oligos to be cloned right before the gRNA scaffolding sequence. The oligos are designed according to the target site of 20 base pairs and requires a 3' NGG PAM sequence (adapted from <https://www.addgene.org/crispr/zhang/>).

Figure S2: Expression map of CRISPR lentivirus

Plasmid expression maps of lentiCRISPR constructs: CRTC1^{A1} and CRTC1^{Scr}

Figure S3: Loss of CRTC1 does not affect viability of GAD67-positive inhibitory neurons in bicuculline-stimulated cultured hippocampal neurons

- (A) Representative confocal micrographs showing the number of GAD67-positive neurons in primary hippocampal neurons across all samples: WT, CRTC1^{Scr} and CRTC1^{A1}. Hippocampal neurons were transduced at 7 DIV and stimulated with bicuculline (40 μ M, 30 minutes) before immunostained at 17 DIV for GAD67 (red), and Hoechst nuclear dye (blue). Scale bar, 20 μ m.
- (B) Quantification showing the total and average number of hippocampal neurons (stained by MAP2) and the number of GAD67-positive cells in a given field. Sample size recorded the number of random images analysed within the same coverslip per condition spread out over three independent experiments.
- (C) Bar graph showing no change in the percentage of the total number of GAD67-positive cells in the total number of MAP2-positive cells across all three conditions in a given field (n.s., not significant).

Figure S4: Loss of CRTC1 reduces number of GAD67 puncta upon bicuculline treatment

Representative confocal micrographs showing reduction of CRTC1 expression in primary hippocampal neurons. Cultures were transduced at 7 DIV and bicuculline stimulated (40 μ M, 30 min) at 17 DIV and thereafter immunostained for MAP2 (cyan), GAD67 (red) and Hoechst nuclear dye (blue). CRTC1^{A1} significantly reduces the number of perisomatic GAD67 GABAergic puncta (**** $p < 0.0001$ relative to CRTC1^{Scr}; n.s. not significant). Scale bar, 5 μ m. Sample size indicated the number of neurons analysed with data collected over three independent experiments.

Figure S5: Loss of CRTC1 reduces number of Gephyrin terminals upon bicuculline treatment

- (A) Representative confocal micrographs showing a reduction of CRTC1 expression in primary hippocampal neurons. Cultures were transduced at 7 DIV, and bicuculline stimulated (40 μM , 30 min) at 17 DIV and thereafter immunostained with MAP2 (cyan) and Gephyrin (red) at 17 DIV. Quantification showing reduction in the average number of Gephyrin-positive puncta in CRTC1^{A1} neurons (**** $p < 0.0001$ relative to CRTC1^{Scr}). Scale bar, 10 μm . Data were collected over three independent experiments.
- (B) Representative confocal micrographs showing a reduction of CRTC1 expression in primary hippocampal neurons. Cultures were transduced at 7 DIV, and bicuculline stimulated (40 μM , 30 min) at 17 DIV and thereafter immunostained with MAP2 (cyan) and Gephyrin (red) at 17 DIV. Quantification showing reduction in the average number of Gephyrin-positive puncta in CRTC1^{A1} neurons (**** $p < 0.0001$ relative to CRTC1^{Scr}). Scale bar, 10 μm . Data were collected over three independent experiments.

Figure S6: Loss of CRTC1 has no effect on expression of synaptic proteins at basal level

Basal

Representative western blot showing total protein lysates of Synaptophysin, PSD95 and CRTC1 from transduced cultures at basal level (without bicuculline stimulation) at 17 DIV. Band intensities were quantified against TuJ1 and normalised against CRTC1^{Scr} (**p < 0.001; n.s., not significant).

Figure S7: qPCR melt curve plots

qPCR primer melt curves from (A) Gabbr1a, (B) Gabbr1b, (C) Gabra1, (D) Glra1, (E) Glrb, (F) Arc, (G) cFos, (H) CRTC1, (I) Cyr61, (J) Egr4, (K) Npas4, (L) Zif268, (M) ATF4, (N) JunB, (O) Nr4a1 and (P) Hprt1.

Figure S8: Proposed illustration for spatial and temporal control of CRISPR/Cas9-mediated genome editing

The proposed illustration shows the Cre-dependent Cas9 Rosa26 targeting vector containing neuron-specific promoter α -CaMKII promoter, LoxP–Stop–LoxP (LSL) cassette, Cas9 endonuclease gene, self-cleaving P2A peptide and the reporter gene (EGFP). The transgene expression cassette was introduced into the Rosa26 locus. For the spatial and temporal control of CRISPR/Cas9-mediated editing of CRTC1, a single AAV vector (integrated with gRNA targeting CRTC1) can be delivered via intratracheal route into the Cre-dependent Cas9 mice. The presence of Cre recombinase enabled the excision of LSL and Cas9 expression in transduced neurons. On the other hand, in non-transduced neurons, this CRTC1 targeting site remains intact.

