Memory HQ: the possible central role of the epigenome in maintaining LTP.

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A thesis submitted for the
degree of Doctor of Philosophy
of the University of Otago,
Dunedin, New Zealand

November, 2016

// Abstract

Plasticity mechanisms such as long-term potentiation (LTP) are believed to underlie the formation and maintenance of memories. LTP induction stimulates downstream signalling pathways that lead to changes in gene expression which are critical to the maintenance of LTP. However, how these changes allow LTP to persist is not currently understood. The epigenetic mechanism, histone acetylation, has been shown to be regulated over the first few hours after LTP induction *in vitro*. Indeed, inhibition of enzymes that negatively regulate histone acetylation, histone deacetylase 1 and 2 (HDAC1 and HDAC2), enhances LTP induced *in vitro*, suggesting that HDAC inhibition supports LTP persistence. However, HDAC1 and HDAC2 have themselves been shown to be upregulated 5 – 24 h post-LTP induction *in vivo* and the effect of inhibiting HDACs over these later time-points has not been investigated. We aimed to identify if changes in HDAC activity played a role in LTP persistence over weeks, a timeframe which can not be studied when LTP is induced *in vitro*. We found that the activity of both HDAC1 and HDAC2 was upregulated 20 min post-LTP induction, returning to near baseline by 5 h and that HDAC1 activity was subsequently upregulated 12 h post-LTP induction. Interestingly, inhibition of the initial increase in HDAC activity, using the HDAC inhibitor Trichostatin A (TSA), had no effect on the induction of LTP, nor on the overall persistence of LTP. However, TSA did enhance the magnitude of LTP expressed between 12 h and 7 days post-induction. This time period has previously been associated with an intermediate form of LTP, LTP2. However, inhibition of the increased HDAC activity 12 h post-LTP by TSA had no effect on the persistence of LTP, nor did it

make the LTP more susceptible to disruption by LTP induction at a competing input onto the same set of cells.

An additional important finding from this work was that HDAC activity and protein expression was regulated in the contralateral non-tetanised hemisphere. This led to the hypothesis that increased HDAC activity may create an environment in which persistent LTP could not be induced. We found, however, that despite heightened HDAC activity, LTP was able to be induced and persisted as normal.

Our findings do not support the hypothesis that LTP persistence is supported by HDAC1 and HDAC2 activity. However, we have identified an intermediate enhancement of plasticity over the first week after induction. This leads to the suggestion that HDAC1 and HDAC2 may regulate genes involved in the early stages of learning and memory formation but not the very long-term consolidation process. Further, interhemispheric communication may occur after LTP induction, though the mechanisms of action remain unclear. We can conclude that temporally and spatially widespread mechanisms underlie the induction and maintenance of LTP and though we are yet to elucidate the maintenance mechanisms for LTP, we are beginning to tease apart the intricate mechanisms involved over 24 h post-LTP induction.

Acknowledgements

I could not be more grateful to my supervisors Dr. Joanna Williams and Prof. Cliff Abraham for your unwavering support of me throughout this process. I count myself incredibly lucky to have had an environment in which I could be so creative with my thoughts and ideas, and to be reined in when needed. Talking about LTP is one of my favourite things to do and I treasure all the conversations we have had. Thank you for the time you have given me, the patience you have had with me, the honest words, all the opportunities you have given me and the many laughs we have had.

Thank you to all the Williams and Abraham lab members. In particular thank you Barb for teaching me so much. Thank you for coming in at night to help me with my 12 h experiments, which was so much to ask of you and yet you did it with a smile on your face! I couldn't have done it all without your support.

Thank you to the paper round crew Owen, Thom, Blake, Jodi and Megab for making me yell, laugh and love science and beer every Thursday afternoon. I love our musings about how neuroscience will be when we grow up and I can't wait for it to happen. Thank you to my wonderful old friends for all your patience, I am sorry I was lost inside this book for so long!

Thank you to the most incredible family in the world. Mum, Dad, Ard, Chlo, Elle, Georgia and the little one coming. I couldn't do anything without you, your love and support picks me up every time I am struggling and you are the delights in my life. I am absolutely the luckiest person in the world to have you all.

Dave, thank you for supporting me in taking this plunge and for all the good times.

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List of abbreviations

acH3	Acetylated Histone 3
acH4	Acetylated Histone 4
arc	Activity-regulated cytoskeleton-associated protein
AFC	7-amino-4-trifluoromethyl coumarin
AKT	Protein kinase B
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
BDNF	Brain derived neurotropic factor
bp	Base pair
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
CaMKII	Calcium calmodulin dependent kinase II
CaMKIIβ	Calcium calmodulin dependent kinase II β
CaMKIV	Calcium calmodulin dependent kinase IV
CaMKK	Calcium calmodulin dependent kinase kinase
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CK2	Casein kinase 2
CoREST	Co-repressor for element-1-silencing transcription factor
CREB	cAMP response element binding protein
DAB	3,3'-diaminobenzidine
DG	Dentate Gyrus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	Entorhinal cortex
E-LTP	Early long-term potentiation
ERK	Extracellular signalling related kinase
FC	Fold change
fEPSP	Field excitatory post-synaptic potential
FGF2	Fibroblast growth factor 2
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDAC1	Histone Deacetylase 1
HDAC2	Histone Deacetylase 2
HDAC2OE	Histone Deacetylase 2 over-expression
HDACi	Histone Deacetylase inhibitor
HFS	High frequency stimulation
Homer1a	Homer scaffolding protein 1a
Homer1b	Homer scaffolding protein 1b
IEG	Immediate early gene
i.p.	Intraperitoneal (injection)
LTD	Long-term depression
LTP	Long-term potentiation
L-LTP	Late long-term potentiation

LTM	Long-Term Memory
L-type VGCC	L-type voltage-gated calcium channel
k	Lysine
MAPK	Ras-mitogen-activated protein kinase
Mg^{2+}	Magnesium
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NaBut	Sodium butyrate
NCoR	Nuclear receptor co-repressor
NE	Norepinephrine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDAR	*N*-methyl-D-aspartate receptors
NuRD	Nucleosome remodelling and deacetylation
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-bufffered saline
pCREB	Phosphorylated CREB
PEG	Polyethylene glycol
PI3K	Phosphophatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKMζ	Protein Kinase M ζ
PLCγ	Phospholipase C γ
PP	Perforant path
PP1	Protein phosphatase I
PRP	Plasticity related protein
PSD	Post-synaptic density
RFU	Relative fluorescent unit
RNA	Ribonucleic acid
SAHA	Suberoylanilide Hydroxamic Acid
SARE	Synaptic activity-response element
SDS	Sodium dodecyl sulfate
shRNA	Short-hairpin RNA
Sin3	Paired amphipathic helix protein
SMRT	Silencing mediator of retinoid and thyroid receptors
STC	Synaptic tag and capture
TF	Transcription factor
TSA	Trichostatin A
trkB	Tropomyosin receptor kinase B

1. Introduction

1.1. The maintenance of memories

The evolution of the brain has developed from merely controlling basic functions such as movement, breathing and eating, to being the seat of a rich tapestry of emotions, desires, critical thought, beliefs, language and choice. We as humans develop rules that govern our behaviour by integrating new information with our past experiences to come to conclusions about the world around us and who we are. The ability to store information by forming stable networks of neurons in the brain, or engrams, allows for the association of multiple sensory inputs, feelings, social cues and value assessment which culminate in specific outputs (D. J. Cai et al., 2016; Dunsmoor, Murty, Davachi, & Phelps, 2015; Ramirez et al., 2013; Ramirez et al., 2015). Indeed, the formation and maintenance of engrams is the basis of memory (Eichenbaum, 2016). Many psychological diseases such as schizophrenia (Uhlhaas, 2013), and neurodegenerative diseases such as Alzheimer's disease (Fornito & Bullmore, 2015) involve disruption to engrams. Understanding how engrams are formed and maintained is critical to understanding the healthy brain and thus having this knowledge will enable us to understand the diseased brain and the best ways to treat neurological conditions.

Altering the connectivity of an engram has now been conclusively shown to alter a memory. Artificially activating an established engram, at the same time as

learning something new, can associate the two engrams and thus create a 'false memory' without the two events ever being associated in 'real life' (Ramirez et al., 2013; Ramirez et al., 2015). Moreover, disrupting the connectivity of an established engram can erase a memory (Hayashi-Takagi et al., 2015; Roy et al., 2016) and re-connecting the neurons can restore it (Roy et al., 2016). The engram, therefore, is dependent upon the connectivity of specific neurons which are regulated by mechanisms that enhance or decrease synaptic transmission, referred to as *synaptic plasticity* (Citri & Malenka, 2008). However, cell to cell communication is not just dependent upon synaptic transmission; it also depends upon transmission of current through dendrites, action potential firing and the transmission of current down axons, leading to activation of the next set of synapses and the continued flow of information through the network. These latter processes reflect the *excitability* of a neuron. Engram formation and maintenance is also critically dependent on modifications to excitability, referred to as *intrinsic plasticity* (Mozzachiodi & Byrne, 2010; W. Zhang & Linden, 2003).

The study of memory, and in particular the underlying molecular mechanisms such as gene expression, have for a long time been almost exclusively attributed to the regulation of synaptic plasticity. Particular focus has been on the prominent mechanism of enhanced synaptic transmission, long-term potentiation (LTP). While gene expression has long been known to be *the* fundamental component of the maintenance of a long-term memory (LTM) the gene expression response is, in fact, downstream of a variety of signalling pathways and its output regulates a number of different synaptic and non-synaptic mechanisms (Benito & Barco, 2010; Gruart, Benito, Delgado-Garcia, &

Barco, 2012). Though gene expression has not yet been shown to be critical to the persistent intrinsic plasticity, protein synthesis has been shown to extend the persistence of intrinsic plasticity (Cohen-Matsliah, Motanis, Rosenblum, & Barkai, 2010) and transcription factors known to be regulated after LTP induction and learning, regulate genes involved in regulating excitability (Mucha et al., 2010; van Loo et al., 2012; J. Zhang et al., 2002).

The prevailing focus of the current memory and plasticity literature is on changes in gene expression over the first few hours after plasticity induction or learning, in an attempt to identify the specific gene or genes underlying maintenance. A particular focus is, therefore, on the production of proteins that maintain the enhanced transmission at potentiated synapses (Alberini & Kandel, 2015; Sweatt, 2016). However, we propose a different hypothesis: that engrams are not maintained at each individual synapse by the expression of specific genes. Instead, the gene expression programme observed following the induction of plasticity i.e. the 'plasticity' programme induces widespread restructuring of an engram by altering synaptic connectivity *and* intrinsic excitability. Inherently, such widespread modifications would be disruptive and detrimental to any previously established engrams involving those same neurons. Therefore, we hypothesise that to maintain an established engram, the 'plasticity' gene expression programme must be inhibited and instead a 'maintenance' programme of gene expression takes over which negatively regulates plasticity, raising the threshold for future changes. Therefore, the maintenance mechanism of an engram, and memory, is not at any one physical location, such as synapses,

but instead in the regulation of the plasticity threshold within neurons involved in an engram.

1.1.a. Classification of Memory

Memories have been classified into a number of categories. Non-declarative or implicit memories are, as their name suggests, automatic responses or outcomes that do not need to be consciously considered. These memories can be further classified into either habits which are a direct association between a sensory input and some kind of automatic response or emotional memories which add a layer of complexity to the input-output of a habit by associating it with a positive or negative consequence (Eichenbaum, 2016; Moscovitch et al., 2005). Alternatively, there are declarative, or explicit, memories. These are memories of events or experiences that can be recalled and consciously thought about, which often have a temporal organisation and join a number of associated experiences (Eichenbaum, 2016; Moscovitch et al., 2005). Implicit memories are associated with brain regions such as the motor cortex with motor programmes (Monfils, Plautz, & Kleim, 2005), the striatum and cerebellum with habits and the amygdala with emotional memories (Eichenbaum, 2016).

Explicit memories, while involving many cortical circuits, are dependent on the hippocampus (Eichenbaum, 2016). These regions do not act alone, but they are focal points where these memories can be studied. In reality, multiple regions are involved in each type of memory, contributing at different times and in different ways, all culminating in the engram (Eichenbaum, 2016). The hippocampi, however, have been a specific focus in the field of learning and

memory since it was discovered that long-term declarative memories cannot be formed in their absence (Penfield & Milner, 1958; Scoville & Milner, 1957). Further, the highly organised neural architecture of the hippocampus, in particular what is referred to as the 'tri-synaptic loop' (Andersen, Bliss, & Skrede, 1971), is conducive for studying the physiology and molecular mechanisms of synaptic plasticity, and thus engram formation.

1.2. The hippocampus

The bilateral removal of the anterior two thirds of Henry Molaison's medial temporal lobes, which included the hippocampi, established a critical role of this brain region in the formation of long-term declarative memory (Penfield & Milner, 1958; Scoville & Milner, 1957). The result holds true in animal studies where silencing of the hippocampi, soon after learning (within the first 2 days) prevents recall of that experience, either at that time (Varela et al., 2016) or at a later 'remote' time point (1-2 months), when the memory is purported to be hippocampus independent (Lesburguères et al., 2011; Varela et al., 2016). If the hippocampus is silenced at a later time point after learning (2 weeks to a month), however, the recall is unaffected (Lesburguères et al., 2011). Contrary to this hypothesis, recent studies have shown that the cornu ammonis 2 and 3 (CA2 and CA3) regions of the hippocampus are activated upon recall of a memory (Rajasethupathy et al., 2015) and cellular adaptations induced by learning persist in the hippocampus for at least a month (Pavlowsky, Wallace, Fenton, & Alarcón, 2016). Further, the dentate gyrus (DG) region of the hippocampus, though not involved typically in the recall process, has been shown to be critical

to the maintenance of memory (Madronal et al., 2016). Thus, though the hippocampus does seem to be heavily involved in the formation and early stages of LTM development, particular subregions may play specific roles in the maintenance and recall of memory.

1.2.a. Anatomy of the hippocampus

The classical description of the hippocampus circuitry is of an excitatory, unidirectional 'tri-synaptic loop' which encompasses the DG region and the CA1, CA2 and CA3 regions (Andersen et al., 1971). Axons from the layer II cells of the entorhinal cortex (EC) form the perforant path (PP) and the first major projection into the hippocampus. PP axons form the first of the 'tri-synaptic' connections with DG granule cells. Axons from DG granule cells, called mossy fibres, in turn project to, and form the second synaptic connection with, CA3 pyramidal cells. Axons from CA3 cells, called Schaffer collaterals, in turn project to, and form the third synaptic connection with, CA1 pyramidal cells (Fig. 1.1). The classical, though simplistic, view of the output from the hippocampus is of axons from CA1 cells projecting to the subiculum and on to the deep layers of the EC (Andersen et al., 1971).

As imaging and labelling techniques have advanced, the classical description of the tri-synaptic loop has been found to be incomplete. In addition to the layer II EC projections to the DG, the layer III EC, projects directly to CA3 and CA1 regions in rats (Steward & Scoville, 1976; Witter, 2007) and monkeys (Witter & Amaral, 1991). These direct projections are essential for appropriate

firing of cells representing spatial information (Brun, 2008) and temporal associations during learning (Suh, Rivest, Nakashiba, Tominaga, & Tonegawa, 2011). Further, the CA3 region, as well as having a considerable array of collateral projections within the CA3 region, also project back to the DG (Ishizuka, Weber, & Amaral, 1990; Scharfman, 2007). The recurrent collaterals within the CA3 are argued to underlie pattern completion and therefore the ability to construct associated memories (Nakashiba, Young, McHugh, Buhl, & Tonegawa, 2008; Renno-Costa, Lisman, & Verschure, 2014).

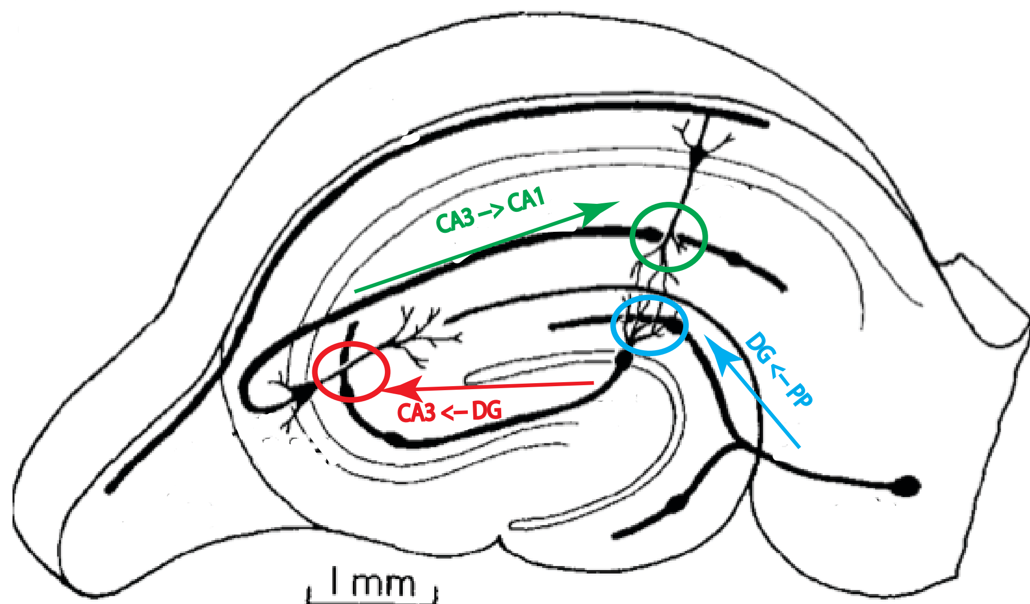

Fig 1.1. The ‘tri-synaptic loop’ of the rat hippocampus. The first set of synapses is formed between the perforant path and the dentate gyrus granule cells. The second set of synapses is formed between the mossy fibres of the dentate gyrus cells and the CA3 pyramidal cells. The third set of synapses is formed between the Schaffer collateral fibres of the CA3 pyramidal cells and the CA1 pyramidal cells. Image adapted from: Andersen et al, 1971.

1.2.b. Anatomy of the dentate gyrus

The DG receives input from the EC, and through its sparse connectivity, works as a pattern separator of incoming information, allowing for seemingly similar experiences to be stored as distinctly different memories (Leutgeb, Leutgeb, Moser, & Moser, 2007). Adult-born neurons, a unique characteristic of the DG though also found in other discrete regions of the brain, play an important role in this process (Clelland, 2009; McAvoy et al., 2016). The DG is often used to study synaptic plasticity *in vivo* in the hippocampus due to the ability for faithful recording from awake, freely moving animals over months (Abraham, Logan, Greenwood, & Dragunow, 2002) and for this reason will be studied in this thesis. Further, though *in vitro* LTP is studied mostly in the CA1 region, the molecular mechanisms of plasticity differ considerably between these regions (French et al., 2001) and thus warrant independent study.

A cross-section of the hippocampus shows the DG as a 'U' or 'V' shaped structure with two blades, the suprapyramidal and infrapyramidal blades (Fig 1.2) (Amaral, Scharfman, & Lavenex, 2007). There are three layers to the DG. The most superficial layer is the molecular layer, which comprises the dendrites of the principal cells, the granule cells. The second, middle layer is the granule cell layer which is comprised of the cell bodies of the granule cells and is about 4-8 cells thick. Finally, the polymorphic layer is the deepest layer and comprises the axons of the granule cells, a vast array of inhibitory interneurons and the excitatory mossy cells (Amaral et al., 2007).

Molecular layer

The molecular layer can be subdivided into three sections, the outer, middle and inner molecular layers. The lateral component of the PP fibre tract projects to the outer and the medial component of the PP fibre tract projects to the middle molecular layers forming distinct synaptic connections (Hjorth-Simonsen, 1972; Hjorth-Simonsen & Jeune, 1972). The inner molecular layer receives inhibitory input from a number of inhibitory interneurons such as basket cells as well as input from excitatory mossy cells (Amaral et al., 2007; Ribak, Seress, & Amaral, 1985). Interestingly, a projection via the ventral hippocampal commissure, from layer II of the EC crosses to the outer middle and outer layers of the contralateral DG molecular layer (Goldowitz, White, Steward, Lynch, & Cotman, 1975; Steward & Scoville, 1976; Witter, 2007). However, the density of this cross-projection differs between species, with very few projections in mice and monkeys but a more prominent projection in rats, rabbits and cats (Witter, 2007). Further, the excitatory mossy cells also cross-project to the contralateral DG granule cells (Amaral et al., 2007; Frotscher, Seress, Schwerdtfeger, & Buhl, 1991; Laurberg & Sørensen, 1981).

Granule cell layer

The granule cell layer contains not only the cell bodies of mature granule cells but also newly differentiated neurons which move into the granule cell layer from the polymorphic layer and project dendrites deep into the molecular layer which may then be integrated into networks (Altman & Das, 1965; Stanfield & Trice, 1988; Toni, 2007).

Polymorphic layer

The polymorphic layer can be divided into the subgranular zone and the hilus. The subgranular zone is where neuronal progenitor cells are found, which are proliferating and differentiating into granule cells (Okano, Pfaff, & Gibbs, 1993) before moving up to the granule cell layer (Kuhn, Dickinson-Anson, & Gage, 1996; Seki & Arai, 1993). One of the main types of interneuron in the DG, basket cells, are also found in this region. These cells form inhibitory synapses with the cell bodies, and most proximal shafts of apical dendrites on the granule cells (Amaral et al., 2007). The hilus is the deepest layer of the dentate gyrus and contains a myriad of interneurons which give the polymorphic layer its name (Amaral, 1978). This region is traversed by the axons of the granule cells, the mossy fibres, which project to the CA3 region. However, these fibres also extend collaterals which innervate the array of inhibitory interneurons which regulate granule cell activity. Mossy cells are the exception to the rule and are in fact excitatory neurons found deep in the hilus. The dendrites of mossy cells are mostly constrained to the polymorphic layer but can extend all the way up to the molecular layer and receive limited input from the perforant path (Amaral et al., 2007; Ribak et al., 1985).