Table 1: RT-qPCR primer pairs used

Gene	Forward Primer	Reverse Primer	Amplicon size (bp)
<i>Gabbr1a</i>	CACACCCAGTCGCTGTG	GAGGTCCCCACCCGTCA	85
<i>Gabbr1b</i>	GGGACCCTGTACCCCGGTG	GGAGTGAGAGGCCACACC	85
<i>Gabra1</i>	CCAAGTCTCCTTCTGGCTCAAC	AAGCCACCTTCGGGAGGGAATT	122
<i>Glr1</i>	CCGCCGTCAACTTTGTGTCT	GGGGAAACCGATGCGAGATA	284
<i>Glr2</i>	GACGCTTGCTAAAGAAAAGTC	CCAGCAGCCTGTTCAAGATATTG	139
<i>Arc</i>	GGTGAGCTGAAGCCACAAAT	GGTGTCATTCTCCTGGCTCT	205
<i>cFos</i>	CACACAGGACTTTTGCGC	GACACGGTCTTCACCATTCC	213
<i>Crtcl</i>	AGCAGCTCGGCTACACAGGC	TGACATCACTGACTCCTGCC	120
<i>Cyr61</i>	CTGCGCTAAACAACCTCAACGA	GCAGATCCCTTTCAGAGCGG	109
<i>Egr4</i>	TATCCTGGAGGCGACTTCTTG	AGATGCCAGACATGAGGTTGA	196
<i>Npas4</i>	AGGGTTTGCTGATGAGTTGC	CCCCTCCACTTCCATCTTC	74
<i>Zif268</i>	AGCGAACAACCCTATGAGCACC	ATGGGAGGCAACCGAGTCGTTT	116
<i>Atf4</i>	AACCTCATGGGTTCTCCAGCGA	CTCCAACATCCAATCTGTCCCG	125
<i>JunB</i>	TCACGACGACTCTTACGCAG	CCTTGAGACCCCGATAGGGA	125
<i>Nr4a1</i>	GTGCAGTCTGTGGTGACAATGC	CAGGCAGATGTACTTGCGCTT	113
<i>Hprt1</i>	TGTTGTTGGATATGCCCTTG	GGCCACAGGACTAGAACACC	180

This table lists all the primers used for the duration of this study.

Table 2: Computational prediction of CREB binding sites (CREs)**GI-LTP**

Symbol	Gene Name	Accession	Chrom.	Strand	Position	CRE Sites (-3Kb to 300b)	Total CRE sites	Proximal CRE (-500b to 300b)	Distal CRE (-3Kb to -500b)	CRE TATA
<i>Arc</i>	activity regulated cytoskeletal-associated protein	NM_018790	chr15	-	74876181	ht_-1813	1	0	1	1
<i>c-Fos</i>	FBJ osteosarcoma oncogene	NM_010234	chr12	+	80146346	ht_-890 H_-349 HT_-301 ht_-138 HT_-75 H_175	6	5	1	3
<i>Cyr61</i>	cysteine rich protein 61	NM_010516	chr3	-	146317756	ht_-2405 ht_-2347 ht_-801 HT_-61 H_225	5	2	1	1
<i>Egr4</i>	early growth response 4	NM_020596	chr6	-	85985121	ht_-720 ht_-223 HT_-155	3	2	1	2
<i>Npas4</i>	bHLH-PAS type transcription factor NXF	NM_153553	chr19	+	5691820	HT_-282 HT_-272 h_258	3	3	0	2
<i>Zif268</i>	early growth response 1	NM_007913	chr18	+	35126324	ht_-309 h_-117	2	2	0	1

mGluR-LTD

Symbol	Gene Name	Accession	Chrom.	Strand	Position	CRE Sites (-3Kb to 300b)	Total CRE sites	Proximal CRE (-500b to 300b)	Distal CRE (-3Kb to -500b)	CRE TATA
<i>Arc</i>	activity regulated cytoskeletal-associated protein	NM_018790	chr15	-	74876181	ht_-1813	1	0	1	1
<i>Atf4</i>	activating transcription factor 4	NM_009716	chr15	+	80499495	h_-2125 ht_-327 ht_-275 ht_-228 HT_-120	5	4	1	4
<i>c-Fos</i>	FBJ osteosarcoma oncogene	NM_010234	chr12	+	80146346	ht_-890 H_-349 HT_-301 ht_-138 HT_-75 H_175	6	5	1	3
<i>Junb</i>	Jun-B oncogene	NM_008416	chr8	-	84265045	ht_-2877 h_-1335 h_-1089 HT_-105	4	1	3	1
<i>Nr4a1</i>	nuclear receptor subfamily 4, group A, member 1	NM_010444	chr15	+	101707146	h_-2529 ht_-1402 H_-247 H_-227 HT_-83 HT_-54	6	4	2	2
<i>Zif268</i>	early growth response 1	NM_007913	chr18	+	35126324	ht_-309 h_-117	2	2	0	1

Chloride channels

Symbol	Gene Name	Accession	Chrom.	Strand	Position	CRE Sites (-3Kb to 300b)	Total CRE sites	Proximal CRE (-500b to 300b)	Distal CRE (-3Kb to -500b)	CRE TATA
<i>Gabra1</i>	gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 1	NM_010250	chr11	-	42710268	ht_-2851 ht_-994	2	0	2	2
<i>Glr1</i>	glycine receptor, alpha 1 subunit	NM_020492	chr11	-	56204385	HT_-465 ht_-543	2	1	0	2
<i>Glr3</i>	glycine receptor, beta subunit	NM_010298	chr3	-	81854522	H_-1528 h_-1433	2	0	2	0

Table lists predicted functional CREs of mouse RefSeq genes. “H/h” refers to half CRE sites with an upper case representing conserved CRE whereas “T/t” at the end means the presence of TATA box less than 300 bp downstream of the CRE. The Chrom./Position are the chromosomal position of the transcription start site, and the strand of the transcript is based on the mm3 mouse genome assembly from UCSC Genome Bioinformatics (<http://genome.ucsc.edu/>). Adapted from CREB Target Gene Database (<http://natural.salk.edu/CREB/>).