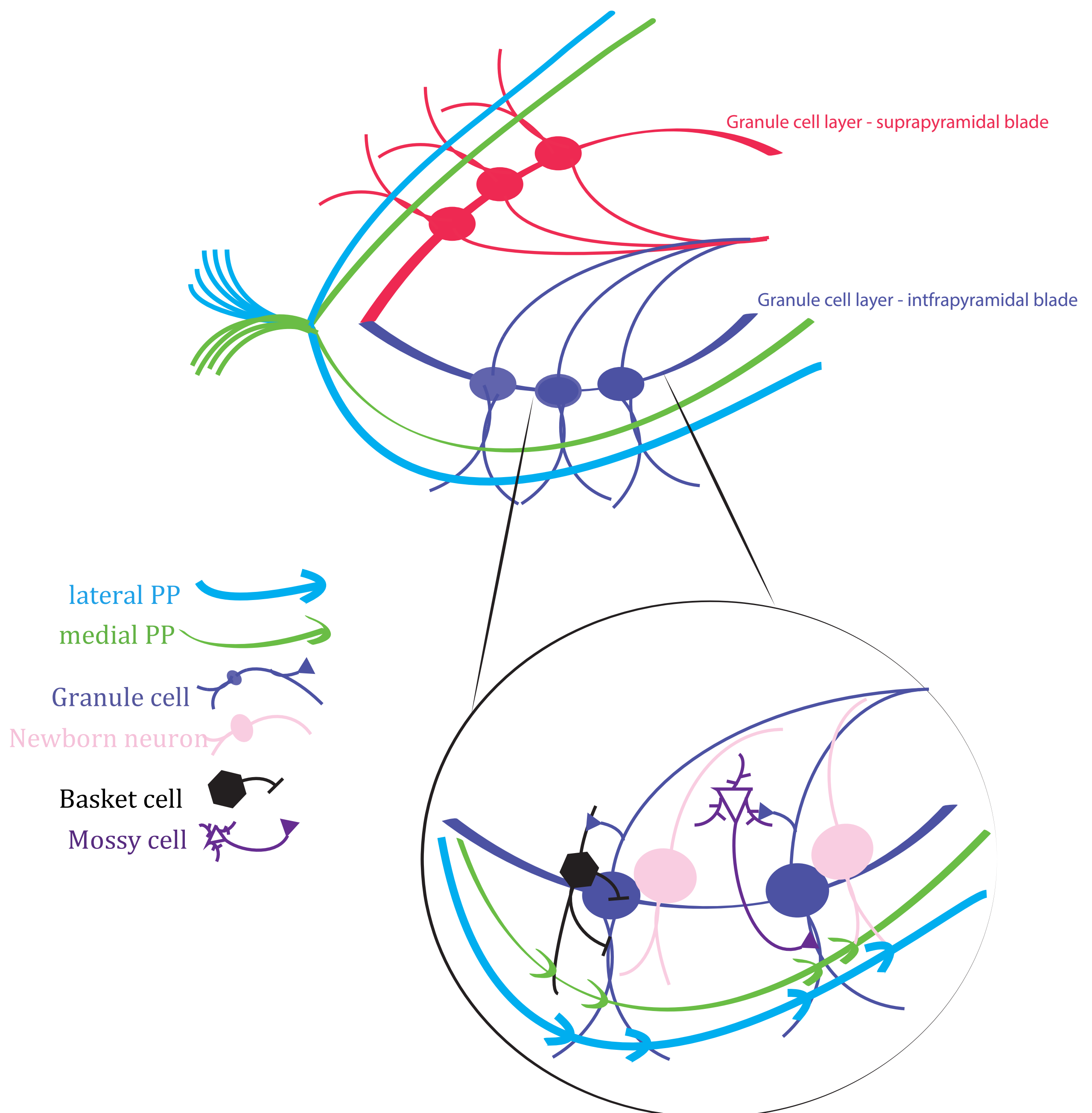

Fig. 1.2. Dentate gyrus anatomy. The lateral PP and medial PP are the major excitatory inputs to the DG. The LPP projects to the outer molecular layer and the MPP projects to the middle molecular layer. The inner molecular layer receives inhibitory input from interneurons such as basket cells and excitatory input from mossy cells. The granule cell layers form two blades, the suprapyramidal and intrapyramidal blades. These blades are comprised of the cell bodies of the granule cells. From the subgranular zone at the deepest edge of the granule cell layer, new neurons differentiate and begin to move into the granule cell layer, extending dendrites up to the molecular layer. The polymorphic layer includes the subgranular zone and the hilus, and contains a vast number of inhibitory interneurons including the basket cells and the excitatory mossy cells, all of which receive excitatory inputs from the mossy fibres.

1.3. Theories of long-term memory

Learning or encoding of a long-term declarative memory involves the formation of an engram, with enhanced connectivity between the hippocampal and cortical neurons involved. The engram, and therefore the memory, is initially labile and susceptible to disruption, but then is consolidated, i.e. made resistant to disruption over time, thus becoming a LTM (Alberini & Kandel, 2015; Dudai, 1996; Frankland & Bontempi, 2005; Medina, Bekinschtein, Cammarota, & Izquierdo, 2008). Consolidation of declarative, hippocampal dependent memories can be broken down into cellular and systems consolidation (Dudai, 2004). Cellular consolidation occurs very rapidly and involves the reinforcement of the structural adaptations that occurred to the cells involved immediately upon learning. Systems consolidation takes weeks to months and involves the restructuring of cortical regions to accommodate the memory, making the memory independent of the hippocampus (Dudai, 2004; Medina et al., 2008). The terminology and definitions of these types of consolidation are perhaps misleading because the distinction between the two is entirely based around the dependency on the hippocampus (Dudai, 2004). However, neurons involved in the engram that are based in the cortex also need to be involved in this initial learning process (Lesburguères et al., 2011; Tse et al., 2011) particularly when the experience is being incorporated into an existing engram (Tse et al., 2011). Further, the cellular mechanisms, such as a change in gene expression, underlying the adaptation of connectivity occur concurrently in both of these regions (Tse et al., 2011). The distinction could, therefore, be focused on understanding the mechanisms which make the connection of cortical neurons

involved in an engram more persistent and the connection of hippocampal neurons, involved in that same engram, possibly less so.

A major concern with the hypothesis of a hippocampal independent, consolidated memory is that updating of that engram is critically dependent on the hippocampus (Debiec, LeDoux, & Nader, 2002). Indeed, projections back from the cortex to the hippocampus, which activate neurons involved in the original engram, have been identified, albeit at a time point where the memory is not yet hippocampal independent (Rajasethupathy et al., 2015). This process, referred to as reconsolidation, initially makes the engram labile again, which allows it to be updated with new information but also puts it at risk of being aberrantly modified or lost (Nader, Schafe, & Le Doux, 2000). The previously described evidence which shows that LTMs can be hippocampus independent have shown that recall can occur in the absence of the hippocampus (Lesburguères et al., 2011; Penfield & Milner, 1958; Scoville & Milner, 1957; Varela et al., 2016). However, it has not been shown that in a healthy, intact brain, recall *does* occur without the hippocampus. Indeed, it has been shown that recall does activate processes that update and then reconsolidate the engram which is dependent on hippocampus (Debiec et al., 2002; Frankland & Bontempi, 2005; Medina et al., 2008). Therefore, it needs to be clarified as to whether cells in the hippocampus are always activated upon recall and if so, whether they are the same cells that were involved in the original trace. If it is indeed the case that the hippocampus is always activated upon recall of a declarative memory, it cannot be said that a memory ever becomes hippocampus-independent, and indeed, the

molecular mechanisms underlying the maintenance of an engram, encompassing all brain regions involved, need to be fully understood.

1.4. What makes neurons plastic and easy to adapt during learning?

1.4.a. Plastic versus stable spines

Dendritic spines are the sites at which classical excitatory synaptic transmission occurs. One side of a synapse is formed by a presynaptic axon varicosity which releases the excitatory neurotransmitter glutamate, into the space between the pre- and postsynaptic neurons, referred to as the synaptic cleft. The opposite side of a synapse, the postsynaptic density (PSD), is a region where the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and *N*-methyl-D-aspartate receptors (NMDAR) glutamate receptors are localised. Both AMPAR and NMDAR are ion channels which allow influx of cations into the cell upon glutamate activation. The ion pore of NMDARs, however, is blocked by magnesium (Mg^{2+}) ions unless the cell is sufficiently depolarised, such as by prior AMPAR activation, at the same time as glutamate is bound (Mayer, Westbrook, & Guthrie, 1984). Most dendritic spines in the DG and CA1 region of the hippocampus are classed as thin (Desmond & Levy, 1985; Harris, Jensen, & Tsao, 1992; Spacek & Harris, 1997) some of which have few if any AMPARs and are thus referred to as silent, though they do contain NMDAR which can be activated with sufficient depolarisation (Matsuzaki et al., 2001; Nusser et al., 1998; Sametsky, Disterhoft, Geinisman, & Nicholson, 2010). A considerably smaller proportion of spines are classed as mushroom shape, which

is where the vast majority of AMPARs are located (Harris et al., 1992; Matsuzaki et al., 2001). Thin spines also lack the machinery required to synthesis protein locally to support these structures, i.e. the spine apparatus (Harris et al., 1992; Spacek & Harris, 1997). Only 15% of spines do have spine apparatus, the majority of which are mushroom shaped spines (Harris et al., 1992).

Long-lasting changes are purported to be more commonly induced on thin spines (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004). Indeed, increasing the number of silent synapses has been shown to enhance plasticity (Arendt, Sarti, & Chen, 2013). By contrast large spines are seemingly more stable and do not undergo the same long-lasting potentiation (Matsuzaki et al., 2004). Further, if these large spines are shrunk or erased, memory is also erased (Hayashi-Takagi et al., 2015). New spines, or the physical growth of spines, take at least 20-40 min to appear after stimulation or learning and thus cannot explain immediate functional increases in synaptic drive, which perhaps occurs on large spines (Cichon & Gan, 2015; Engert & Bonhoeffer, 1999; Matsuzaki et al., 2004). Recently it was found that in response to LTP in the DG there was an increase in the number, or cluster, of mushroom spines per pre-synaptic bouton 24 h post-stimulation (Medvedev et al., 2014). Further, the coordination of depolarisation of discrete dendritic segments, causing dendritic calcium (Ca^{2+}) spikes, and NMDAR activation at synapses has been shown to be needed to make long-term enhancements of spines and to maintain learning (Cichon & Gan, 2015). Together, this evidence supports the notion that thin, silent spines need strong activation at nearby mushroom spines to depolarise dendritic segments causing dendritic Ca^{2+} spikes, at the same time as activation of silent synapses trigger the

voltage-dependent NMDARs, allowing these channels to become active and for potentiation of these synapses and spines (Liao, Hessler, & Malinow, 1995). This clustered synapse hypothesis is well supported as an integrative model for memory (Kastellakis, Cai, Mednick, Silva, & Poirazi, 2015).

LTP (Engert & Bonhoeffer, 1999) and learning induce at least a transient increase in spine density (O'Malley, O'Connell, Murphy, & Regan, 2000; O'Malley, O'Connell, & Regan, 1998). Learning increases the rate of new spine formation, and these new spines can be maintained for months (A. J. Holtmaat et al., 2005; T. Xu et al., 2009) and even when the memory is apparently forgotten spine stability can persist and the forgotten memories can be 're-learned' more readily on the same spines (Hofer, Mrsic-Flogel, Bonhoeffer, & Hübener, 2009). Interestingly, increases in spine density observed after contextual fear conditioning show a transient increase in the hippocampus but only appear much later (>30 days) in the anterior cingulate cortex (Restivo, Vetere, Bontempi, & Ammassari-Teule, 2009). If the hippocampus is inactivated after learning, this late increase in spine density in the anterior cingulate cortex is not observed, suggesting the hippocampus drives this process (Restivo et al., 2009). Additionally, subsequent learning does not destabilize the spines that were formed during the original learning experience (T. Xu et al., 2009). This new spine development only outweighs spine elimination over the first few days. Over this time the amount of spine elimination gradually increases so that the total number of spines is rebalanced (T. Xu et al., 2009). This elimination includes the vast majority of the growth after learning, with only a very small proportion being maintained (G. Yang, Pan, & Gan, 2009) and is critical to the

maintenance of LTM (T. Xu et al., 2009). Elimination is hypothesised to remove 'noise' in an engram so that only those very specific connections are maintained (G. Yang et al., 2009).

While evidence supports the notion that thin spines may be learning spines that are turned into mushroom spines during the consolidation process, thus forming LTMs (Bourne & Harris, 2007), there has been debate arising from *in vivo* imaging of spines. For the CA1 spines it has been predicted that there is an almost complete turnover of all spines in this region every 3-6 weeks (Attardo, Fitzgerald, & Schnitzer, 2015). However, in cortical regions identification of the rate of spine turnover has been hampered by the effects of preparation of the skull for imaging (H. T. Xu, Pan, Yang, & Gan, 2007). Thinning the skull, rather than implanting of a cranial window, ameliorates this problem and shows that the fast turnover associated with the cranial window preparation slows from 20% - ~ 2% spine turnover over 1 week and 50% - 6% turnover over a month (A. J. Holtmaat et al., 2005; A. Holtmaat, Wilbrecht, Knott, Welker, & Svoboda, 2006; Trachtenberg et al., 2002; H. T. Xu et al., 2007; Zuo, Lin, Chang, & Gan, 2005).

1.4.b. A plastic synapse

Donald Hebb's neurophysiological postulate (Hebb, 1949) inspired synaptic plasticity and engram research. He proposed that when a cell repeatedly and persistently causes another to fire, a growth at the connection between those two cells, which he referred to as a synaptic knob, would develop to allow for more efficient communication between those cells in the future (Hebb, 1949).

Thus, his cell assembly model further proposed that neurons which simultaneously converge to stimulate a common neuron, would both undergo development of the 'synaptic knobs' to allow for future coordination, and therefore the development of neuronal networks (Hebb, 1949). This concept has gained much support since and a number of spine (perhaps instead of knobs) and synaptic plasticity mechanisms, which modulate the efficiency of communication between two neurons, have been identified.

LTP and the opposing mechanism long-term depression (LTD), a persistent decrease in synaptic drive, are synaptic plasticity mechanisms widely regarded to underlie the formation and maintenance of memory. Though this statement has been debated over time, evidence such as the fact that LTP occurs at the same time as learning, that learning occludes further electrically induced LTP (Rioult-Pedotti, Donoghue, & Dunaevsky, 2007; Whitlock, Heynen, Shuler, & Bear, 2006) and most conclusively the fact that abolishing the potentiation of the synapses involved in a previously established engram, and then restoring the potentiation, will erase and then restore the memory respectively (Nabavi et al., 2014) strongly support this statement. Synaptic remodelling has been reported after LTP, with an increase in the number of segmented perforated synapses (i.e. the synapse has split and formed two PSD with a protrusion of the spine between the two contacts) and a decrease in the number of horseshoe shaped partitioned synapses (i.e. a protrusion of the spine but not separating the PSD so there is still only one contact) (Geinisman, 2000). However, this has been disputed and instead it has been suggested that it is not one synapse that has perforated but in fact multiple mushroom spines grouping around one presynaptic varicosity

(Medvedev et al., 2014). Indeed, these experiments have not shown that this kind of synapse is any stronger or more stable than any other and thus, synapse type still needs further investigation.

1.5. LTP induction

Depending on the cell type and brain region under investigation, there are a number of different pathways involved in the induction of LTP. However, central to most is a rise in intracellular free Ca^{2+}. Typically, this is achieved by activation of NMDARs where upon removal of the Mg^{2+} blockade the ion channel becomes Ca^{2+} permeable (Bliss & Collingridge, 1993; Collingridge, 1985). With D-serine also bound to the NMDAR, the current is significantly augmented, and LTP can be induced (Henneberger, Papouin, Oliet, & Rusakov, 2010).

NMDAR independent forms of LTP can also be induced such as at the DG mossy fibre- CA3 synapses of the hippocampus (Johnston, Williams, Jaffe, & Gray, 1992). Additionally, a slow developing form of LTP can be induced in the CA1 that is also independent of NMDARs (Grover & Teyler, 1990) but instead is dependent upon a rise in intracellular Ca^{2+} through voltage gated Ca^{2+} channels (Bayazitov, Richardson, Fricke, & Zakharenko, 2007). Finally, a form of LTP can also be induced by a release of Ca^{2+} from intracellular stores such as after metabotropic glutamate receptor activation (Bortolotto & Collingridge, 1993). How all of these mechanisms work together, or individually during learning is yet to be elucidated. Some or all of them may be activated in response to learning and may together contribute to the total LTP response.

The activation of specific downstream signalling pathways, can also induce a form of LTP referred to as chemical LTP (Aniksztejn & Ben-Ari, 1991; Lu et al., 2001; Reymann, Matthies, Frey, Vorobyev, & Matthies, 1986). Additionally, application of brain derived neurotropic factor (BDNF) can increase synaptic drive (H. J. Kang & E. M. Schuman, 1995; H. Kang & E. M. Schuman, 1995). These types of LTP induction protocols rely on bath application of drugs and thus have the potential to activate all synapses at the same time. This leads to a loss of input specificity. The specificity of connectivity is essential to this thesis and thus this type of LTP will not be discussed in detail.

LTP, when examined *in vitro* in the CA1 region, is separated into two major forms, referred to as early LTP (E-LTP) and late LTP (L-LTP). Alternatively, when describing *in vivo* LTP experiments (mostly in the DG), these types of LTP have been referred to as LTP1 (instead of E-LTP) and LTP2 and LTP3 (instead of L-LTP), where LTP3 can last months rather than hours *in vitro* (Abraham, 2003). E-LTP/LTP1 is independent of protein synthesis and gene expression (Nguyen, Abel, & Kandel, 1994), and relies on post-translational modifications to proteins already present at the synapses (Benke, Luthi, Isaac, & Collingridge, 1998; Shirke & Malinow, 1997) particularly by protein kinases such as protein kinase C (PKC) and calcium calmodulin dependent kinase II (CaMKII) (Malinow, Schulman, & Tsien, 1989). These kinases can increase the conductance of AMPAR and NMDAR currents (Soderling & Derkach, 2000). Both LTP2 and LTP3 depend on a number of the same mechanisms as E-LTP, such as kinase activity, with the addition of the stimulation of pathways leading to protein synthesis and gene expression (Nguyen et al., 1994). LTP2 and 3, which have been studied *in vivo* in the DG, are

time and mechanistically distinct where LTP2 lasts ~3.5 days and is dependent on protein synthesis and LTP3 lasts ~20.5 days which also is dependent on protein synthesis but additionally, dependent on gene expression (Abraham et al., 1993; Abraham & Williams, 2008).

Though E-LTP occurs immediately after LTP induction and decays rapidly, the molecular pathways leading to gene expression and protein synthesis needed for L-LTP are induced at the time as E-LTP is induced (Abraham, Dragunow, & Tate, 1991; Benito & Barco, 2015; Bito, Deisseroth, & Tsien, 1996; Cole, Saffen, Baraban, & Worley, 1989; Raymond & Redman, 2006). This suggests that L-LTP is not merely a continuation of E-LTP. Instead, as the stimulation intensity increases L-LTP related mechanisms are activated (Abraham et al., 1993; Raymond & Redman, 2006). Thus, L-LTP induction occurs at the same time as the induction as E-LTP, but they are distinct mechanisms which are not necessarily dependent upon one another (Raymond & Redman, 2006). The physiological separation of these processes is difficult when measuring synaptic drive, as the distinction between E-LTP and L-LTP is not to do with the physiology measured *per se* but instead the underlying molecular mechanisms which make or maintain these changes. The critical distinction between E-LTP and L-LTP, or more specifically LTP1 and LTP3, are the changes in gene expression. Therefore, for the purpose of trying to decipher the maintenance mechanisms of LTP, understanding LTP induced gene expression and its regulation, from the point at which L-LTP is induced is critical to understanding the maintenance of LTP and memory.

1.5.a. Managing LTP induction – neuromodulation and metaplasticity

The theoretical potential to enhance synaptic transmission at all synapses not only renders cells susceptible to excitotoxicity but, without a limit to the number of synapses that can potentiate, the specificity of inputs that underpins engrams or neuronal networks would be lost (Abraham, 2008). The ability to induce synaptic plasticity must be regulated to counteract these issues. This can be achieved by neuromodulators, whose activity at the time of induction can alter the extent or duration of the plasticity induced (Abraham, 2008). Alternatively, prior activity within a cell can cause a shift in the threshold for plasticity, and is termed *metaplasticity* (Abraham, 2008; Abraham & Bear, 1996). Critically, metaplasticity differs from neuromodulation because the event that caused the threshold shift is temporally distinct from the induction of plasticity. Further, metaplasticity mechanisms may be induced at the same time, or as a result of plasticity mechanisms (Abraham, 2008). Indeed LTP induction and learning can, for a time, block any further plasticity within the same cells (Nabavi et al., 2014; Whitlock et al., 2006) even if the plasticity is at a different set of synapses on the same cell (Abraham, Mason-Parker, Bear, Webb, & Tate, 2001). There is evidence from the motor cortex that this blockade is maintained for at least 23 days before being readjusted and the dynamic range of plasticity recovered (Rioult-Pedotti et al., 2007). This increase in the threshold for plasticity may not only protect the neurons from toxicity but maintain the engram by blocking other inputs from making competing alterations. Sufficiently strong competing inputs have been shown to detrimentally affect the persistence of LTP. Indeed, inducing LTP at one set of synapses on the DG granule cells can

cause a previously established LTP at a different set of synapses on the same cells to decay rapidly (Abraham, Mason-Parker, Irvine, Logan, & Gill, 2006). Further, established LTP in the DG can also be rapidly degraded by exposing animals to an enriched environment (Abraham et al., 2002; Irvine, Logan, Eckert, & Abraham, 2006). Together this evidence suggests that metaplastic mechanisms can inhibit the ability to induce subsequent LTP and this may protect the initial plasticity from decay, though what these mechanisms are is currently unknown.

1.5.b. Morphological changes upon LTP induction

An interpretation of how synaptic plasticity might fit into the structural modifications to spines and synapses discussed above is that LTP1, and perhaps LTP2, occur on mushroom spines and LTP3 develops later at thin spines. It has been reported that long term changes are induced at thin spines which, at the time of induction, lack spine apparatus (Matsuzaki et al., 2004) and the formation of new spines takes 20 – 40 min to develop after stimulation (Cichon & Gan, 2015; Engert & Bonhoeffer, 1999; Matsuzaki et al., 2004). After learning, newly synthesised GluA1 is found in mushroom spines but this change does not persist any longer than 72 h (N. Matsuo, Reijmers, & Mayford, 2008). After LTP *in vivo s*hort-term, immediate changes in AMAPR and NMDAR subunit protein expression on the cell surface has been reported (Williams et al., 2007). GluA1 and GluA3 increased immediately, and remained high over the first 20 min but were back to basal levels by 4 h whereas GluA2 did not increase until 20 min but remained elevated at 4 h (Williams et al., 2007). However, by 48 h post-induction there were no changes in surface expression of AMPAR subunits, instead only

increased expression in whole cell extracts (Kennard, Guevremont, Mason-Parker, Abraham, & Williams, 2009), in particular in regions near potentiated synapses (Kennard, Guevremont, Mason-Parker, Abraham, & Williams, 2014). These changes were back to baseline 2 weeks later (Kennard et al., 2009). Interestingly, the delivery of the AMPAR subunit GluA1 after LTP induction in hippocampal cell cultures has been shown to be directed to two locations, towards synapses and to cluster in dendritic shafts at the base of spines, which the authors suggest may be related to the location of spine apparatus (Shi et al., 1999). Together, these results suggest that though there is an immediate increase in AMPAR and NMDAR subunits at the cell surface and there is a protracted increase in the synthesis of AMPAR and NMDAR subunits near to potentiated synapses but not to the cell surface, none of these changes persist as long as the measured LTP. Together with the results of potentiation at specific spine types, this raises the possibility that LTP1 and LTP2 may be induced on mushroom spines, but the potentiation is not maintained there (Matsuzaki et al., 2004). Instead, long-term changes, such as LTP3 may be inducible on thin, plastic spines which are purported to turn into large, mushroom, memory spines with spine apparatus and become the physical trace of LTM. This does not exclude mechanisms related to LTP1, such as receptor trafficking, occurring on thin spines, or indeed LTP2 after protein synthesis at either more distant locations or after the morphological development of the thin spines to include protein synthesis machinery. Indeed, local protein synthesis, underlying the intermediate phase of LTP2, may be critical to the maintenance of pre-existing spines which only temporarily undergo potentiation while thin spines are

undergoing development of long-term adaptations which are maintained (Hill & Zito, 2013).

In hippocampal organotypic slice cultures, 'phase 1' of LTP, between 1 – 7 min, has been described as a reorganisation stage, where the amount of actin increases and polymerization begins, creating F-Actin, further there are decreases in proteins known to stabilise the structure such as CaMKII α and β isoforms (Bosch et al., 2014). A subsequent, re-stabilization step is found between 7 and 60 min where proteins such as profilin IIA as well as CaMKII α and β return to baseline levels to associate with F-actin to restructure the stable PSD, referred to as 'phase 2' (Bosch et al., 2014). Finally, 'phase 3' was identified, starting after 60 min where proteins such as Homer scaffolding protein 1b (Homer1b) and Shank1b assist in changing the spine volume to reflect the change in the PSD and these changes will persist over time (Bosch et al., 2014). A view over a longer time-frame *in vivo* supports these findings by suggesting that first synaptic growth occurs 1–2 h after learning, followed by strengthening of specific synapses over 12–18 h and the elimination of spines over the following 1–2 days (Caroni, Chowdhury, & Lahr, 2014). Together the evidence suggests that there is dynamic spine and synapse restructuring over time after LTP and learning, with both increases in growth and restructuring as well as pruning. However, the vast majority of *in vivo* electrophysiological experiments do not look at specific synapses which are undergoing potentiation, as they rely on the use of field potential recordings. Therefore, understanding whether the molecular mechanisms under investigation are attributable to specific spines or synapses with electrophysiological recordings done this way is unattainable.

Thus, a major question in the LTP field is whether the physiological changes in synaptic drive and molecular mechanisms that we measure as LTP occur at the same synapses and even on the same spines. Indeed, the synapses at which LTP is induced may not be the synapses at which LTP is maintained if new spine growth is essential and yet the induction occurred at pre-existing sites.

Despite the focus on LTP, there are other synaptic plasticity mechanisms induced by learning and in response to LTP-inducing stimulation, such as heterosynaptic depression and depotentiation (Caroni et al., 2014; Lynch, Dunwiddie, & Gribkoff, 1977; Nakayama et al., 2015). Further, a neuron's ability to fire an action potential in response to a given input, or its intrinsic excitability, is modified by changes to ion channel composition throughout the dendritic tree and at the soma. This affects the ability for a given synaptic input to induce action potential firing (Beck & Yaari, 2008; Hausser, Spruston, & Stuart, 2000; Kastellakis et al., 2015; Larkum & Nevian, 2008) and indeed adaptations to these intrinsic excitability mechanisms are also induced by learning, or LTP-inducing stimulation (Andersen, Sundberg, Sveen, Swann, & Wigstrom, 1980; D. J. Cai et al., 2016; McKay, Matthews, Oliveira, & Disterhoft, 2009; Moyer, Thompson, & Disterhoft, 1996).

1.6. Plasticity related gene expression

Arguably the most important discovery in understanding the molecular mechanisms of LTM was that, for a memory to persist, new protein synthesis and gene expression are essential (H. P. Davis & Squire, 1984; Goelet, Castellucci,

Schacher, & Kandel, 1986) though how changes in gene expression can maintain memory is not well understood. Experiments have shown LTM and L-LTP to be dependent upon a transcription- and translation-dependent stage beginning immediately after learning or induction (Goelet et al., 1986). The expression of a group of genes termed immediate early genes (IEGs) is upregulated immediately after the induction of L-LTP/LTP3. A number of these IEGs include inducible transcription factors (TFs) such as *c-fos* (Sheng, Thompson, & Greenberg, 1991), *zif/268 (egr1)* (Abraham et al., 1993; Cole et al., 1989) *c-jun* and *jun-B* (Abraham et al., 1993; Cole et al., 1989; Demmer et al., 1993) and *krox20 (egr2)* (Williams et al., 1995) (Fig. 1.3).

1.6.a. CREB

One of the most critical and well-studied regulators of gene expression in L-LTP and LTM is the constitutively expressed transcription factor cAMP response element binding protein (CREB) (Bourtchuladze et al., 1994; Guzowski & McGaugh, 1997). CREB is phosphorylated (pCREB) at serine 133 by protein kinase A (PKA) (Gonzalez & Montminy, 1989), calcium calmodulin dependent kinase IV (CaMKIV) and mitogen-activated protein kinase/extraceullular-regulated kinase (MAPK/ERK) (S. Davis, Vanhoutte, Pagès, Caboche, & Laroche, 2000; Wu, Deisseroth, & Tsien, 2001). Phosphorylation of CREB occurs immediately upon L-LTP induction (Bito et al., 1996; Deisseroth, Bito, & Tsien, 1996) and learning (Impey et al., 1998). pCREB leads to an increase in the expression of a number of the IEGs described above (Benito & Barco, 2010; Benito, Valor, Jimenez-Minchan, Huber, & Barco, 2011; S. Davis et al., 2000;

Sajikumar & Korte, 2011) as well as IEGs related to synaptic structure and function such as *activity-regulated cytoskeleton-associated protein* (*arc)* (Kawashima et al., 2009) after learning and LTP (Guzowski, McNaughton, Barnes, & Worley, 1999; Link et al., 1995; Lyford et al., 1995) and *bdnf* (Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998) which incidentally also plays a role in inducing further CREB-driven gene expression (Finkbeiner et al., 1997) (Fig. 1.3).

Biphasic changes in pCREB have been identified *in vivo*, first within 5 min of stimulation which lasted for 30 min but was gone by 1 h, followed by a second wave which appeared 2 h post-stimulation, and was sustained for 24 h (Schulz, Siemer, Krug, & Höllt, 1999). Somewhat surprisingly, in a different set of *in vivo* experiments, no change in pCREB was identified even though the normal increase in *zif/268* was observed (Walton et al., 1999). Further, despite increases in *bdnf* mRNA, the concomitant increases in BDNF protein was not observed (Walton et al., 1999). There could be a number of different explanations for these discrepancies such as differences in the stimulation protocol and the potential for seizure activity during LTP induction. However, a major difference between the two protocols was that Walton et al (1999) compared stimulated and non-stimulated hemispheres whereas Schulz et al (1999) compared stimulated animals and control animals. Indeed, Schulz et al (1999) observed enhanced levels of pCREB in both the stimulated hemisphere and non-stimulated hemisphere which would perhaps explain why no difference was seen when measuring between hemispheres (Walton et al., 1999). BDNF has been found to be upregulated after L-LTP induction in just the stimulated hemisphere within 30 min but levels returned to baseline by 4 h (Dragunow et al., 1993). BDNF has

also been shown to increase in both hemispheres between 6 and 24 h post-induction (Bramham, Southard, Sarvey, Herkenham, & Brady, 1996). Interestingly, it was again not identified in the recent gene expression profiles which measured changes between stimulated and unstimulated hemispheres, though this was examined 20 min post-induction and thus may have been prior to a significant increase (Ryan et al., 2012). Nevertheless, the discrepancy between results raises the possibility that unilateral induction of LTP may in fact cause bilateral changes in gene expression and thus, interpretation of results needs to take this into consideration.

pCREB-driven transcription can promote the induction of L-LTP (Barco, Alarcón, & Kandel, 2002) and LTM (Viosca, Lopez de Armentia, Jancic, & Barco, 2009). Conversely, inhibition of CREB inhibits the induction of L-LTP (Jancic, Lopez de Armentia, Valor, Olivares, & Barco, 2009). In correlation with the ability of CREB to increase L-LTP induction, CREB can also increase excitability (Lopez de Armentia et al., 2007; Viosca et al., 2009; Zhou et al., 2009) and increase dendritic spine density (Sargin et al., 2013). Learning alone has been shown to increase excitability (McKay et al., 2009; Moyer et al., 1996) and spine density (Restivo et al., 2009) which serves as a means of linking similar learning experiences, and their underlying engrams, occurring within close temporal proximity (<5 h) (D. J. Cai et al., 2016). Overexpressing CREB also increases the likelihood of affected neurons being incorporated into an engram (Han et al., 2007; Zhou et al., 2009) though it is still unclear how exactly CREB does this (Benito & Barco, 2010). However, these results suggest that CREB drives gene expression and increases excitability which renders the connectivity of neurons

readily adaptable, enhancing morphological restructuring (Gruart et al., 2012). Interestingly, the enriched environment experiment described above, which causes previously established LTP to decay, also increased the excitability of those cells (Irvine et al., 2006). Together, this data suggest that increased excitability, CREB activity, and increased spine density, lead to enhanced plasticity (Fig. 1.3). While these may be critical elements for establishing new synaptic plasticity, they potentially render already established LTP, and thus engrams, susceptible to disruption.

Though the intensity of the stimulus used to induce LTP correlates well with its persistence and with the expression of IEGs, the relationship between IEG expression and LTP persistence is not clear-cut (Abraham et al., 1993). IEG response, *in vivo*, generally increases with the number of high-frequency stimulus trains given. Little change is seen after 10 or 20 trains, but with 30 trains and above there is a significant increase in expression. This coincides with a switch from LTP1 and 2 to LTP3 induction, after 30 trains or more (Abraham et al., 1993). However although 10 trains cannot induce LTP3, 50% of the cells stimulated show some increase in z*if/268* expression (Abraham et al., 1993). After 50 trains, all dentate gyri showed increased expression *zif/268*, even though only 73% showed LTP3 (Abraham et al., 1993). Thus, although changes in IEG expression are indicative of persistent plasticity, they are not an exact correlate. Potentially, these IEGs may actually regulate activity driven increases in the expression of genes that enhance the ability to make long-term changes, such as by changing connectivity or excitability, leading to the potential for rewiring of an engram, but not the consolidation or maintenance of these

processes *per se.* Indeed, CREB is also phosphorylated in response to stimulation that induces LTD and therefore the expression of IEGs as a result may not be specific to potentiation (Deisseroth et al., 1996). This suggests that other, subsequent and as-yet unidentified, gene expression profiles, perhaps made given specificity by the combination of transcription factors activated, may be the critical maintenance programmes for LTM (Fig. 1.3).

1.6.b. How does altered gene expression regulate synaptic restructuring?

Gene expression enables the sustained potentiation of any synapse that is activated to a sufficient degree (U. Frey & Morris, 1997; Sajikumar, Li, Abraham, & Xiao, 2009). A proposed mechanism for this is referred to as synaptic tag and capture (STC) whereby a 'tag' is set at activated synapses which can then 'capture' newly synthesised plasticity related proteins (PRPs) (U. Frey & Morris, 1997). Interestingly, the tag does not have to be set by a stimulation protocol that would induce L-LTP (U. Frey & Morris, 1997). The STC concept has also been identified in memory studies where a short-lasting memory can be converted to a LTM as long as the synthesis of new proteins occurs around the time of learning (Ballarini, Moncada, Martinez, Alen, & Viola, 2009). PRPs can also be synthesised locally, at the stimulated dendrites, in response to electrical stimulation which induces L-LTP, a process which may be indicative of LTP2 (Alarcón, Barco, & Kandel, 2006; U. Frey & Morris, 1997; Sajikumar & Korte, 2011). Additionally, PRPs can be produced by gene expression in response to Ca^{2+} signalling pathways, such as Ca^{2+}/Calmodulin-dependent kinase kinase

(CaMKK) activation, leading to transcription by pCREB (Redondo et al., 2010), muscarinic or β-adrenergic receptor activation in the DG (S. Frey, Bergado-Rosado, Seidenbecher, Pape, & Frey, 2001), dopamine in the CA1 (Sajikumar & Frey, 2004) or simply by cell firing within CA1 (Dudek & Fields, 2002). Further, the 'tag' does not necessarily need to be set via E-LTP (Sajikumar et al., 2009), nor does it need to be there at the time of the production of PRPs (Redondo et al., 2010) (Fig. 1.3).

The ability for the central gene response to interact with synaptically located tags raises the possibility that gene expression may also interact with other kinds of tags, related to other forms of plasticity. In fact, an 'inverse tag' mechanism has already been identified which allows for the depotentiation or depression of other, non-potentiated synapses, after L-LTP induction perhaps to enhance the salience of the potentiated synapses (S. Frey & Frey, 2008; Okuno et al., 2012; Sajikumar & Frey, 2004). This suggests that rearrangement or restructuring of the connectivity of a given cell, or perhaps changes to intrinsic excitability (though no 'tag' mechanisms have been identified) is vulnerable to long-term alteration during periods of heightened gene transcription and protein synthesis. Further, it supports the notion that the IEGs and genes involved in widespread structural rearrangement upon LTP induction and learning could instead be referred to as a plasticity transcriptome, while a separate long-term maintenance transcriptome may be involved in stability.

Tags and plasticity related proteins

Several PRPs have been identified and shown to be essential for L-LTP. Protein kinase Mζ (PKMζ), a constitutively active isoform of PKC, is captured at active synapses after LTP induction (Sajikumar, Navakkode, Sacktor, & Frey, 2005) and incorporates active synapses into engrams during learning (Sajikumar & Korte, 2011; Tsokas et al., 2016). This has been challenged using PKMζ knock out mice where no effects of the knockout were identified (A. M. Lee et al., 2013; Volk, Bachman, Johnson, Yu, & Huganir, 2013). However, this has been explained by identification of a compensatory mechanism, where a different PKC isoform PKCι/λ can perform the same role as PKMζ as a PRP in knockout animals (Tsokas et al., 2016). PKMζ expression increases significantly after the induction of L-LTP (Osten, Valsamis, Harris, & Sacktor, 1996) and works to cluster a major scaffolding protein in the PSD, PSD-95 (Shao, Sondhi, van de Nes, & Sacktor, 2012). This clustering appears to aid in the increase in spine size and the movement of the GluA2 containing AMPAR into the potentiated synapse (Shao et al., 2012), indeed perfusion of PKMζ alone can enhance the synaptic AMPAR current (Ling, Benardo et al. 2002). CaMKII plays a critical role in the tag setting process, such that PRPs, perhaps PKMζ, cannot be captured when it is inhibited (Redondo, Okuno et al. 2010) (Fig. 1.3).

The expression of *bdnf* increases after LTP induction (Dragunow et al., 1993; Patterson, Grover, Schwartzkroin, & Bothwell, 1992), as does the receptor it acts on, *tropomyosin receptor kinase B* (*trkB*) (Dragunow, Hughes, Mason-Parker, Lawlor, & Abraham, 1997). BDNF activation of the trkB receptor is essential for L-LTP (Korte, Kang, Bonhoeffer, & Schuman, 1998), specifically at the synapse

which has been potentiated, a process dependent upon CaMKII (Harward et al., 2016). Somewhat inexplicably, BDNF expression at the time of learning is critical to the formation of LTM but inhibition of BDNF expression immediately after learning has no effect on the persistence of the memory (J. L. C. Lee, Everitt, & Thomas, 2004). BDNF alone can induce a persistent increase in synaptic efficacy, much like L-LTP induction, that is dependent on protein synthesis or gene expression (H. Kang & E. M. Schuman, 1995; Kang & Schuman, 1996; Messaoudi et al., 2007; Messaoudi, Ying, Kanhema, Croll, & Bramham, 2002), it can rescue L-LTP which had seemingly been lost after the application of a protein synthesis inhibitor and it can convert E-LTP to L-LTP (Pang, Teng et al. 2004). This rescue of L-LTP is dependent on PKMζ (Mei, Nagappan et al. 2011). BDNF activation of trkB receptors stimulates a number of downstream signalling cascades including the MAPK pathway, which leads to MAPK/ERK dependent transcription, as well as the phosphotidylinositol 3-kinase (PI3K)-protein kinase B (Akt) pathway and the phospholipase C γ (PLCγ)-Ca^{2+} pathway, which lead to CREB dependent transcription (Minichiello, 2009). BDNF has been found to be essential to that enhanced L-LTP observed in mice with a constitutively active form of CREB (Barco et al., 2005). Together, these results suggest that CREB driven BDNF and trkB expression leads to a subsequent BDNF/trkB driven wave of CREB driven gene expression. Interestingly though, the BDNF rescue of L-LTP can be achieved in the presence of a protein synthesis inhibitor (Mei, Nagappan, Ke, Sacktor, & Lu, 2011). Further, it seems to play a role in not only the regulation of STC (Barco et al., 2005; Sajikumar & Korte, 2011) but also cross-tagging, where the maintenance of LTD can be enhanced by previous production of BDNF (Sajikumar & Korte, 2011) (Fig. 1.3). The IEG *arc* appears similar in its ability to

regulate non-LTP related mechanisms after LTP induction. *Arc* has been shown to increase rapidly after LTP induction (Messaoudi et al., 2007; Ryan, Mason-Parker, Tate, Abraham, & Williams, 2011) and inhibition of *arc* expression, prior to or post-LTP induction, causes LTP to rapidly decay to baseline (Guzowski et al., 2000; Messaoudi et al., 2007). However, it would appear that it interacts with the β isoform of CaMKII (CaMKIIβ) which has been shown to set a tag at recently potentiated, but inactive synapses, termed an inverse tag (Okuno et al., 2012). After BDNF application and electrically induced LTP, CaMKIIβ and Arc have been shown to interact, allowing Arc to remove the AMPA receptor subunit GluA1, resulting in depotentiation of those synapses (Okuno et al., 2012) (Fig. 1.3). Thus, though *arc* and *BDNF* are IEGs which are critical to the maintenance of LTP, they may be doing so by either removing inactive synapses, or enhancing depressed synapses rather than being involved at the potentiated synapses, or be playing multiple roles working at both potentiated and non-potentiated synapses.

1.7. Subsequent waves of gene expression – a maintenance programme?

The ongoing process of gene expression after L-LTP induction is also evident in LTM. A second wave of enhanced translation (12-18 h after learning) and transcription (24 h after learning) (Bekinschtein et al., 2007; Bekinschtein et al., 2010; Katche et al., 2010) has been shown to be critical for consolidation of LTM. Indeed, LTM is often only assessed 24 h post-learning, a time point when the consolidation process is clearly ongoing. The few investigations into the mechanisms occurring at these later times do indeed find LTM deficits if

transcription and translation are inhibited 12 and 24 h post-learning respectively, when assessed 7 days after learning (Bekinschtein et al., 2007; Katche et al., 2010; Katche, Goldin, Gonzalez, Bekinschtein, & Medina, 2012). BDNF has also been identified as a regulator of mechanisms which occur 12 h post-learning (Bekinschtein et al., 2007). Again, the amnesic effects of a protein synthesis inhibitor, 12 h post-learning, can be mitigated by application of BDNF (Bekinschtein et al., 2008). In fact, learning which would not normally lead to the formation of a LTM, can be transformed to a LTM with the application of BDNF 12 h post-learning (Bekinschtein et al., 2008). Again, the ERK pathway is crucial for the formation of LTM, both at the time of learning and again at 12 hours after induction (Bekinschtein et al., 2008). Interestingly, however, inhibition of the other two pathways downstream of BDNF, PI3K and mammalian target of rapamycin (mTOR) only affects consolidation if delivered during training, not during the 12 h phase (Bekinschtein et al., 2008). Further, the transcription factors CREB and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) have both been shown to continue to regulate gene expression at 5 h and 24 h post-LTP induction respectively (Ryan et al., 2012). Thus, mechanisms regulating gene expression are ongoing, over at least 24 h post-learning and LTP.

The activity and expression of a number of proteins have been identified to occur in multiple waves after learning. The expression of the IEG *c-fos,* which has been shown to be critical to consolidation (Feldman, Shapiro, & Nalbantoglu, 2010), increases again 12 h post-learning which is critical for 7 day but not 2 day recall (Katche et al., 2010). *Zif/268* expression also increases again 12 h post-learning (Bekinschtein et al., 2007; Bekinschtein et al., 2010; Katche et al., 2010).

However inhibiting *zif/268* 12 hour post-learning did not appear to affect LTM at 7 days (Katche et al., 2010; Katche et al., 2012). A BDNF driven wave of a*rc* expression has also been identified 12 h (Nakayama et al., 2015). This second wave of *arc* expression after learning has been shown to drive the elimination of small mushroom spines, perhaps pruning unwanted connection and streamlining the engram (Nakayama et al., 2015) (Fig. 1.3). Interestingly, expression of *arc* can be driven by different response elements within the promoter region (Fukuchi et al., 2015). Specifically, the synaptic activity-response element (SARE), located -7 kbp upstream of the *arc* transcription start site, is responsive to NMDA, BDNF and fibroblast growth factor 2 (FGF2) but a proximal promoter region, -1679 from the transcription start site is only responsive to BDNF and FGF2 (Fukuchi et al., 2015). Together, this suggests that the 12 h wave of BDNF driven *arc* expression after learning (Nakayama et al., 2015) may be regulated by different mechanisms than the initial NMDAR driven *arc* expression. Interestingly, CaMKIIβ, the 'tag' with which arc interacts, has also been shown to be critical to long term recall (10 day) but not short term (1 day) (Cho, Cao, Wang, & Tsien, 2007). By 24 h post-learning Homer scaffolding protein 1a (Homer1a) protein expression levels peak (Bekinschtein et al., 2010); moreover, CaMKIIα, ERK-2 and Akt, three protein kinases known to be involved in memory and plasticity, are upregulated 24 hours after learning, with CaMKIIα also increasing at 18 h (Bekinschtein et al., 2010). Thus, the expression of a number of the genes involved in the initial changes after learning, involved in tag and capture mechanisms and inverse tagging, increase again between 12 and 24 h post-learning and many of these changes seem to be involved in a longer term mechanism of maintenance not evident at 24 h post-learning but apparent by 7 days post-learning.

The sequential activation of TFs after L-LTP induction (Williams et al., 2000) leads to the formation of a temporal profile of gene expression. Profiles of gene expression have been identified over 24 h following LTP induction (Ryan et al., 2012). Microarray analysis of gene expression following *in vivo* LTP, further analysed using Ingenuity Pathway Analysis software, has enabled an integrated approach to understanding how networks of genes work together over 24 h post-induction in the DG. Using this approach, central hub molecules of gene networks have been identified as potential controllers of key processes. Through analysis of the pathways regulated by the networks, gene expression itself was found to be a major function of the genes regulated 20 minutes post-LTP, as were cell growth and development, centrally controlled by genes such as *zif/268 and krox20*, which encode inducible transcription factors (Ryan et al., 2011; Ryan et al., 2012) (Fig. 1.3). Indeed, previous microarray analysis 40 min and 2 h post-LTP induction has also suggested widespread restructuring of neurons during these early time-points (Havik et al., 2007). Together, the microarray data support the spine and synaptic restructuring literature discussed above as well as triggering subsequent transcription, again as has previously been suggested by the expression of TFs. By 5 h post-LTP the networks differ markedly from those identified at 20 min, with the focus moving towards Ca^{2+} dynamics and G-protein signalling in addition to gene expression, which are processes essential to the induction of plasticity. Important networks at this time are proposed to be centrally controlled by genes such as *histone deacetylase 1* (*HDAC1*) which is a negative regulator of gene expression and a number of genes encoding CaMK's, which are involved in Ca^{2+} signalling pathways. Thus these delayed gene expression profiles may be indicative of metaplastic regulation of future

plasticity induction (Ryan et al., 2012). Finally, by 24 h the main roles identified were inhibition of protein synthesis and epigenetic negative regulation of gene expression, proposed to be centrally regulated by genes such as *histone deacetylase 2* (*HDAC2*) which, much like HDAC1, is an epigenetic negative regulator of gene expression. Indeed, of all genes regulated at 24 h, the vast majority were downregulated, whereas at 20 min and 5 h the vast majority were upregulated (Ryan et al., 2012). These gene profiles suggest very divergent, but specific roles played by waves of gene expression over time. Indeed, it would appear that there is a gradual increase in negative regulation of gene expression over time (Ryan et al., 2012). Further computational analysis of these profiles has shown that the strength, or stability of these networks also increases over time, where the 20 min networks are unstable, and easily disrupted but the 24 h networks are robust (Nido, Ryan, Benuskova, & Williams, 2015). These networks point towards the importance of high level regulators of gene expression and thus the identification of 'master regulators' would greatly assist in understanding the maintenance of LTP and memory.

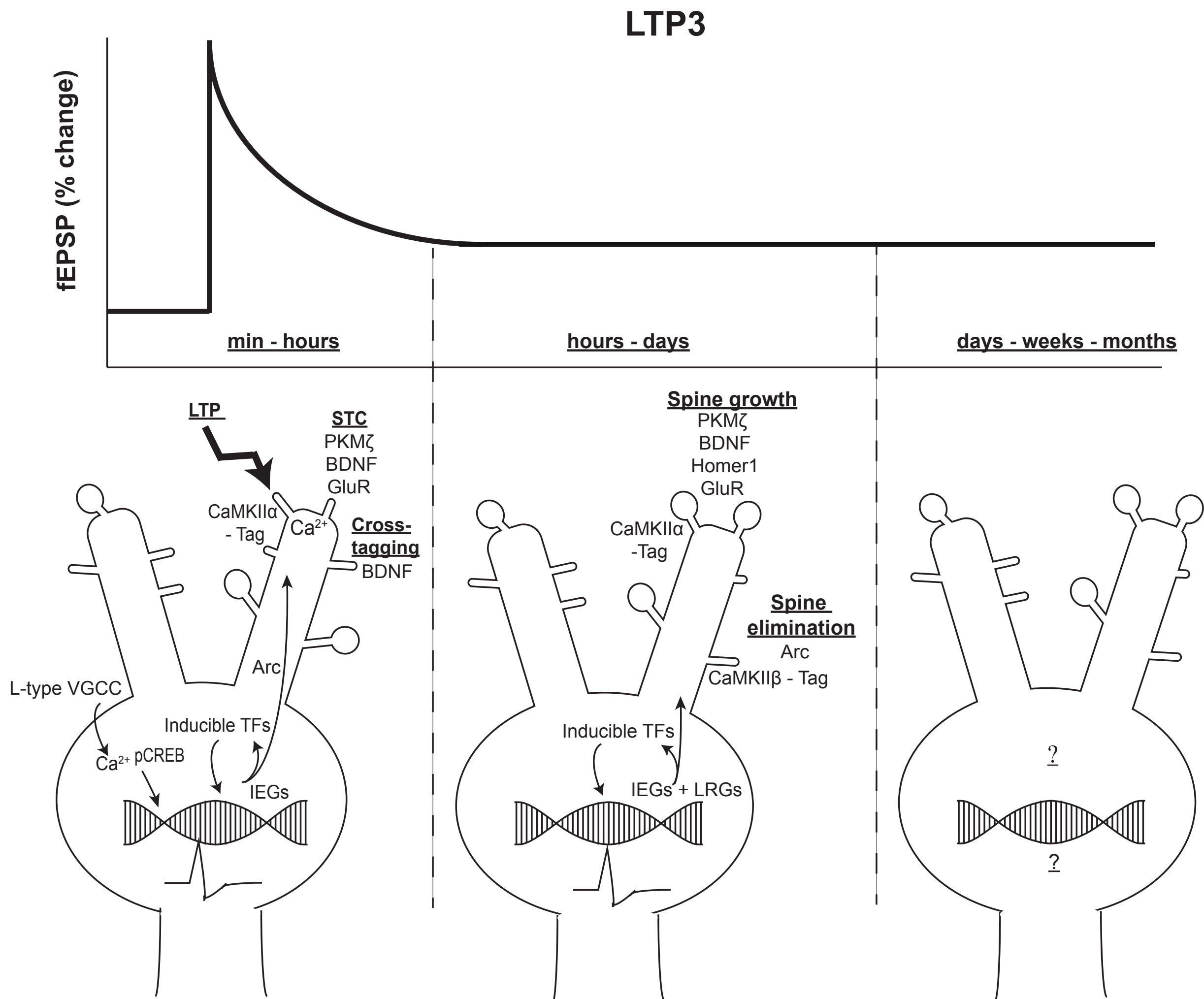

Fig. 1.3. Molecular event underling induction of LTP3. Left panel: Upon LTP induction, pCREB stimulates the expression of a number of IEG's some of which are involved in STC and cross-tagging while others are inducible TFs which stimulate subsequent waves of gene expression. Activation of CREB is also known to increase excitability (depicted as action potential waveform). Middle panel: Over hours to days, subsequent waves of gene expression are involved in spine growth as well as spine elimination. Excitability remains high for at least 5 h (depicted as action potential waveform). Right panel: LTP persists for months, yet how changes in gene expression are regulated and how this regulates functional processes of the neuron over the long term is currently unknown. GluR – subunits of NMDAR and AMPAR.

1.7.a. What drives subsequent gene expression?

A number of neuromodulators have been shown to be necessary and sufficient to drive the second wave of gene expression critical to LTM (Rossato, Bevilaqua, Izquierdo, Medina, & Cammarota, 2009). The second wave of *c-fos*

expression, 12 h post-learning, is critical to LTM but does not necessarily need to be induced by the initial learning experience (Katche et al., 2010). A learning experience which would not normally lead to a LTM, can be promoted to one with infusion of norepinephrine (NE) 12 h post-learning, which drives the critically important *c-fos* expression (Katche et al., 2010). Similarly, corticosterone, can promote a learning experience to a LTM if injected 12 h post-learning (C. Yang et al., 2013). Both NE and corticosterone are signalling molecules involved in the stress response and indeed, stress alone can also promote LTM (C. Yang et al., 2013). Dopaminergic input to the hippocampus from the ventral tegmental area is also critical, and sufficient, for the 12 h wave of transcription (Rossato et al., 2009). This dopamine input, via activation of the D1 dopamine receptor, leads to cyclic adenosine monophosphate (cAMP) activation of PKA, which drives the critical transcription of BDNF (Rossato et al., 2009). Thus, much like the regulation of expression at the time of learning or LTP induction, the second wave of transcription can be driven by a number of stimuli, not just by the induction of L-LTP.

1.7.b. Reconsolidation and gene expression

Reconsolidation is mechanistically very similar to consolidation. It depends on NMDAR activation, protein synthesis and gene expression which target synapses using the STC mechanisms (Alberini, 1999; Cassini et al., 2013; Nader & Hardt, 2009; Nader et al., 2000; Przybyslawski & Sara, 1997). However, it takes three times the dose of a protein synthesis inhibitor to block reconsolidation, immediately after reactivation, compared to that needed to block consolidation

immediately after learning (Debiec et al., 2002). Further, it takes only two days to become hippocampal independent during reconsolidation, rather than the number of weeks it takes during consolidation (Debiec et al., 2002) suggesting that only partial reactivation of process involved in consolidation are activated and thus disrupted, leading to a more efficient and quicker reconsolidation process. Indeed, the protein expression profiles are also somewhat different where consolidation is dependent upon BDNF, but not zif/268, (though see Jones et al., 2001), whereas the opposite is true for reconsolidation where zif/268 is critical but not bdnf (J. L. C. Lee et al., 2004). Further, corticosterone and stress, which drive BDNF expression and which enhance LTM when delivered 12 h post-learning, both instead eliminated LTM if delivered 12 h post-reactivation (i.e. during reconsolidation) (C. Yang et al., 2013). Indeed, the level of IEGs expression, which increases dramatically after LTP3 induction, is significantly less if the same stimulation is given again a day later (Abraham, Mason-Parker, Williams, & Dragunow, 1995). This suggests that IEG expression is in some way dampened by this prior activity. Together, these results suggest that updating and reconsolidating an engram have different gene expression profiles than learning and consolidation. Thus, the prior learning experience seems to have some metaplastic influence on the subsequent gene expression response.

1.8. Plasticity versus maintenance summary

There is strong evidence for independent 'plastic' and 'maintenance' mechanisms within a given cell. The structure of a spine plays a role, with thin spines being more plastic and mushroom spines more stable. Further, spine

density and excitability of a cell can seemingly determine the propensity of the cell to be incorporated into an engram to form a LTM. Modifications to these plasticity mechanisms can be achieved by a central gene expression response, with CREB seeming to play a significant role in this process, particularly in driving the expression of genes which make structural rearrangements, not only of the potentiated synapses but also other, inactive synapses. However, this plasticity related gene expression response is critical but insufficient to maintain LTP. Subsequent waves of gene expression have been shown to occur in the hippocampus after learning and LTP induction and these waves are just as important to the persistence of the memory, but as yet have not been critically linked to LTP. The nature of these gene programmes and how they are regulated are still being discovered. However, I argue that the plasticity related, perhaps pCREB driven, gene expression programme needs to be metaplastically inhibited, potentially by genes involved in the maintenance programme, to enable for the structural integrity of an engram to be maintained and thus a memory to be maintained. This may be for the lifetime of the memory, or alternatively for a specific period of time while the memory is being consolidated and moving out of the hippocampus.

1.9. Epigenetics

The human genome contains over 20,000 protein-coding genes that need to be tightly regulated in every cell throughout the body (Harrow et al., 2012). Constitutively expressed TFs, such as CREB and NF-κB, each have the potential to bind to 19,000 loci in the human genome (Van Steensel, 2005). However, less

than half of the genes that could be transcribed are expressed when these TFs are activated (Van Steensel, 2005). Thus, additional regulatory mechanisms are needed to ensure that there is appropriate basal gene expression within each cell and appropriate changes in response to specific stimuli (Riccio, 2010; Tammen, Friso, & Choi, 2013; Van Bortle & Corces, 2012). These are termed epigenetic mechanisms, the precise definition of which is debated continuously over many disciplines (Deans & Maggert, 2015). The general consensus in neuroscience is that epigenetic mechanisms control the way genes are expressed, without making changes to the DNA sequence itself (Borrelli, Nestler, Allis, & Sassone-Corsi, 2008). In the brain, learning experiences can cause a number of epigenetic changes to occur rapidly or slowly and can be short- or long-lasting and thus do not fit some of the more restrictive definitions of epigenetics (Borrelli et al., 2008; Liu, van Groen, Kadish, & Tollefsbol, 2009; Riccio, 2010; Tammen et al., 2013). Traditional epigenetic mechanisms include histone turnover (Maze et al., 2015), DNA methylation and a number of histone modifications including phosphorylation, methylation, ubiquitination, SUMOylation and acetylation (Gräff, Kim, Dobbin, & Li-Huei, 2011). Histone acetylation is one mechanism that has been a major focus of epigenetic research in learning and memory.

Histone proteins are the core component of chromatin, the structure which folds and packages DNA to confine it to the nucleus. A pair of each of the core histone molecules, H2A, H2B, H3 and H4, make up a nucleosome, around which wraps 147 base pairs (bp) of DNA (Borrelli et al., 2008; Gräff et al., 2011; Kouzarides, 2007). In addition, H1 is a linker histone involved in packaging of nucleosomes (Fischer, Sananbenesi, Mungenast, & Tsai, 2010; Gräff et al., 2011).

Each core histone has an NH_2-terminal tail upon which modifications including methylation, acetylation, phosphorylation, ubiquitination and SUMOylation can occur (Gräff et al., 2011). When an acetyl-group added to a lysine (K) residue on a histone tail, the positively charged ε-amino group of the lysine is neutralised (Gräff et al., 2011). This decreases the electrostatic affinity between the lysine and negatively charged DNA, opening the chromatin and positively regulating gene transcription (Gräff et al., 2011).

Histone acetyl transferases (HATs) acetylate histone tails, opening the chromatin structure and allowing TFs more access to their target genes (Gräff et al., 2011; Stilling & Fischer, 2011). The acetylated tail then provides a binding site for proteins that contain bromodomains which assist in the grouping of the transcription machinery (Hargreaves, Horng, & Medzhitov, 2009). There are 18 HATs, a number of which have additional functions. CREB binding protein (CBP), for example, has HAT activity but can also act as a transcriptional co-activator by binding with other proteins of the transcriptional machinery (Kalkhoven, 2004; Kelly & Cowley, 2013).

In contrast to HATs, histone deacetylases (HDACs) remove acetyl groups from histone tails resulting in the closure of the chromatin structure decreased gene transcription (Gräff et al., 2011). There are 11 HDAC proteins in a zinc-dependent superfamily of HDAC enzymes, classified into three classes (I, II and IV). A fourth zinc-indecent, nicotinamide adenine dinucleotide$^+$ dependent class (III), called sirtuins, comprises of 7 proteins (de Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003; Seto & Yoshida, 2014). Class I HDACs, which are

found in the nucleus, consist of HDAC1, 2, 3 and 8 (de Ruijter et al., 2003; Seto & Yoshida, 2014). Class II HDACs, which shuttle between the cytoplasm and nucleus in response to signals such as those from a number of CaMKs, consist of HDAC4 to HDAC7 and also HDAC9 and 10 (de Ruijter et al., 2003; Seto & Yoshida, 2014). Finally, Class IV consists of HDAC11 alone, which is found in the nucleus but relatively less is known about its function (de Ruijter et al., 2003; Seto & Yoshida, 2014). HDAC1 and HDAC2 have been strongly linked to learning and memory. Both are expressed extensively in the rat hippocampus (Broide et al., 2007) and form homo- or heterodimer cores of the co-repressor complexes paired amphipathic helix protein (Sin3), nucleosome remodelling and deacetylation (NuRD), co-repressor for element-1-silencing transcription factor (CoREST), and silencing mediator of retinoid and thyroid receptors (SMRT)/ nuclear receptor co-repressor (NCoR) which work together to inhibit gene transcription (Kelly & Cowley, 2013).

HDAC1 and 2 are of particular importance to this thesis due to the finding that mRNA expression of both was dynamically regulated following LTP induction in the DG (Ryan et al., 2012). Further, expression of HDAC2 has been found to be increased in post-mortem human brain of patients with Alzheimer's disease and in rat models of this disease (Gräff et al., 2012). Thus, decreasing HDAC2 over-expression has been proposed as a potential target for treatment of the condition. Increasingly, the idea of using HDAC inhibitors (HDACis) to treat dementia as well as numerous other neurological conditions (Gray, 2011) is taking hold; however, negative results have been obtained from phase 3 clinical trials so far (Fleisher et al., 2011; Tariot et al., 2011)

1.9.a. An epigenetic environment for learning and plasticity

Modifications to acetylation prior to LTP induction.

The necessity of gene transcription for the persistence of LTP leads to the hypothesis that creating an epigenetic environment that promotes gene expression will also promote the persistence of LTP. With regards to acetylation, this would be an environment of increased acetylation, and therefore increased DNA accessibility for transcription factors. Indeed, pre-treatment with the HDACis trichostatin A (TSA) or sodium butyrate (NaBut) (Levenson et al., 2004) or by knocking out HDAC2 in mice (HDAC2KO) increases the magnitude of the LTP induced, and the level at which it persists (Levenson et al., 2004; Morris, Mahgoub, Na, Pranav, & Monteggia, 2013). The enhancement of both the magnitude of LTP induced and its persistence is dependent upon gene transcription, suggesting that the HDACis may cause some increase in basal transcription of genes related to synaptic structure, possibly in addition to enhancing the transcription in response to stimulation. Stimulation that would normally induce E-LTP alone induces L-LTP, if the stimulation is given in the presence of TSA (Vecsey et al., 2007) or in slices taken from HDAC2KO animals (Guan et al., 2009). Again, gene transcription, particularly via activation of CREB, is essential for this enhancement, as TSA is unable to reproduce this phenomenon in CREB mutant mice in which the α and Δ isoforms are deleted (CREBαΔ), which inhibits the ability of CREB to bind with CBP (Vecsey et al.,

2007). The opposite also holds true, where decreasing acetylation, presumably leading to decreased transcription, inhibits L-LTP. As stated above CBP, as well as grouping transcriptional machinery, has HAT capabilities and can therefore manage levels of acetylation (Kalkhoven, 2004). Accordingly, L-LTP cannot be induced in mice lacking CBP ($cbp^{+/-}$) (Alarcón et al., 2004) nor in mice which lack the machinery which groups CBP and CREB (Vecsey et al., 2007). The $cbp^{+/-}$ mice do indeed show reduced levels of H2B acetylation which can be restored to normal levels using the HDACi Suberoylanilide Hydroxamic Acid (SAHA), which additionally restores L-LTP (Alarcón et al., 2004). Further, by modifying mice to overexpress HDAC2 (HDAC2OE) or in a mouse model of Alzheimer's disease which leads to increased HDAC2 expression (overexpression of cyclin-dependent kinase 5 regulatory subunit 1 which, when cleaved, leads to overexpression of the neurotoxic p25 isoform; CK-p25), L-LTP cannot be induced (Gräff et al., 2012; Guan et al., 2009) but this can be rescued by inhibiting HDAC activity (Gräff et al., 2012). Thus, modifications to acetylation, either by enhancing or diminishing the impact of HATs and HDACs, show that an open state of enhanced acetylation leads to CREB-driven gene expression, perhaps expression of the plasticity related gene programme and the induction of L-LTP.

What does the modification of acetylation do to neurons prior to LTP induction?

Chronic HDAC regulation results in structural modifications to neurons which promote plasticity. Both HDAC2OE animals and CK-p25 mice have decreased spine density (Gräff et al., 2012; Guan et al., 2009) and decreased number of presynaptic terminals (Fischer, Sananbenesi, Wang, Dobbin, & Tsai,

2007; Guan et al., 2009). The opposite holds true for HDAC2KO animals where spine density and presynaptic terminals are increased. Interestingly though, the total number classified as mushroom spines is decreased, suggesting a greater than normal proportion of thin spines (Guan et al., 2009), though this is not always found (Morris et al., 2013). Interestingly, HDAC2KO animals also display decreased synaptic transmission (Morris et al., 2013). The decreased synaptic transmission, despite greater overall spine density, suggests that the spines contain silent synapses or ones with fewer AMPARs (Matsuzaki et al., 2004). This would account for decreased basal transmission while allowing for enhanced LTP through the rapid insertion of AMPARs upon stimulation, at thin spines (Matsuzaki et al., 2004). Further, increasing the number of silent synapses can enhance LTP induction (Arendt et al., 2013). Furthermore, as there are fewer mushroom spines in HDAC2KO animals (Guan et al., 2009), it would suggest that the knockout of HDAC2 enhances plasticity, perhaps at the expense of maintaining stable mushroom spines. This idea is supported by the fact that HDAC2 negatively regulates the expression of genes associated with spine growth and synapse formation, leading to enhanced learning perhaps at the expense of maintaining stable mushroom spines.

1.9.b. HDAC1 and 2 regulate distinct sets of genes

While HDAC1 and HDAC2 are both class I HDACs, they can regulate the expression of distinct regions of the DNA and distinct genes (Wang et al., 2009).

HDAC1

In relation to learning, memory and plasticity, little is known about the genes regulated by HDAC1. An investigation using CK-p25 mice, independent of the investigation into HDAC2 (Gräff et al., 2012), has shown that HDAC1 regulates the promoter region of genes involved in cell cycle such as *p21/WAF1* and *E2F1* which play critical roles in cell survival (Kim et al., 2008). However, HDAC1OE can also lead to increases in acetylation at genes such as *c-fos* and *creb* (Guan et al., 2009) and has recently been shown to negatively regulate expression of *arc* via a specific region of the promoter (Fukuchi et al., 2015).

HDAC2

HDAC2 can be found at the promoter region of genes that are involved in synaptic structure, such as *nrxn1, nrxn3, SVP, shank3, synapsin2, psd-95*, and *agrin* and genes which play a role in synaptic plasticity such as, *GluA1, GluN2B, GluN2A, CaMKIIa, PKMζ, CREB1, crebbp, cbp, cdk5r1, cdk5, homer1* (Guan et al., 2009). Similarly, in CK-p25 mice the expression of a number of genes are downregulated by increased HDAC2 at the promoter region of these genes, deacetylating lysine residues and limiting access for transcriptional machinery (Gräff et al., 2012). These genes include IEGs which encode transcription factors such as *zif/268,* genes encoding proteins involved in synapse structure such as *arc* and *homer1* and genes which encode glutamate receptor subunits *GluA1, GluA2, GluN2A and GluN2B* (Gräff et al., 2012) and indeed a general decrease in H3 and H4 acetylation (Fischer et al., 2007). The concomitant regulation of acetylation is generally found at the promoter regions of these genes, where acetylation is increased in HDAC2KO animals (Guan et al., 2009) and decreased in HDAC2OE (Guan et al., 2009) and CK-p25 mice, which could be recovered to

normal by inhibiting HDAC2 (Gräff et al., 2012). However, acetylation is actually increased at the promoter region of *creb, c-fos, GluA1* and *actb* in HDAC2OE animals (Guan et al., 2009) which suggests that dysregulation of HDAC2 may engage the activity of other regulators of acetylation such as HATs or other HDACs.

1.9.c. Acetylation modifications prior to learning

HDAC2KO animals display enhanced fear conditioning and spatial learning measured 24 h after learning, whereas HDAC2OE and the CK-p25 mice display the opposite effect (Gräff et al., 2012; Guan et al., 2009), though this can be recovered by inhibiting HDAC activity, much like the LTP results discussed above (Gräff et al., 2012). Interestingly, although HDAC2KO animals have enhanced contextual conditioning at 24 h post-learning, this enhancement is not maintained at 48 and 72 h post-learning (Morris & Monteggia, 2013). Additionally, *cued* fear conditioning was not enhanced at 24 h and was actually decreased 48 and 72 h post-learning, an effect which the authors argued to be due to enhanced fear extinction (Morris et al., 2013) though it could also be that LTM was poorly encoded. Again, the opposite holds true with decreased acetylation. The $cbp^{+/-}$ animals (Alarcón et al., 2004) as well as mutant mice which lack the HAT ability of CBP (CPB HAT^{-}) (Korzus, Rosenfeld, & Mayford, 2004), and the CREBαΔ mutant animals (Vecsey et al., 2007), all show impaired LTM formation. The HDACi SAHA can increase acetylation and recover memory to control levels in the $cbp^{+/-}$ mice (Alarcón et al., 2004). Similarly, the memory impairment in the CPB HAT^{-} animals could be rectified by recovery of HAT activity (Korzus et al., 2004). Perhaps somewhat surprisingly, more intensive

training in the tasks could also restore memory deficits in the $cbp^{+/-}$ (Alarcón et al., 2004) and CPB HAT^{-} animals (Korzus et al., 2004). However, none of these treatments were able to restore the LTM capacity of the CREBαΔ animals (Vecsey et al., 2007). Further, glucocorticoids enhance memory consolidation object recognition and object location memory, at the same time as increasing H3K14 acetylation, by driving the interaction of pCREB and CBP, thereby causing the HAT activity of CBP to increase acetylation (Roozendaal et al., 2010). Indeed, the CPB HAT^{-} animals were not impaired in contextual fear conditioning at all which is another hippocampus dependent learning task involving a stressor (Korzus et al., 2004). Thus the permissive, open state of the chromatin can, seemingly, be brought about via a number of mechanisms that regulate the balance of HAT and HDAC activity. However, it would seem that increased acetylation is needed to drive the CREB dependent transcription that is critical to LTM. Intriguingly, however, inhibiting the HAT activity of CBP has suggested that in fact HAT activity, rather than HDAC, negatively regulates fear extinction memory consolidation in the prefrontal cortex (Marek et al., 2011). This needs considerably more investigation but perhaps suggests regional differences in epigenetic programmes throughout the brain.

HDAC1 seems to play a considerably different role than HDAC2 in learning and memory processes (Bahari-Javan et al., 2012). Overexpression of HDAC1 enhances fear extinction, but has no effect on behavioural tests of depression and anxiety, as well as working memory, novel object recognition and contextual fear conditioning (Bahari-Javan et al., 2012). A gradual decrease in *c-fos* expression after each fear extinction trial corresponds to an increase in

HDAC1 expression and a decrease in acH3K9 at the promoter region of the *c-fos* gene (Bahari-Javan et al., 2012). Thus, the lack of reinforcement of the shock somehow leads to an increase in HDAC1 activity, negatively regulating the expression of *c-fos* and altering the reconsolidation. Alternatively, HDAC1 activity may be critical for a gradual decrease in *c-fos* expression which is critical to the consolidation of extinction and the decrease in fear response. This reinforces the differences identified between HDAC1OE and HDAC2OE, where HDAC1OE had no effect on fear conditioning, whereas HDAC2OE does (Guan et al., 2009).

1.9.d. Acetylation after learning or LTP

An early investigation into epigenetic regulation of gene transcription highlighted dynamic changes in histone acetylation after learning (Levenson et al., 2004). A transient NMDAR- and ERK- dependent increase in acetylation of H3K14 was found 1 h after fear conditioning in the CA1 but it returned to baseline by 24 hours (Levenson et al., 2004). This transient change in acetylation appears to be critical as aged animals have apparently normal basal acetylation levels, but the increase in H4K12 acetylation after learning did not occur in these animals, which correlated with their inability to form LTMs (Peleg et al., 2010). The epigenetic regulation of specific plasticity related genes has been investigated in detail. *Homer1* is critical to LTM (Feldman et al., 2010) and L-LTP (R. Matsuo, Murayama, Saitoh, Sakaki, & Inokuchi, 2000) and a transient increase in expression occurs 2 h after fear conditioning in the hippocampus and amygdala (Mahan et al., 2012). BDNF signalling, via the MEK - ERK pathway, has

been found to be responsible for this increase in expression and to a concomitant increase in H3 acetylation at *homer1* promoter region (Mahan et al., 2012). This suggests that BDNF, implicated by Vecsey et al 2007 as being affected by HDAC2, may in turn affect the epigenetic environment itself.

HDACis delivered immediately post-learning can also enhance memory. The HDACis vorinostat, NaBut or TSA can enhance fear conditioning and object recognition when tested 24 h post-learning (Fujita et al., 2012; Stefanko, Barrett, Ly, Reolon, & Wood, 2009; Vecsey et al., 2007). Vorinostat also enhances fear extinction 24 h after learning (Fujita et al., 2012) and both TSA and vorinostat seemingly lead to this enhancement via increased acetylation and CREB driven gene expression (Fujita et al., 2012; Vecsey et al., 2007) in one case increasing pCREB at the promoter region of the gene encoding the NDMAR subunit *GluN2B* (Fujita et al., 2012). Further, a learning experience that would normally only lead to short-term memory formation can be converted into a LTM with the injection of TSA or NaBut at the time of learning and memory tested 24 h (Stefanko et al., 2009; Vecsey et al., 2007) and 7 days post-learning (Stefanko et al., 2009). However, there was no enhancement of a short-term memory test, 90 minutes post-learning (Stefanko et al., 2009). Further, the inhibitor only affected the consolidation of the memory, not the retrieval, because injection at 24 h, immediately before retrieval had no effect (Vecsey et al., 2007). Rats treated with the HDACi vorinostat have shown enhanced fear conditioning memory, object recognition retention and fear extinction 24 h post-injection (Fujita et al., 2012). In line with the previously discussed differences between HDAC1 and 2, however inhibition of HDAC1 after training actually impairs fear extinction (Bahari-Javan

et al., 2012). This is an intriguing result as it suggests, in contrast to other work, that deacetylation may indeed have a role to play in consolidation (Bredy & Barad, 2008; Bredy et al., 2007).

1.9.e. Evidence of ongoing epigenetic regulation after learning

The evidence presented so far supports the notion that HDAC2 in particular regulates the expression of genes involved in structural rearrangements of synapses after learning or the induction of L-LTP, i.e. the plasticity transcriptome. However, there is evidence that dynamic gene expression occurs for at least 24 h post-learning and L-LTP induction and few studies have investigated long-term HDAC modifications. Recently however, the HDACi TSA was shown to only enhance the transcription of *arc* by the proximal promoter region, a region regulated by HDAC1, after BDNF or FGF2 application (Fukuchi et al., 2015). Interestingly, BDNF drives a second wave of *arc* expression 12 h post-learning which is essential for LTM and is believed to be responsible for pruning of spines (Nakayama et al., 2015). Further, TSA actually inhibited NMDAR-driven *arc* expression (Fukuchi et al., 2015), suggesting that TSA could potentially reduce HDAC1 inhibition of the proximal promoter region of *arc* leading to BDNF driven *arc* expression at 12 h post-learning if present during that timeframe. Indeed, HDACi driven gene expression may be changing the 'plasticity' transcriptome and, therefore, the specificity of the genes expressed may be lost. Arc has recently been shown to have epigenetic functions. In cell cultures arc was found to interact with Tip60, a HAT, leading to increased acH4K12 after NMDAR activation (Wee et al., 2014). Thus, in addition to the

previously described movement of arc out of the nucleus towards active spines after LTP induction and learning (Steward, Farris, Pirbhoy, Darnell, & Driesche, 2014; Steward, Wallace, Lyford, & Worley, 1998), the ongoing waves of arc expression, dynamically regulated by various signalling mechanisms, may be indicative of vastly different roles arc may be playing in LTP and memory.

1.9.f. HDACs and metaplasticity

Ocular dominance columns in the visual cortex are well characterised examples of very stable neuronal networks. After a critical period of plasticity during early postnatal days, the visual cortex becomes relatively rigid and signalling pathways, which would normally induce the expression of plasticity genes, are unable to do so (Putignano et al., 2007). Evidence suggests that though the intracellular pathways involved in the stimulation of gene expression are still fully functional, the gene expression response is supressed, and this suppression is regulated by either increased HDAC activity or decreased HAT activity (Baroncelli et al., 2016; Putignano et al., 2007). Restoring the acetylation with an HDACi, and therefore presumably gene transcription, leads to the destabilization of the networks forming the ocular dominance columns thus allowing for the restructuring of their connectivity (Baroncelli et al., 2016; Lennartsson et al., 2015; Putignano et al., 2007). Similarly, it has been shown that a fear memory, 24 h post-learning, is labile and can be readily and persistently updated via new learning, i.e., to no longer elicit a fear response, with fear extinction training (Gräff et al., 2014). Further, acetylation of H3K9/K14 is transiently increased 1 h after recall of this recently formed memory (Gräff et al., 2014). However, a

consolidated fear memory, recalled 30 d post-learning, elicits no such increase in acetylation, and the memory can only be updated with training for a short period of time but the updating not maintained (Gräff et al., 2014). These two examples suggest that negative regulation of acetylation coincides with negative regulation of restructuring of connectivity or plasticity. Indeed, *Arc* and *c-fos*, previously described as being critical to long-term plasticity, are negatively regulated when recalling a LTM, but inhibiting HDAC2 before the recall allows for their expression (Gräff et al., 2014).

1.10. Summary and hypotheses

There are a number of synaptic and non-synaptic plasticity mechanisms which are induced upon learning or LTP induction. The central integrating point, and the critical component of LTM and LTP, is changes in gene expression (Beck & Yaari, 2008; D. J. Cai et al., 2016; Cohen-Matsliah et al., 2010; Geinisman, 2000). These changes in gene expression, not only allow for the restructuring of synaptic connectivity between neurons but also changes in excitability. Indeed, evidence suggests that a 'plastic state' within a cell is one of increased intrinsic excitability. How this is achieved is unclear although increases in the density of thin spines and CREB activity are correlated with these changes. To control such diverse modifications, the genome must produce specific outputs based on the relevant incoming information. Further, long-term modifications to this output can be achieved by long-term changes to the epigenetic state, driven by prior activity (i.e. metaplasticity). This metaplastic mechanism could regulate the cells'

position in an engram, and the maintenance of the engram, by altering the threshold for subsequent plasticity.

Though the role of gene expression in learning and plasticity is well documented, the current literature focuses on changes in gene expression over the first few hours after plasticity induction or learning and addresses how changes in expression of specific genes regulate synaptic structural plasticity (Alberini & Kandel, 2015; Sweatt, 2016). Indeed, the STC hypothesis is a well-documented phenomenon in which expression of PRPs such as PKMζ and BDNF are critical to the restructuring of synapses within the first few hour of induction. Further, expression of IEGs such as *arc*, *c-fos* and *homer1* have been shown to be important in the early stages of consolidation. However, the fact that gene expression profiles continue to change for at least 24 h post-learning and LTP induction, suggest that there may be a diverse range of processes underlying the consolidation process. Indeed, the changes in structural plasticity, which continue over days after learning, go from growth and development to pruning and stabilization of structure. Further, excitability increases over the first few hours post-learning to allow for the association of multiple experiences, thus expanding the engram and promoting plasticity before recovering to stable levels. Analysis of the gene expression networks has indicated that the gene profiles expressed immediately following learning are volatile and easily disrupted whereas over time they become more stable (Nido et al., 2015). Finally, memories become gradually more stable and resistant to disruption over time. Thus, the enhancement of plasticity, while extremely interesting and indeed needed for the formation of LTM may be, if maintained, counterproductive for the long-term maintenance of memory (Fig. 1.4).

LTP3

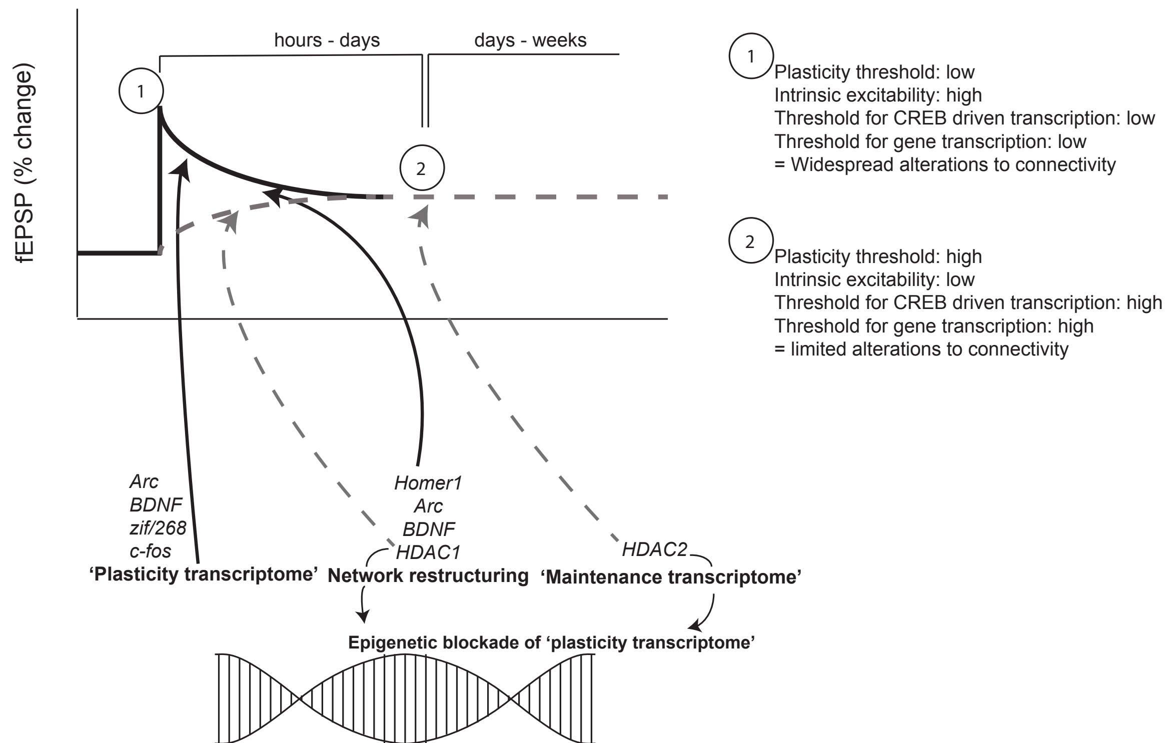

Fig. 1.4. Hypothesised metaplastic inhibition of plasticity by HDAC1 and HDAC2 post-LTP3 induction. Prior to LTP3 induction, the threshold for change is relatively low. The threshold for change is underpinned by the ability to induce the plasticity transcriptome which makes the initial changes to the synaptic drive. Over time the maintenance transcriptome, particularly the expression and subsequent activity of HDAC2, indirectly maintains the increased synaptic drive by resisting future expression of the plasticity transcriptome.

1.10.a. Hypotheses

I hypothesise that the gene expression profiles critical to the maintenance of the engram, i.e., the 'maintenance' transcriptome, are not the same as the genes needed during the first few hours post-learning, the 'plasticity' transcriptome. These distinct profiles are regulated at different time points post-LTP induction in that the 'plasticity' profile is upregulated immediately upon LTP induction but

over time becomes negatively regulated by HDAC1 and HDAC2 and that the 'maintenance' profile, which includes HDAC1 and 2 as negative regulators of gene expression, increases over time post-induction. Further, I hypothesise that the induction of an epigenetic programme coincides with the induction of L-LTP, and this programme initially creates a plastic environment in which expression of plasticity genes are expressed (i.e. decreased HDAC activity) but gradually, over hours to days, the epigenetic programme leads to the negative regulation of plasticity related genes (i.e. increased HDAC activity). It is this later metaplastic shift in the epigenetic regulation of gene expression that I hypothesise to be critical to the maintenance of LTP by suppressing induction and consolidation of competing synaptic changes.

1.11. Aims

Epigenetic regulation of gene expression has been shown to play a role in the induction of persistent LTP, however, all LTP work has been done *in vitro*, in which it not possible to follow the effect of HDAC inhibition for longer than a few hours. This means the LTP experiments in the literature so far have stopped well before the time where the increase in HDAC1 expression is seen (5 h), or where increased HDAC2 expression (24 h) has been identified (Ryan et al., 2012). Furthermore, HDAC inhibition has only been tested before, or at the time of learning, rather than these later time points where we have identified changes in HDAC expression. These issues will be addressed by:

- Profiling the activity and expression of HDAC1 and HDAC2 over time (20 min, 5 h, 12 h and 24 h) after LTP induction *in vivo*. This will enable us to pinpoint times of enhanced action, rather than just the change in mRNA expression.
- Inhibiting HDAC1 and 2 activity at time-points of enhanced HDAC action, which will allow us to assess the necessity of HDAC activity and establish whether these HDACs play a role in the maintenance of LTP.
- Testing whether these HDACs have metaplastic control over subsequent plasticity, including the maintenance of LTP.

2. Methods

2.1. Animal preparation and Surgery.

2.1.a. Animals

Male Sprague Dawley rats (3-6 months old) were used for all experiments. They were housed on a standard 12 h light/dark cycle. Animals used in chapter 3 had access to food and water *ad libitum*. The animals used for the rest of the project were on a restricted diet so as to maintain a bodyweight of ~500 g, while still having access to water *ad libitum*. Animals on food restriction were found to recover faster from surgery and were more active and alert both in their home cages and during recordings. This led of an overall increase in health and well-being of the animals as well as more consistent recordings, as the animals remained more alert during the experiments. Further, the amount of each drug used in the experiments was more consistent as the animals were all of similar weight. For all of the experiments, the animals were surgically implanted with bilateral stimulating and recording electrodes to enable the induction of LTP at PP – DG synapses. All animal work was completed in accordance with the University of Otago Animal Welfare Office training programmes and approved by the University of Otago Animal Ethics Committee (AEC number: 78/12, 103/14).

2.1.b. Surgical preparation

Rats were anesthetised, via subcutaneous injection, with a combination of ketamine (75 mg/kg , PhoenixPharm, New Zealand), domitor (0.5 mg/kg, Pfizer,

Orion Corporation, Finland) and atropine (0.065 mg/kg, provided by the University of Otago Animal Welfare Office). Once the animals were fully anesthetised, as determined by the lack of paw reflex, the heads were shaved and the animals were injected subcutaneously, along the incision line, with the local anaesthetic lopaine (3 mg/kg, provided by the University of Otago Animal Welfare Office). Further, subcutaneous injections of the analgesic carprieve (5 mg/kg, provided by the University of Otago Animal Welfare Office), the antibiotic amphoprim (12 mg, provided by the University of Otago Animal Welfare Office) and saline (10 ml, Baxter, United States of America) were administered. The animals were then placed in the stereotaxic frame (David Kopf Instruments, United States of America) with the tooth bar set to -3 mm, as utilised by the Paxinos and Watson Rat Brain Atlas (Paxinos & Watson, 1982). Paraffin liquid (Home Essentials, New Zealand) and a gauze patch was used to cover the eyes for protection from light exposure under the heat lamp.

Electrodes

Stimulating and recording electrodes were made by soldering stainless steel wires (0.003” bare and 0.0055” coated, A-M Systems Inc., United States of America) to gold pins (Allied Electronics, United States of America). Ground and reference electrodes as well as electrodes used as the negative pole for the stimulating electrodes, were also constructed this way (0.005” bare, 0.008” coated, A-M Systems Inc., United States of America) but additionally soldered to a carbon steel miniature screw (J & G Hardware Supermarket, United States of America) with resistance of no more than 3 Ω.

2.1.c. Surgery

The scalp was incised to expose the top of the skull, which was thoroughly cleaned. Lambda and Bregma were identified, marked and electrode positions were calculated. Recording electrodes were positioned bilaterally at the DG (2.5 mm lateral and 3.8 mm posterior to Bregma). Stimulating electrodes were positioned bilaterally to stimulate a mixed (medial and lateral) PP fibre tract (4.5 mm lateral to Lambda (Fig. 2.1) or unilaterally to stimulate medial and lateral PP fibre tracts individually (4 mm and 5 mm lateral to Lambda respectively) (Fig. 2.2). Holes were drilled through the skull at the marked positions. Further holes were drilled for screw electrodes: 1 reference, 1 ground and the negative pole for each stimulating electrode (2 in total). Another 4 holes were drilled and carbon steel miniature anchoring screws attached (J & G Hardware Supermarket, United States of America).

Ground, reference and recording electrode signal are amplified 200 times (P511, Grass Instrument Company, United States of America), with a low pass filter of 3 kHz and high pass filter initially of 300 Hz, before being passed through an analogue-digital converter (BCN-2110, National Instruments, United States of America). Recording electrodes were lowered through the brain while observing multi-unit activity on a two channel oscilloscope (TDS 210 or TDS 1002, Tektronix, United States of America). As the electrode passed through the sparse neuronal populations of the cortex and reached the densely packed CA1 region, the activity and frequency observed increased considerably. This position was noted, and the electrode was lowered more until the activity changed to the distinctly slower activity of the DG region. The electrodes were left in position

(~3 mm below surface) but were further optimised when evoked responses were initiated. The high pass filter was decreased to 0.1 Hz to accommodate the increased duration of evoked responses. The depth of the stimulating electrodes was optimised with evoked responses, around a starting depth of ~3 mm below surface. A custom built Labview-based software programme (Scavenger) was used to deliver stimulus pulses via custom built programmable constant-current stimulators, unilaterally every 15 seconds. Electrode positions eliciting the maximum field excitatory postsynaptic potential (fEPSP) slope and population spike size were established in both hemispheres, for mixed path stimulation (Fig. 2.1). Electrode positions which elicited distinct medial and lateral PP responses (as determined by a test of convergence and a test of paired pulse responses) were optimized for stimulation of medial and lateral PP individually (Fig. 2.2).

With the electrodes in place, the wires were connected to a 9 pin head plug (#GS09PLG-220, Grinder Scientific, Canada) which was held in place with dental cement. The animal was then injected, subcutaneously with antisedan (2.5 mg/kg, Pfizer, Orion Corporation, Finland) to reverse the domitor. A further injection of saline was given (10 ml, Baxter, United States of America). The animals were then taken to a recovery area where they were covered by a towel and placed under a heat lamp while regaining consciousness. The animals were given a mixture of mashed food pellets and sugar soaked in water, and put back into individual cages. Each animal's recovery was monitored for 10 days. A second dose of the analgesic carprieve (5 mg/kg, provided by the University of Otago Animal Welfare Office) was administered 24 h after surgery.

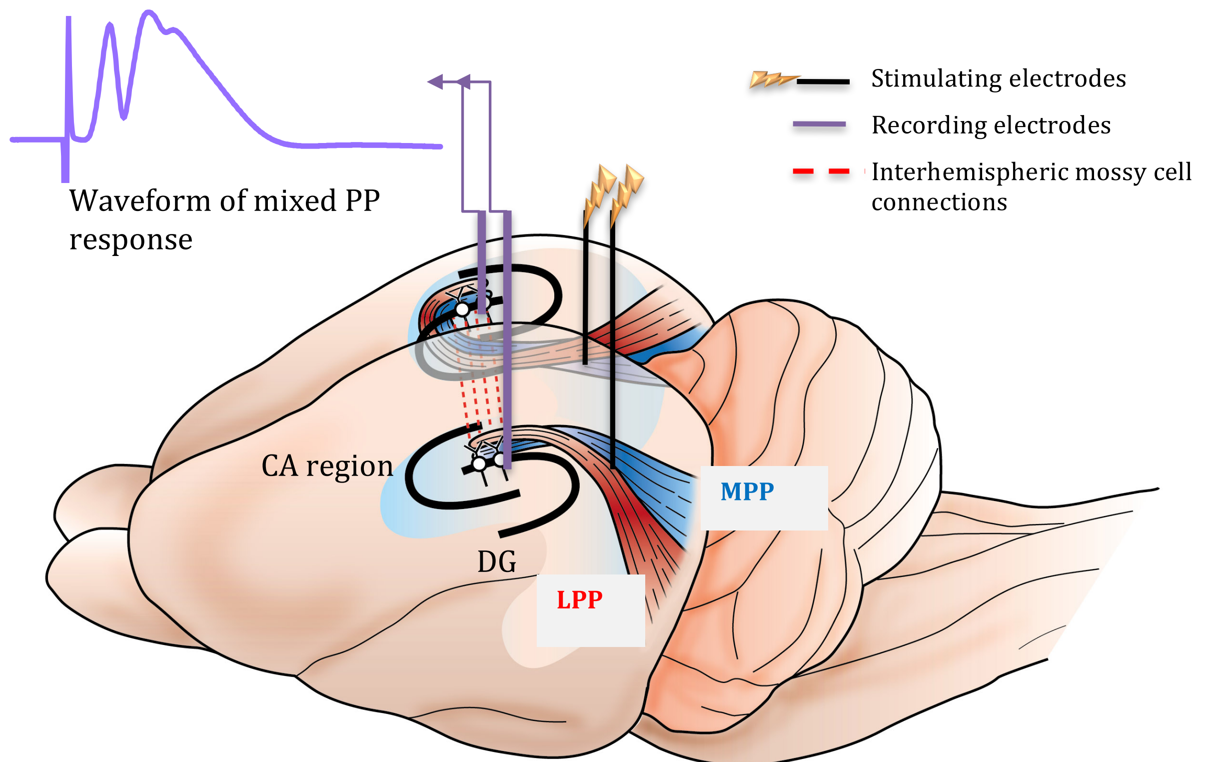

Fig. 2.1. Diagram of electrode placement in the brain for *in vivo* LTP in awake, freely moving animals for bilateral, mixed PP stimulation. Bilateral recording electrodes were placed in the hilus of the DG to record waveforms from the mixed pathway when stimulated. Inset: representative waveform of local field potential evoked by mixed PP stimulation. Adapted from: palomar.edu by Robbie McPhee, University of Otago

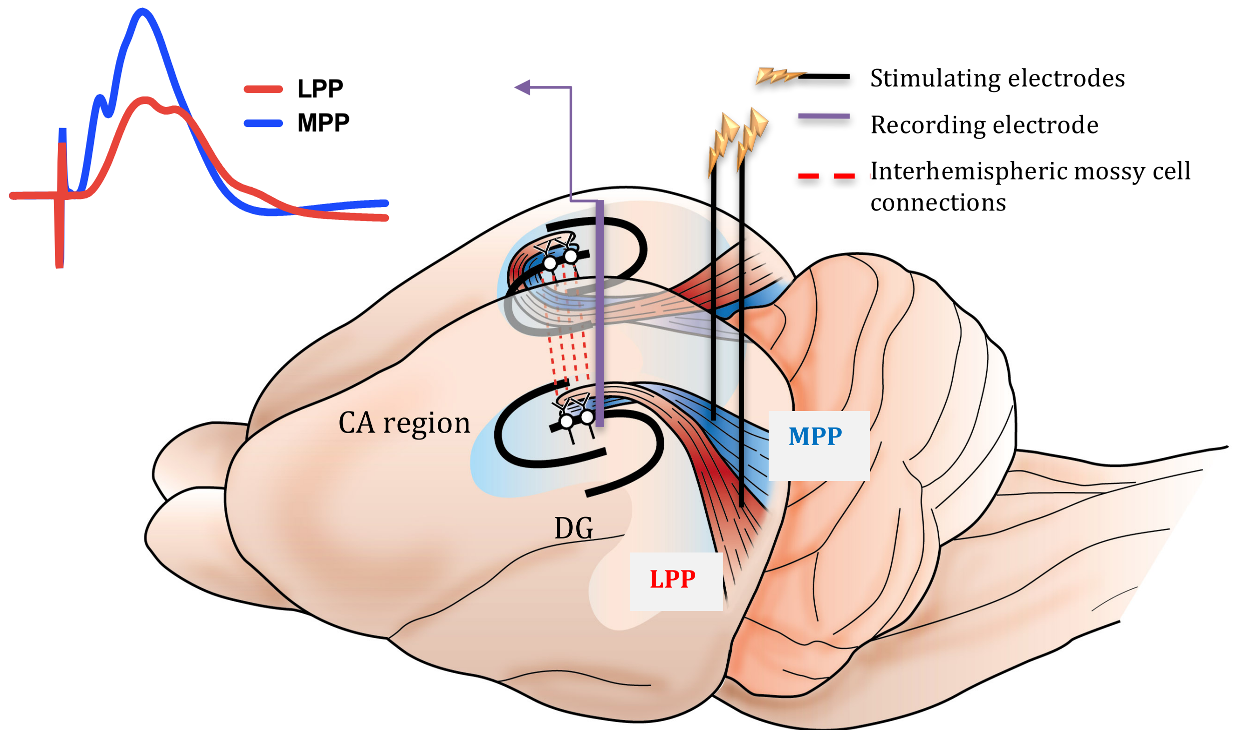

Fig. 2.2. Diagram of electrode placements in the brain for *in vivo* LTP in awake, freely moving animals for separate medial PP (MPP) and lateral PP (LPP) stimulation. Unilateral stimulating electrodes were placed to activate medial and lateral PP fibres individually. Unilateral recording electrodes were placed in the cell body layer of the DG granule cells to record waveforms of each pathway when stimulated. Distinct evoked response waveforms were identified when stimulating the MPP and the LPP. Inset: representative waveforms of local field potential evoked by separate MPP and LPP stimulation. Adapted from: palomar.edu by Robbie McPhee, University of Otago.

2.2. Long-Term Potentiation

2.2.a. Baseline recordings

Awake animal recording set-up

An attachable plug connected wires between the implanted electrodes in the animal to the stimulating and recording equipment. Wires ran through Field Electrode Transistors (9V) to remove high impedance noise from recording electrode wires. This removed the need to use shielded wires which would have restricted the flexibility of the cord. The stimulators and the preamplifiers were the same as was used for surgery. Due to the position of the recording electrodes in the hilus of the DG (Fig. 2.3), measurements of synaptic drive were determined by measuring the slope of the initial positive going component of the waveform and the population spike was measured as the amplitude of the negative going component of the waveform (Fig. 2.4).

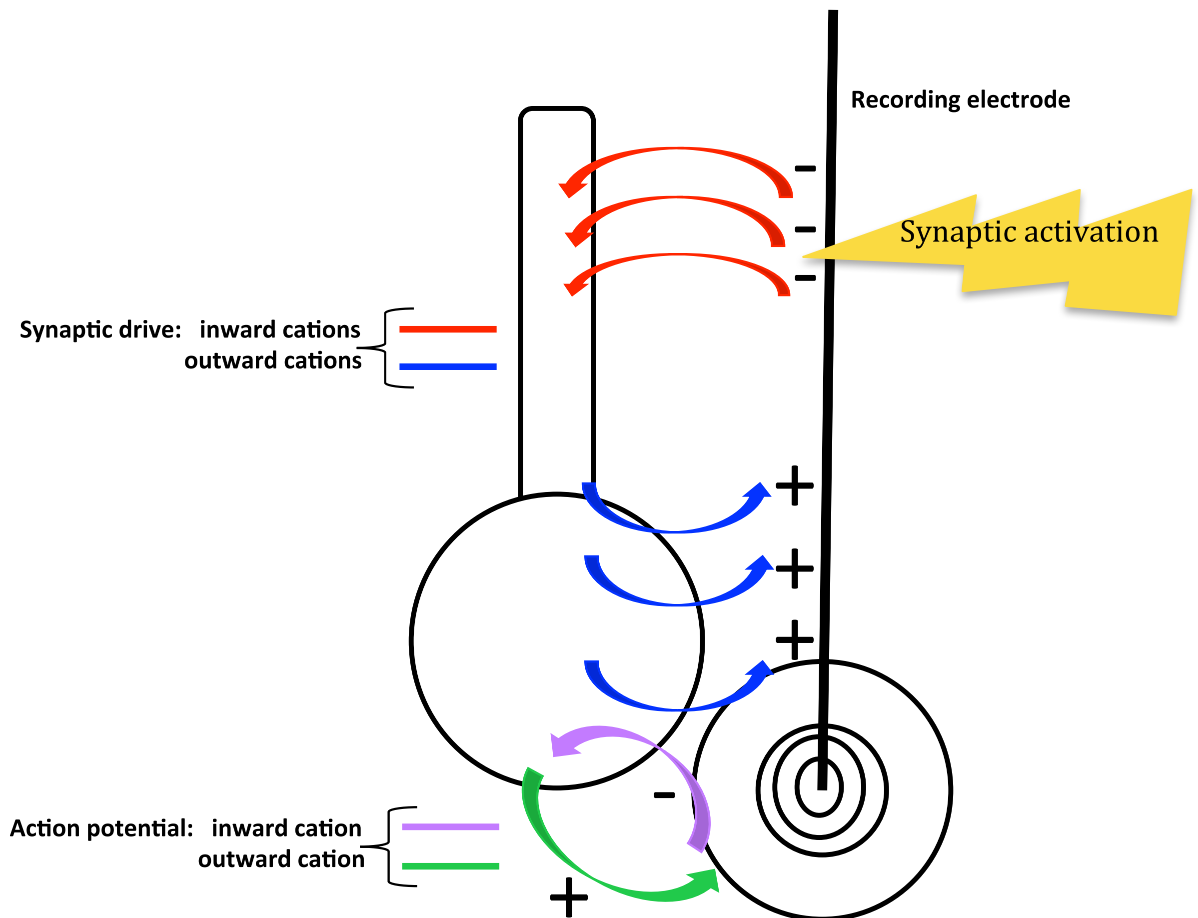

Fig. 2.3. Waveform components during field potential recordings. As cations move away from the field electrode and into neurons they leave negative sinks, whereas as cations move out of the cell and towards the field electrode they create a positive source recorded. Recording electrodes were positioned in the hilus of the DG, thus recording positive going synaptic drive as cations move back out of the cell after synaptic activation. Population spike measurements of action potential firing were measured as an initial negative going potentials as sodium moved into the cell to initiate the action potential firing, before potassium moved out of the cell, thus returning to positive going recording.

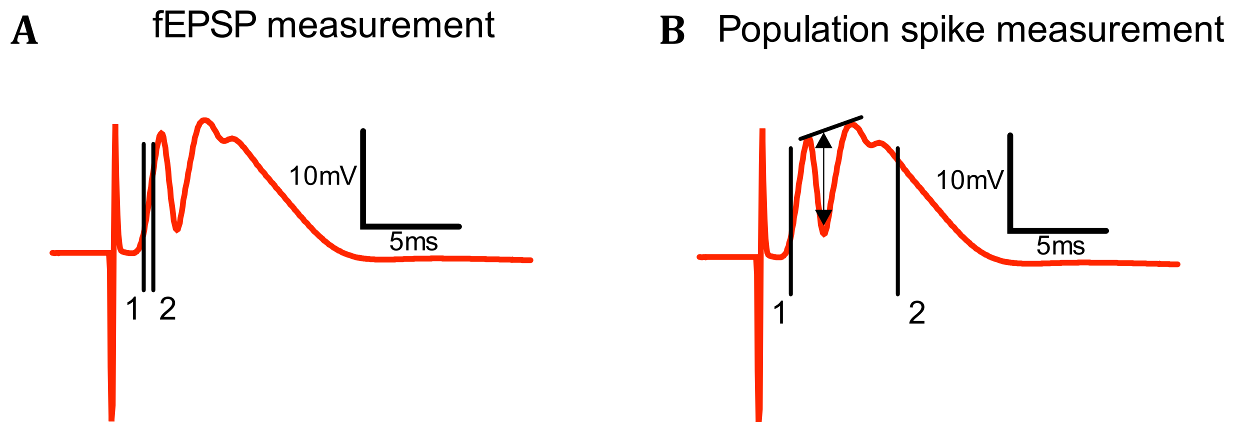

Fig. 2.4. Measurements used to determine potentiation. Using the analysis Labview program Cruncher, cursers were positioned at the start of the measurement area (1) and the end of the measurement area (2) before measurements were made of **A,** fEPSP slope, a measure of synaptic drive. **B,** Population spike amplitude, a measure of action potential firing.

Input-Output curves

After 2 weeks recovery from surgery, an input-output curve was assessed by measuring the slope of the fEPSP (Fig. 2.3a) and size of the population spike (Fig. 2.3 B) elicited by stimuli ranging from 10 to 700 μA. The stimulation intensity which elicited a population spike that was 30% of the maximal was used as the stimulation intensity from then on. The population spike was used as the criterion in order to ensure that the same proportion of neurons from the population was being stimulated in each animal.

Baseline

After the baseline stimulation intensity had been established, animals underwent 5 – 6 baseline recording sessions, each session 2 – 3 days apart, or until baseline recordings were stable (change of no more than 10% in any single

direction over the last 4 baseline sessions). Using the stimulation intensity established above, each hemisphere was stimulated (150 μs pulse duration) alternately, every 15 seconds for 30 minutes. The animals were taken to the same recording chamber, at the same time (between 7 – 9 am) every session.

2.2.b. LTP induction

On the day of LTP induction, animals were taken to the same room and set-up for a final 30 min baseline recording. After that, LTP was induced using a high-frequency stimulation (HFS) protocol that has previously been shown to induce LTP3 (Abraham et al., 2002). This protocol consisted of 10 sets of 5 trains at 1 Hz. Each train consisted of 10 pulses at 400 Hz. There was 1 min between sets. A further 1 h recording (same protocol as baseline) was taken after the HFS stimulation, apart from those animals which were sacrificed at 20 min, in which case they were removed immediately from the recording chamber 20 min post-LTP. For the remaining animals, a final 30 min recording (same protocol as baseline) was taken immediately prior to sacrifice.

2.2.c. LTP persistence

The persistence of LTP was monitored either until the time point of sacrifice, for experiments in which tissue was collected, or until the population spike amplitude and fEPSP slope had returned to baseline levels. To determine this, baseline recordings were taken in exactly the same manner as they were taken prior to LTP induction, every 2 – 3 days.

2.2.d. LTP analysis

LTP was determined by measuring the average percent change in the slope of the fEPSP (Fig. 3. A) from the last 15 min of the baseline prior to LTP induction. A threshold of 15% change from baseline needed to be met to determine that LTP had been induced. Further, the percent change in the size of the population spike (Fig. 3. B) was also measured. However, because the population spike is a measurement of cell firing, which is not necessarily just driven by changes in synaptic efficacy, it was not used as a measure of 'LTP' per se but was still included in analysis.

2.2.e. Statistics

For experiments where tissue was collected animals were only included if the 15% LTP threshold was met. Therefore, no statistics were needed for the LTP section of these experiments. For the study of the persistence of LTP, ANOVA with repeated measures were used. Mauchly's test of sphericity was employed to assess sphericity of samples and if significant, *p* was adjusted using Greenhouse-Geisser correction. $p<0.05$ was considered to be statistically significant.

2.3. HDAC1 and HDAC2 immunoprecipitation and Activity Assay

2.3.a. Tissue collection

The dorsal DG was dissected (n=5-7/ time point) after LTP induction by Barbara Logan (Abraham laboratory, Department of Psychology, University of Otago). The animals were first anesthetised using isoflurane until unconscious,

and then immediately decapitated with a guillotine. The brain was removed and chilled in ice cold Phosphate Buffer Solution (PBS) for 1 min, before the dissection of each hemisphere. The tissue was collected 20 min, 5 h, 12 h and 24 h post-LTP induction. The tissue was collected from the hemisphere in which LTP was induced (HFS) and the contralateral hemisphere in which LTP was not induced (Non-HFS). Further, dorsal DG tissue was collected from two sets of control animals which had gone through exactly the same procedure as described, but one group received test pulses and the other did not. Neither group received the LTP induction protocol (control). We investigated whether there were any differences in HDAC1 and HDAC2 activity between animals that received test pulses and those that did not, and found none. Therefore all control animals were pooled.

2.3.b. HDAC1 and HDAC2 immunoprecipitation

HDAC1 and HDAC2 Immunoprecipitation and Activity Assay Kits (#K342-25 and cat. #K341-25 respectively, BioVision, United States of America) were used to measure HDAC1 and HDAC2 activity 20 min, 5 h, 12 h and 24 h post-LTP induction. The amount of protein used with these kits was further optimised once it was received for our specific needs.

Protein extraction

Dissected tissue, placed in pre-weighed microtubes (1.7 ml, 14-222-171, Axygen, United States of America), was immediately frozen in a liquid N_2 bath and ground to a fine powder using a plastic pestle. The tubes were subsequently

weighed again to determine the weight of tissue collected. Samples were then stored in a -80°C freezer until time of use.

Proprietary Lysis Buffer (BioVision) was supplemented with the protease inhibitor (Complete EDTA-free, 11873580001, Roche, Switzerland) by dissolving 1 tablet (25 x concentrate) in 25 ml of the buffer. This was aliquoted and stored at -20°C until needed. Lysis Buffer containing protease inhibitor (300 µl per 5 mg of tissue) was added to each sample to break down the cells, enabling protein extraction. This was then mixed on a rocking platform (1 h at 4°C). The samples were then sheared by passing the lysate through a needle (25 ga) 3 times before the sample was centrifuged (5 min, 4°C at 10,000 g) to precipitate cell debris. Protein-rich supernatant from each sample was transferred to new tubes and held on wet ice while the protein concentration was determined using the Bradford protein assay.

2.3.c. Bradford Protein Assay

A Bovine Serum Albumin (BSA) (#A4503 – 50G, Sigma-Aldrich, United States of America) standard curve, in triplicate, was created (0 µg, 2 µg, 4 µg, 8 µg and 12 µg) to determine the amount of protein in each tissue sample. This was performed on a clear, flat bottom Microtest 96-well ELISA plate (#353279, BD Falcon, United States of America). Each sample (2 µl) was added to the plate in triplicate. A 1:4 dilution of BioRad protein assay reagent (#500 – 0006, BioRad, United States of America) was made and was added (200 µl) to each well. This was left to develop (5 min) and then read by a microplate reader (version 550,

BioRad, United States of America), using Microplate Manager Software (4.0, BioRad, United States of America) with absorbance at 595 nm.

2.3.d. Immunoprecipitation

To immunoprecipitate HDAC1 or HDAC2 from protein extracts, 200 µg of each sample was added to new, pre-chilled tubes to be used with HDAC1, HDAC2 and control Rabbit IgG antibodies (10 µl of undisclosed concentration, BioVision, United States of America). IgG antibody was used as a control for non-specific pull-down of protein. PBS (25 ml) was supplemented with a protease inhibitor (25 x concentrate tablet, Complete EDTA-free, 11873580001, Roche, Switzerland). The sample-antibody reactions were adjusted to a volume of 500 µl, using the PBS containing protease inhibitor and incubated overnight (4°C, on a rotary mixer).

A 1000 µl pipette was used when working with the Protein-A/G beads (provided with the kit as a 50% slurry) so as to ensure the pipette tips were wide enough to accommodate the beads. The necessity of pre-blocking the beads was assessed and determined to remove significant background noise. Therefore, the beads were pre-blocked using BSA (3 µg/µl) for 1 h at room temperature. The beads (25 µl per reaction) were then washed (10 s at 14,000 g) three times with PBS (1 ml). The beads were then resuspended in a 50% slurry with PBS (25 µl per reaction).

To precipitate the antibody-antigen complexes 25 µl of the blocked, washed and resuspended slurry was added to each sample tube and incubated (1 hour at 4°C). Protein A/G-antibody-antigen complexes were collected by centrifugation (10 seconds, 4°C at 14,000 g). The bead complexes were washed to remove non-cognate protein (3 times, 1 ml PBS, inverting each sample 8 times to mix, and centrifuged at 14,000 g for 10 s).

2.3.e. HDAC activity assay

The level of HDAC1 or HDAC2 activity in each sample was measured using an HDAC pseudo-substrate which could be deacetylated. Deacetylation of the substrate produced a fluorophore, which was cleaved by a developer to be measured. Therefore, the amount of fluorescence measured was indicative of the amount of deacetylase activity within each sample. The HDAC pseudo-substrate (4 µl per sample) was mixed with HDAC Assay Buffer (156 µl per sample, pre-warmed to 37°C), added to each test sample and incubated on a rocker (1 h at 37°C). The Developer (20 µl), was then added to the reaction and incubated (30 min at 37°C). Samples were collected (14,000 g for 2 min) and added (100 µl) to individual wells on a 96-well Spectraplate™ -96 (P12-106-041, PerkinElmer, United States of America). A standard curve (0-500 pmol) was created to enable a quantitative measure of HDAC activity using a 7-amino-4-trifluoromethyl coumarin standard (AFC, 1mM) in duplicate, on the same plate as the samples. The plate was then read at an excitation length of 360 nm and emission length of 528 nm using a Fluorescence plate reader (Synergy 2, BioTek, United States of America) using reader software (Gen5, 1.11, BioTek, United States of America).

2.3.f. Statistics

Paired t-tests were used to test between HFS and non-HFS hemispheres. Non-paired t-tests were used to test between each hemisphere of the test animals with the control samples. This analysis was chosen because of the mixed within animal and between animal samples. Further, due to this being an exploratory experiment we decided that a type II error was of more concern than a type I as further experiments were planned to test the results. Thus, $p<0.05$ was considered to be statistically significant, although an adjusted $p<0.017$ (0.05 divided by 3 questions) was also assessed to understand the range of probability we were testing.

2.4. Western blot Analysis

2.4.a. Materials

All materials used for western blot analysis were BioRad products, unless otherwise stated. The same antibodies against HDAC1 and HDAC2, which were used for HDAC1 and 2 activity assays, were used in these experiment (#3601-100 and #3602-100 respectively, BioVision, United States of America).

2.4.b. Western blot

In order to measure the levels of HDAC1 and HDAC2 protein, the samples (section; 2.3) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and transferred to nitrocellulose membranes. Resolving gels (15%) were prepared (appendix A), loaded into a cassette (Gel

Cassettes mini, 1.0 mm, NC2010, Life Technologies, United States of America) and allowed to set. A stacking gel (5%) was prepared (appendix A) and added on top of the resolving gel, with a 15 lane comb and allowed to set. Following removal of the comb and flushing of the wells with 1 x running buffer, the cassettes were then added to a gel electrophoresis cell and covered with running buffer (appendix A). Samples (15 µl, 5 µg) were denatured in 5 µl of loading buffer (appendix A) (99°C for 10 min) and then loaded to individual lanes with Rainbow ladder (2 µl, GE Healthcare Life Sciences, United States of America) loaded to one lane to indicate protein size (kDa). Gels were run at a constant 125 V for ~ 2 h, or until ladder was spread down the resolving gel. A 20% methanol transfer buffer was prepared (appendix A) and chilled. After removing gels from the cassettes each were stacked against a nitrocellulose membrane. The gel-membrane pair were then stacked between 2 sheets of filter paper and 2 pairs of sponges and placed into transfer chambers. Current was driven from the cathode, through the gel, transferring proteins from the gel to the nitrocellulose membrane which was on the anode side on the gel. They were then placed in electrophoresis cells and covered with the pre-chilled transfer buffer. The transfer was run at a constant 100 mA for 2 h. Nitrocellulose membranes were then air-dried overnight.

To prevent non-specific binding, dried nitrocellulose membranes were with blocked with 5% fat-free milk powder PBS/TWEEN-20 solution (1 h, RT). Membranes were rinsed in PBS/TWEEN before incubation with HDAC1 or HDAC2 primary antibodies (BioVision, United States of America) used in the immunoprecipitation experiments (rabbit, 1:1000 with 0.1% BSA, 0.1% NGS

PBS/TWEEN-20, 4 °C, overnight) and tubulin (#ab-7291, Abcam, United Kingdom; mouse, 1:10,000, 0.1% BSA, 0.1% NGS PBS/TWEEN-20, 4 °C, overnight). Membranes were then washed (4 x 5 min, gentle rocking, PBS/TWEEN-20) before incubation with appropriate secondary antibodies (1 h, RT). Membranes were washed again (4 x 5 min, gentle rocking, PBS/TWEEN-20) before being rinsed in PBS and dried away from direct light.

2.4.c. Imaging and analysis

Secondary antibodies were used which fluoresce at wavelengths of either 680 (rabbit, 1:10000) or 800 (mouse, 1:15000) λ , so as to detect tubulin and the HDAC of interest separately (RDye 680RD Goat anti-Rabbit, 925-68071, and RDye 800CW Goat anti-Mouse, 925-32210, LI-COR, United States of America). Antibody binding was visualised and imaged using an Odyssey Clx Infrared Imaging System (LI-COR, United States of America), scanning in both fluorescence channels (700nm red and 800 nm green). Images were analysed using Image Studio software (LI-COR, United States of America). HDAC signal intensities were expressed relative to the tubulin signal intensities for each sample. Normalised values were used in analysis. Each western blot consisted of 5 pairs of samples from test animals (HFS and non-HFS hemispheres) and the 3 median control samples, which had been determined by previous western blot analysis of control samples. Data from each test hemisphere was expressed as a fold change from the average of the control samples.

2.4.d. Statistics

All test samples were normalised to control samples. One sample t-tests were then used to assess HFS and non-HFS hemispheres. Again, due to this being an exploratory experiment we decided that a type II error was of more concern than a type I as further experiments were planned to test the results. Thus, $p<0.05$ was considered to be statistically significant, although an adjusted $p<0.017$ (0.05 divided by 3 questions) was also assessed to understand the range of probability we were testing.

2.5. TSA treated *in vivo* LTP.

Three experiments were designed to establish the effect of inhibiting HDAC activity on the persistence of LTP. First, however, the injection time and concentration of TSA was established by assessing the change in histone acetylation after TSA injection.

2.5.a. TSA action

Trichostatin A

TSA (#T8552-5mg, Sigma-Aldrich, United States of America) was dissolved in dimethyl sulfoxide (DMSO) (1% of final volume) with polyethylene glycol (PEG) (30% of final volume) in PBS. Injections of 1% DMSO and 30% PEG in PBS were given as the control vehicle.

TSA injection and tissue collection

Animals were injected (i.p.) with either 2 mg/kg, 4 mg/kg or control vehicle 30 min, 1 h or 4 h prior to sacrifice (n=1 per condition apart from 2mg/kg and 4mg/kg 4 h prior to sacrifice where n=3 per condition). Cardiac perfusion was performed, under isoflurane, with 50 ml of ice cold PBS before 50 ml of paraformaldehyde (4%) to fix the tissue. Animals were then decapitated and brains removed and stored in 50 ml of paraformaldehyde (overnight at 4°C). Brains were then removed from paraformaldehyde and placed in 30% sucrose solution until no longer floating (4°C). Brains were then snapped frozen using liquid nitrogen and sliced (40 µm thick sections) using a freezing microtome. Slices were stored in storage solution (0.1% sodium Azide in 0.1 M PB) until use (4°C). Each well contained a slice from a TSA treated animal and the relevant control animal (up to a maximum of 6 per well) and wells were repeated in triplicate. Measurements of each TSA treated slice was measured as a fold change (FC) from the control slice within that well before the triplicates were averaged.

Immunohistochemistry

Slices were prepared for 3,3'-diaminobenzidine (DAB) immunohistochemistry. DAB can be conjugated to an antibody of interest and will stain brown when oxidised and can, therefore, be used as a marker for immunohistochemistry. To block any endogenous peroxidase activity, which would oxidise DAB, prior to incubation with an antibody slices were incubated with hydrogen peroxide (15 min, 1%) before being thoroughly washed (3 x 10 min, PB/TX, rocking platform). Further, slices were pre-blocked to reduce non-

specific binding of the antibody of interest (60 min, NGS in PB/TX). Slices were then probed with primary antibodies (overnight, 4°C, rocking platform). Antibodies tested were Acetyl-Histone H4 (Lys12) antibody (#2591, Cell Signaling Technology, United States of America) and Acetyl-Histone H3 (Lys9/Lys14) antibody (#9677, Cell Signaling Technology, United States of America) (1:500 in 10% NGS PB/TX). The following morning, slices were again washed thoroughly (4 x 10 min, rocking platform, PB) before being probed with a secondary biotinylated anti-rabbit antibody (1:500 in 10% NGS, PB/TX) (#B7389, Sigma-Aldrich, United States of America) and incubated (2 h, room temperature, rocking platform). Slices were washed (4 x 10 min, PB) before binding with avidin:biotinylated enzyme complex kit (#PK-4000, Vector Laboratories, United States of America) and incubation (2 h, room temperature). Slices were then washed (3 x 10 min, 0.1 M PB) before the DAB reaction (6 min, rocking platform). Finally, slices were washed again (3 x 5 min, 0.1 M PB) and mounted on gelatine coated slides (appendix B) and left to dry overnight. Slices were then dehydrated in ethanol before being cleared using xylene and coverslipped.

Analysis

Analysis was completed by comparison of samples within each well, rather than between wells, to limit well-to-well variability. Imaging of slides was all completed in one session, with the brightness of the Axioskop microscope (Zeiss, United States of America) on the same setting (5). Images were captured using VisiCapture software (Visionet, United States of America) and measured using ImageJ. Software (National Institutes of Health, Unite States of America).

Images were taken of both DG from each slice, as well as the corpus callosum which was used to measure the background signal from the slice. An area without any cell bodies within the CC was selected and the mean grey value measured. The DG region was selected and the mean grey value was measured. The mean grey value of the CC was subtracted from the mean grey value of both DG on each slice. The FC was calculated between the TSA slices and control slices within each well, in triplicate.

Statistics

Statistics was only completed on the samples injected 4 h prior to sacrifice with a dose of 2 mg/kg and 4 mg/kg. Unpaired t-tests were used to compare the mean grey value between vehicle treated and 2 mg/kg pairs and vehicle treated and 4 mg/kg pairs; $p<0.05$ was considered statistically significant.

2.5.b. The effect of pre-treatment with TSA on persistent LTP.

Animals

All surgery, recovery and baseline and LTP recordings were the same as previously described.

20 min post-LTP induction

To assess whether pre-treatment with TSA would enhance the induction and maintenance of LTP, TSA was injected to target 20 min post-LTP induction. The optimal dose and time course, as determined in exp. IV, was identified as 2mg/kg injected 4 h pre-sacrifice. Therefore, injections (i.p. TSA or vehicle) were

given 4 h prior to the time point being targeted (20 min post-LTP). LTP was followed for at least 21 days post-induction, with recording sessions (same protocol as baseline recordings) taken every 2 – 3 days post-LTP.

12 h post-LTP induction

I next wanted to assess whether inhibition of HDAC activity, 12 h post-LTP induction, had an effect on LTP persistence. Therefore, animals were injected (i.p. TSA or vehicle) 8 h post-LTP induction (to have the maximum effect 12 h post-LTP induction). LTP was followed for at least 21 days post-induction, with recording sessions (same protocol as baseline recordings) taken every 2 – 3 days post-LTP.

2 pathway assessment of HDAC stabilization of LTP.

Finally, I wanted to assess whether increased HDAC activity may protect a previously established LTP from decay via competing inputs onto the same cells. To assess the electrode placement into independent medial and lateral PP pathways, I performed a convergence test and a PP test at the start of baseline recording.

Convergence test. A stimulation intensity which would elicit a response in each pathway that was just sub-threshold for cell firing, was set. Timing of the stimulation of each pathway was set so that the latency until population spike onset was matched. The lateral PP stimulating electrode was positioned further away from the recording site than the medial PP electrode. Thus, the time from stimulation of the lateral PP to recording of a synaptic response, and indeed cell

firing, was greater than the medial PP. Further, the medial PP fibres form synaptic connections closer to the cell body of the DG cells, whereas the lateral PP fibres form synaptic connections in more distal regions of the dendrites of the DG cells. Therefore, the time it takes for stimulation of an lateral PP EPSP to elicit a population spike is longer than it is for the medial PP EPSP. Once the timing was set to accommodate these differences, stimulation of both pathways was initiated so that the summation of both synaptic inputs would be enough to drive cell firing and thus population spike generation, where each pathway alone would not.

Paired Pulse test. Pairing of test pulses 50 ms apart elicits paired pulse facilitation of the lateral PP synapses but paired pulse depression, or no change, of the medial PP synapses (McNaughton, 1980; Petersen et al., 2013). Therefore, each pathway was tested using a paired pulse paradigm to determine the specificity of the pathway stimulated.

Once the electrode positions had been assessed, baseline recordings and LTP induction were initiated. LTP was induced in the medial PP (with the same LTP induction protocol previously described) before inducing LTP in the lateral PP 12 h later (with the same LTP induction protocol previously described). Prior to lateral PP induction, animals were injected (TSA or vehicle, i.p.) so that HDAC activity was inhibited at the time when lateral PP LTP was induced (4 h pre-lateral PP HFS). LTP was followed for at least 21 days post-induction, with recording sessions (same protocol as baseline recordings) taken every 2 – 3 days post-LTP induction.

2.6. Interhemispheric effects of LTP induction.

2.6.a. Animals

Surgery as described above. The animal care, baseline recordings, LTP induction and follow of LTP decay was completed by myself and Aimee Smith as part of her 4th year honours project. The LTP induction protocol and analysis were the only component of the experiment that varied from the previously described protocol.

2.6.b. LTP induction

LTP was induced, as previously described, unilaterally (LTP_{hemi1}) and followed for 20 min. Following this, LTP was induced in the contralateral hemisphere (LTP_{hemi2}) again using the same protocol as previously described. The LTP in both hemispheres were followed for at least 21 days, with testing every 2 – 3 days, again, as previously described. LTP was induced unilaterally only in a control group of animals (LTP_{unilat}).

2.6.c. LTP analysis

The first LTP (LTP_{hemi1}), and the single LTP in control animals (LTP_{unilat}), were assessed as previously described. The second LTP (LTP_{hemi2}) was measured as a percent change from baseline where the baseline was taken from the average size of the fEPSP slope during the 20 min post-HFS1.

2.6.d. Statistics

Statistics for experiments where LTP persistence was measured were completed using ANOVA with repeated measures. Mauchly's test of sphericity was used to assess sphericity of samples and if significant, p was adjusted using Greenhouse-Geisser correction. Time*treatment interactions and tests of between subject effects were assessed $p<0.05$ was considered statistically significant. In experiments with more than two treatments, Tukey's post-hoc analysis was completed.

3. Temporal profile of HDAC1 and 2 activity and expression following LTP induction.

3.1. Introduction

The consolidation of LTM depends upon a coordinated set of mechanisms across time, rather than a single change upon learning which is then maintained (Caroni et al., 2014). Changes in expression of specific genes, and the synthesis of specific proteins, at discrete time points has been shown to be critical to the maintenance of LTM (Bekinschtein et al., 2010; Katche et al., 2010; Katche et al., 2012). In accordance with this, our group has demonstrated distinct networks of genes which are regulated over 24 hours post-LTP induction, with distinct functions (Ryan et al., 2012). Regulation of gene expression itself is regulated over this timeframe, suggesting that master-regulators of specific groups of genes must themselves be tightly controlled. At 20 min post-LTP induction the majority of the regulated genes are upregulated and, in addition to vast structural modifications at synapses which these genes are responsible for, a major function of these networks is regulation of gene expression (Ryan et al., 2011; Ryan et al., 2012). By 24 h post-LTP induction, the majority of the regulated genes are downregulated and a major function of these networks is the negative regulation of gene expression (Ryan et al., 2012). Further, the stability of these networks has also been predicted to increase over this time, where the 24 h networks are much more stable and resistant to disruption than the 20 min networks (Nido et al., 2015). This suggests that not only are there temporally

dynamic changes in gene expression, but there may be temporally dynamic changes to higher level regulatory mechanisms. Over time these regulatory mechanisms appear to have stronger control over gene profiles, producing more robust and reliable gene responses, as well as regulating the environment, or threshold of activity required for gene expression.

Epigenetic mechanisms regulate the condensation of chromatin, leading to an increase, or decrease, in the permissiveness to gene expression (Kouzarides, 2007). This can assist in regulating gene expression profiles by altering the ease at which gene expression can occur at specific regions of the genome (Kouzarides, 2007). One epigenetic mechanism associated with LTP and LTM is histone acetylation and its opposing mechanism histone deacetylation (Alarcón et al., 2004; Levenson et al., 2004). Acetyl groups can be added to lysine residues by HATs or removed by HDACs, which increase or decrease transcription factors access to the DNA respectively and therefore leads to an overall positive or negative regulation of gene expression (Stilling & Fischer, 2011). Our group has found mRNA expression of class 1 HDACs, HDAC1 and 2, to be upregulated post-LTP induction (Ryan et al., 2012). HDAC1 was upregulated 5 h post-LTP induction and predicted to be the hub of a major network of genes with predicted interactions with genes regulating Ca^{2+} signalling such as *CamK1* and *CamK2* and genes regulating gene expression itself such as *SMARCA4* and *RUNX1T1* (Ryan et al., 2012). HDAC2 was upregulated at 24 h post-LTP, and was also predicted to be the hub of a major network of genes (Ryan et al., 2012). Thus, we hypothesised that HDAC1 and 2 may work as higher lever regulators of gene expression profiles after LTP induction.

HDAC2 has previously been shown to act specifically at the promoter region of a number of LTP and memory-related genes such as *arc, homer1, CaMKII* as well as number of AMPA receptor subunits, such as *GluA1 and GluA2* (Gräff et al., 2012; Guan et al., 2009). While HDAC1 has been shown to interact with *c-fos* (Bahari-Javan et al., 2012), an IEG regulated after learning and LTP induction, it has mostly been associated with genes involved in cell cycle (Kim et al., 2008). An extension of our preliminary work showing mRNA expression was needed to gain a full overview of HDAC1 and HDAC2 activity across 24 h post-LTP induction. Thus we aimed to assess whether concomitant changes in protein and activity levels of HDAC1 and HDAC2 could be discerned.

3.2. Hypotheses

1) HDAC1 activity would increase 5 h post-LTP, in conjunction with the increase in mRNA previously identified, or later (12 h) after the mRNA had been translated to protein and then stabilize at a higher level than baseline by 24 h. This increased level at 24 h is hypothesised to negatively regulate Ca^{2+} signalling mechanisms and gene expression programmes, previously predicted to be regulated by HDAC1 (Ryan et al., 2012), needed for future plasticity.
2) HDAC2 activity would decrease 20 min post-LTP induction, given that it negatively regulates IEGs that we know to be upregulated at this time, but it would gradually increase over 24 h, reaching a stable level, higher than baseline, by 24 h so as to increase the threshold for future IEG expression and thus future morphological change.

3) Changes in protein expression of these HDACs would coincide with changes in their activity and increase in mRNA expression.

3.3. Results

3.3.a. HFS induced robust LTP at PP-DG synapses.

In order to investigate HDAC1 and HDAC2 activity and protein levels, LTP was induced at PP-DG synapses (>15% change in fEPSP slope, n = 5-7 per time-point). The HFS protocol induced LTP that persisted until the time-point of sacrifice for each group (Fig. 3.1 A). No change in perforant path synaptic drive occurred in the non-HFS hemispheres of the same animals (Fig 3.1 B).

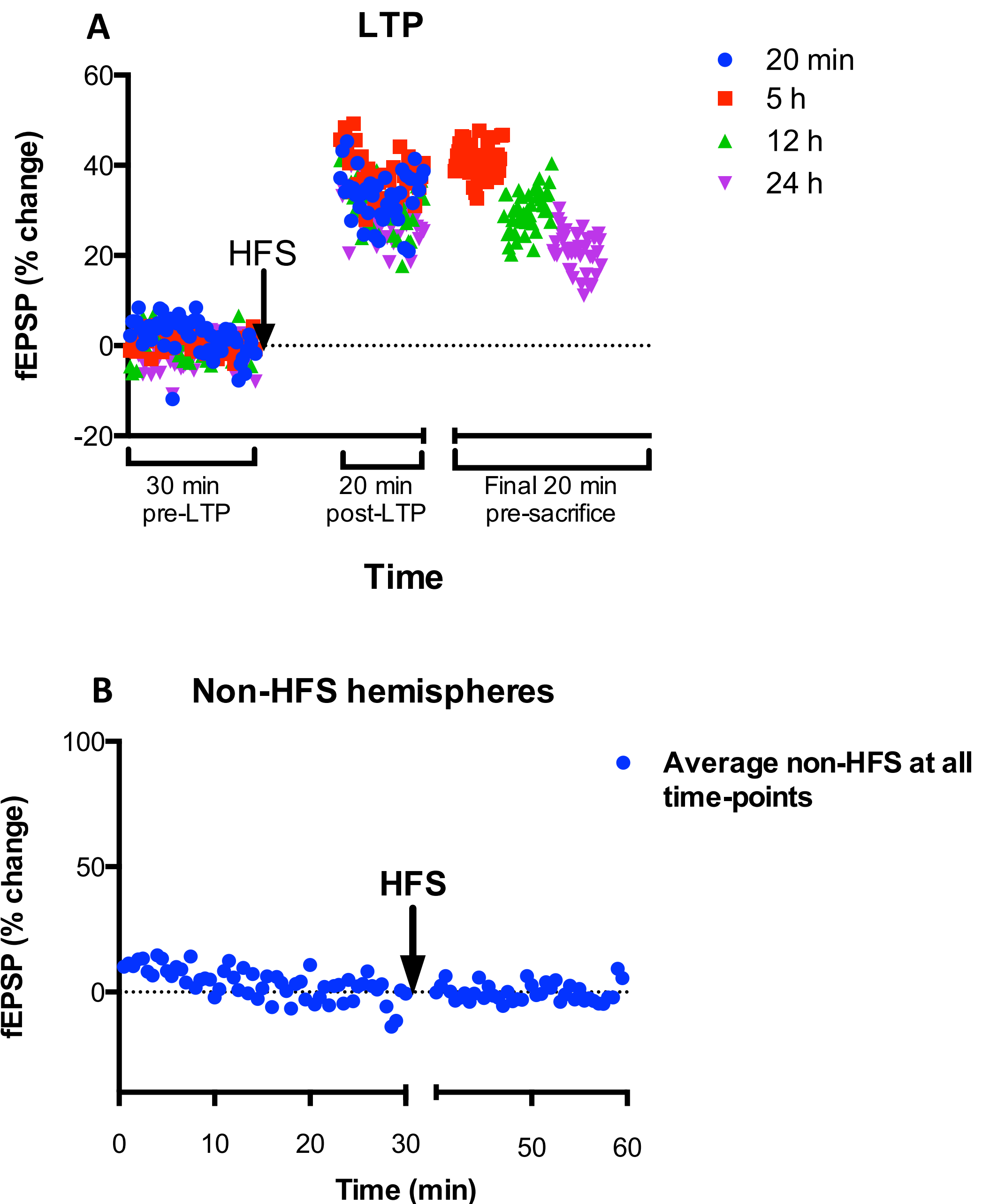

Fig 3.1. LTP was induced unilaterally and persisted until sacrifice. **A**, LTP (>15% change in fEPSP slope) was induced (measured during 20 min post-LTP induction), and persisted until sacrifice (measured during final 20 min pre-sacrifice) at 20 min, 5 h, 12 h and 24 h post-LTP (*n* = 5-7 per time-point). **B**, There was no change in synaptic drive induced in the non-HFS hemisphere. Results are plotted as a group average for each time-point.

3.3.b. HDAC1 and 2 activity assay

Optimization

HDAC activity assays have been used for some time (Dangond et al., 1998). The process involves incubating test samples with a pseudosubstrate, which will be deacetylated by any HDAC present. A developer is then added which will cleave any deacetylated substrate to produce a fluorophore (fluorometric method) or chromophore (colorimetric method) which can be read on an appropriate plate reader for the method type. The higher the fluorescence or absorbance reading, the more deacetylated substrate is present and therefore the more HDAC activity. This can be quantified using a standard curve on each plate with a known fluorophore or chromophore concentration. By immunoprecipitating the samples with HDAC1 or HDAC2 antibodies, prior to the activity assay, the activity measurement was specific to the HDAC of interest.

Using a fluorometric version of HDAC activity kits, which is purported to be more sensitive than the colorimetric version, we optimised the amount of protein. This enabled the use of very small samples (10 – 20 mg of dorsal DG tissue) so as to limit the collection of tissue to only the region in which LTP had been induced. The protocol provided with the kit suggested the use of 50 – 100 μg of extracted protein with each antibody. However, once the protein had been immunoprecipitated, and only HDAC1 and HDAC2 protein remained, we found that increasing the amount of protein to 200 μg was needed to sufficiently record HDAC activity reliably above background noise (Fig 3.2).

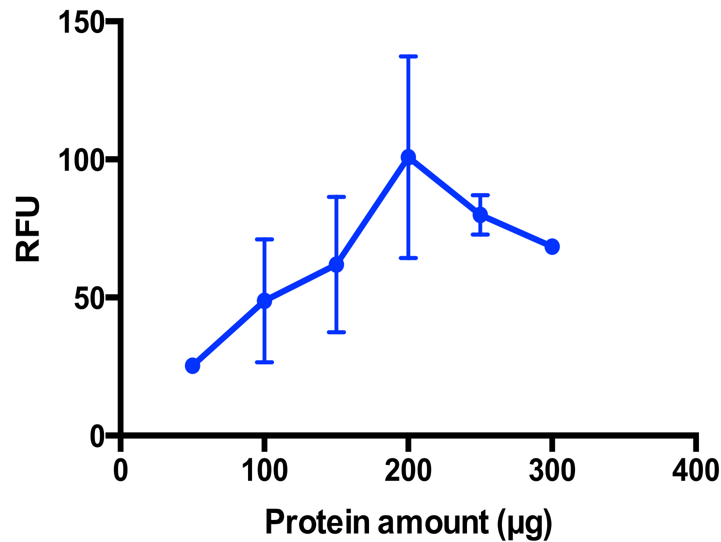

Fig 3.2 Protein concentration optimised for HDAC activity assays. The amounts tested ranged from 50 – 300 µg (n = 12) with 200 µg showing the greatest amount of fluorescence, measured an arbitrary relative fluorescent unit (RFU). Mean + S.E.M.

Temporal profile of HDAC1 and HDAC2 activity post-LTP

Dorsal DG tissue was taken 20 min, 5 h, 12 h and 24 h post-LTP induction and used in the HDAC1 and HDAC2 immunoprecipitation and activity assays. Previously, DG tissue from the contralateral, non-HFS hemisphere, was used as a within-animal control. However, when starting the work, the 20 min samples were found to have consistently more HDAC activity in both hemispheres, than samples used when establishing the protocol. The samples used to establish the protocol had gone through the same procedure as the samples used for the experiment, apart from having no LTP induced (average 20 min HFS = 126.5 ± 42, Non-HFS = 119.4 ± 15.6, test sample used to establish protocol = 100.5 ± 51.6). Therefore we investigated the level of HDAC activity in a control group of animals which had undergone surgery and recording sessions in exactly the

same manner as the test animals but without any HFS. We found no change in HDAC1 nor HDAC2 activity in the HFS hemisphere, in which LTP had been induced, at any time point when measured as a fold change between the HFS hemisphere and the contralateral, non-HFS hemispheres of the test animals (Paired t-test; $p>0.05$. Fig. 3.3).

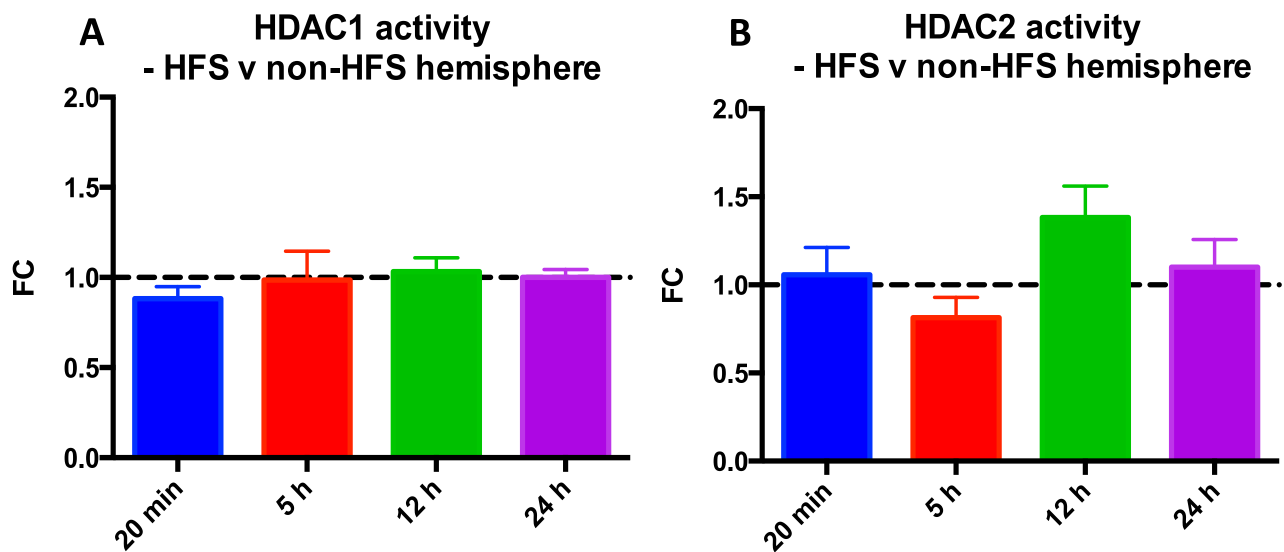

Fig 3.3 HDAC1 and 2 activity over 24 h post-LTP induction, relative to control hemisphere. HDAC activity was not regulated over time post-LTP induction when measured as a fold change (FC) between the hemisphere in which LTP was induced and the contralateral, non-LTP hemisphere. **A,** There is no difference in HDAC1 activity between hemispheres at any time point after LTP induction. **B,** There is no difference in HDAC2 activity between hemispheres of test animals at any time point after LTP induction. Paired sample t-test. Mean + S.E.M. (n = 5-7 per time-point).

When HDAC1 and HDAC2 activity was measured as a fold change from control animals, both HDAC1 (2.5 ± 0.58, n = 6, one-sample t-test; $p<0.0001$. Fig. 3.4) and HDAC2 (1.97 ± 0.66, n = 7, one-sample t-test; $p=0.003$. Fig, 3.4) activity was significantly increased in the HFS hemisphere, 20 min post-LTP induction. HDAC2 was significantly decreased, 5 h post-LTP induction (0.64 ± 0.16, n = 7,

one-sample t-test; p=0.004. Fig. 3.4). Further, HDAC1 was significantly increased 12 h post-LTP induction (1.22 ± 0.18, n = 6, one-sample t-test; p=0.04. Fig. 3.4). This result fell outside the more restrictive criteria of the adjusted p<0.017 for repeated measures. However, due to the exploratory nature of these experiments we continued with this result as a type 2 statistical error would have been more detrimental to the study than a type 1.

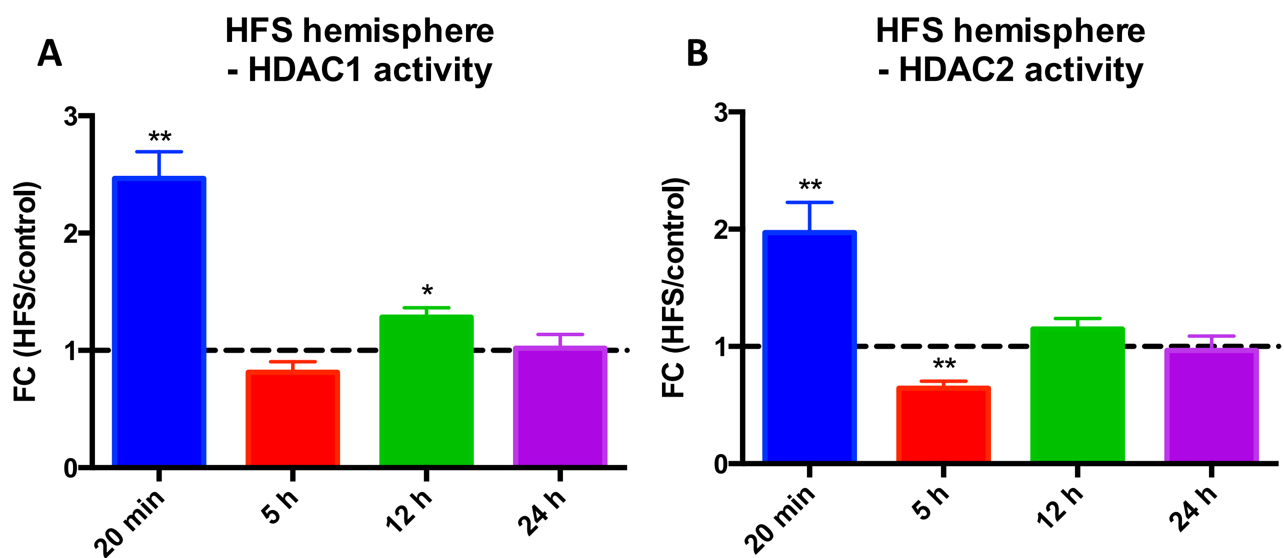

Fig 3.4 HDAC1 and 2 activity over 24 h post-LTP induction, relative to control animals. HDAC activity was modulated over time when measured as a fold change (FC) between the hemisphere in which LTP was induced and control animals. **A,** HDAC1 activity was upregulated 20 min and 12 h post-LTP induction. **B,** HDAC2 activity was upregulated 20 min post-LTP induction and downregulated 5 h post-LTP induction. One sample t-test. Mean + S.E.M. *p<0.05 **p<0.01.

Temporal profile of HDAC1 and HDAC2 activity in the contralateral non-HFS hemisphere post-LTP

The addition of a control group allowed for the measurement of HDAC1 and 2 activity in the contralateral, non-HFS group in which LTP had not been

induced. In this non-LTP hemisphere HDAC1 (2.7 ± 0.47, n = 6, one-sample t-test; p<0.0001) and HDAC2 (1.9 ± 0.24, n = 7, one-sample t-test; p<0.0001) activity was significantly increased 20 min post-LTP induction (Fig. 3.5). HDAC1 showed a strong trend towards a significant increase 12 h post-LTP (1.22 ± 0.07, n = 6, one-sample t-test; p=0.067; Fig 3.5). Further, HDAC2 activity was, very slightly but significantly, decreased 5 h post-LTP induction (0.81 ± 0.07, n = 7, one-sample t-test; p<0.05; Fig 3.5). We identified no physiological change in perforant path-mediated synaptic drive (Fig 3.1) in the non-LTP hemisphere to which we can attribute these changes.

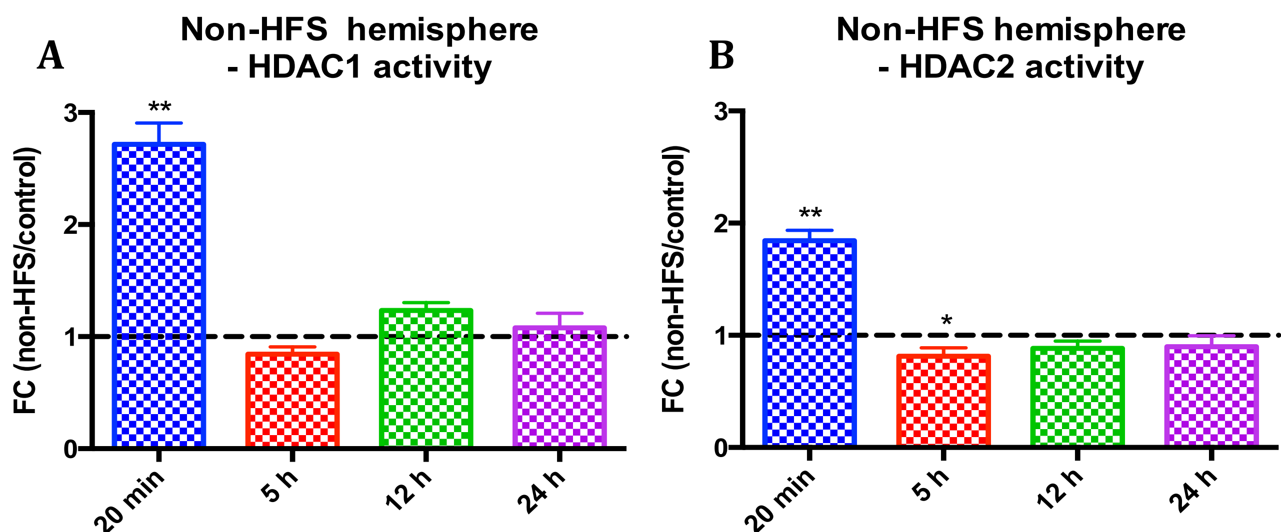

Fig. 3.5 HDAC1 and 2 activity over 24 h post-LTP induction in non-LTP hemisphere, relative to control animals. HDAC activity was modulated over time when measured as a fold change (FC) between the hemisphere in which no obvious LTP was induced and control animals. **A,** HDAC1 activity was upregulated 20 min post-LTP induction. **B,** HDAC2 activity was upregulated 20 min post-LTP induction and downregulated 5 h post-LTP induction. One sample t-test. Mean + S.E.M. *p<0.05 **p<0.01.

3.3.c. HDAC1 and HDAC2 protein expression

Optimizing western blot analysis

Western blotting can comparatively measure the amount of protein present in given samples. Signals of HDAC1 and HDAC2 protein expression (800; red channel) were measured relative to tubulin protein expression (680; green channel) in the same samples, as LTP does not alter tubulin expression (Kennard et al., 2014). Rainbow ladder was used on each blot to assess protein size (kDa). The predicted molecular weight of HDAC1 is 62 kDa and HDAC2 is 55 kDa. There were bands for both HDACs at their predicted sizes. However, there were also particularly strong bands at ~ 38kDa for both, as well as some much larger bands detected with the HDAC1 antibody. These bands could either be indicative of non-specific binding of the antibody or of partial degradation of the proteins of interest. The band at ~ 38 kDa on each blot and the band at the predicted weights of ~ 62 kDa for HDAC1 and ~ 55 kDa for HDAC2 suggested that the HDAC proteins may have degraded. Nevertheless, due to the clear bands just above 52 kDa for both HDAC1 and HDAC2, and due to the fact that standard curves created to test both HDAC1 and HDAC2 antibodies were near linear, the antibodies were deemed suitable for use to assess HDAC1 and 2 protein levels from our LTP samples (Fig 3.6).

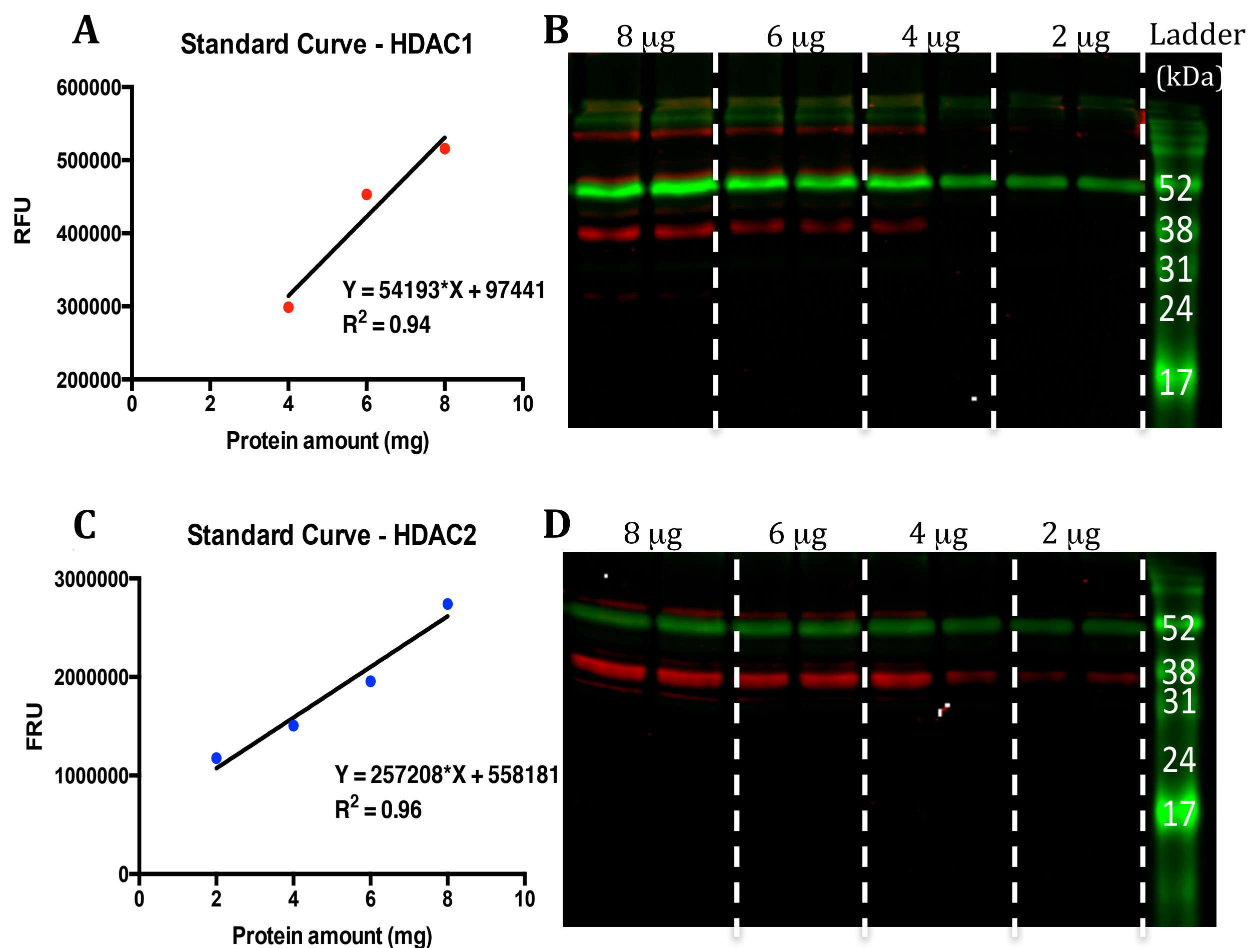

Fig. 3.6 Standard curve analysis of HDAC1 and HDAC2 antibodies. Detecting specific bands and increasing signal intensity, measured as a relative fluorescent unit (RFU) in a linear fashion indicated the reliability of the antibodies to detect change in protein amounts. **A,** Standard curve of 4, 6 and 8 mg of protein, used with HDAC1 antibody. The smallest amount, 2mg was undetectable above background and thus not included. Plotted as average of 2 wells. **B,** Western blot image of HDAC1 standard curve. Red (800) channel measuring HDAC1 antibody, green (680) channel measuring Tubulin antibody. **C,** Standard curve of 2, 4, 6 and 8 μg of protein, used with HDAC2 antibody. **D,** Western blot image of HDAC2 standard curve. Red (800) channel measuring HDAC2 antibody, green (680) channel measuring tubulin antibody.

3.3.d. Temporal profile of HDAC1 and HDAC2 protein expression post-LTP

Some of the protein extracted for the activity assays (section 3.3.b) from tissue dissected 20 min, 5 h, 12 h and 24 h post-LTP induction was also used used for HDAC1 and HDAC2 western blot analysis. When measured as a fold change between the LTP and contralateral, non-LTP hemispheres, as would normally be measured, HDAC1 protein expression was significantly decreased 24 h post-LTP (0.68 ± 0.11, n = 4, paired t-test; p=0.02. Fig 3.7) and HDAC2 protein expression was significantly increased 12 h post-LTP (1.81 ± 0.55, n = 5, paired t-test; p=0.01. Fig. 3.7).

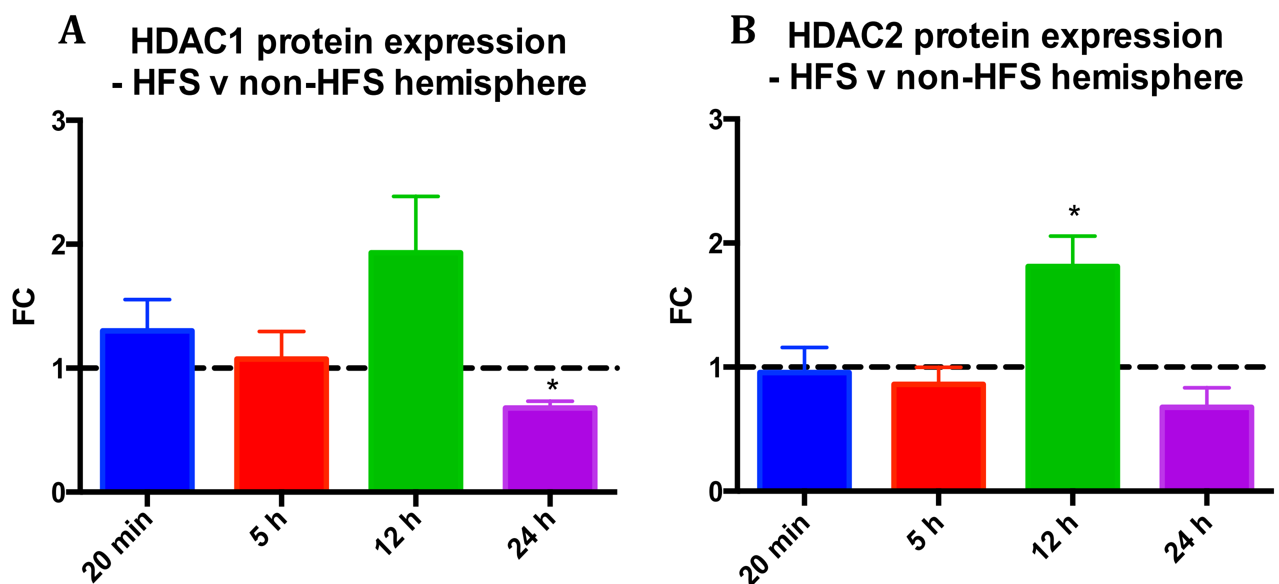

Fig. 3.7 HDAC1 and 2 protein expression over 24 h post-LTP induction, relative to control hemisphere. **A,** HDAC1 protein was downregulated 24h post-LTP induction. **B,** HDAC2 protein was upregulated 12h post-LTP induction. Paired t-test. Mean + S.E.M. *p<0.05.

When HDAC1 and HDAC2 protein expression was measured as a fold change from control animals, there was no change in expression of HDAC1 in the hemisphere in which LTP was induced but there was a significant downregulation of HDAC2 protein expression 20 min post-LTP induction (0.83 ± 0.11, n = 5, one-sample t-test; p=0.03. Fig. 3.8).

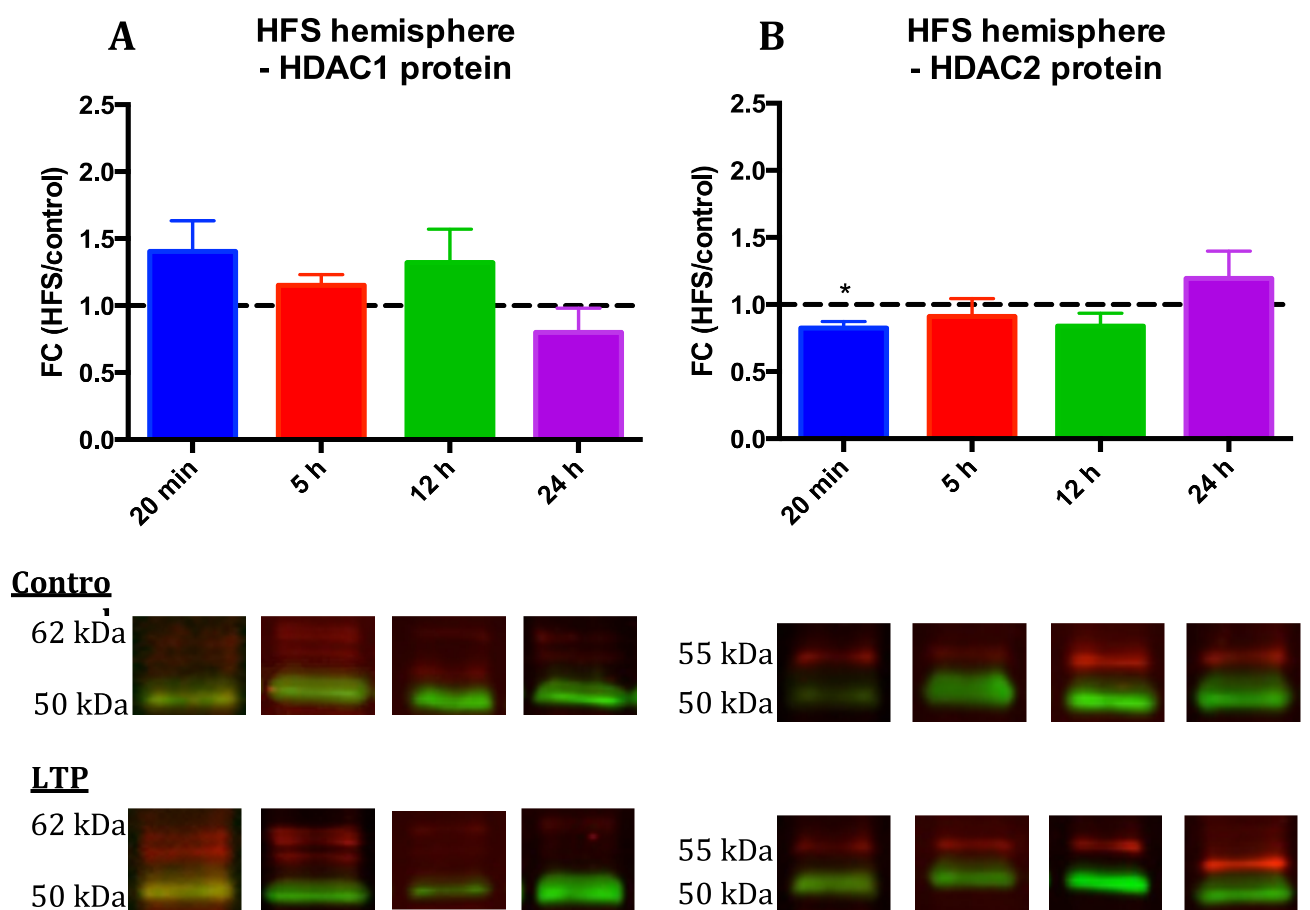

Fig. 3.8 HDAC1 and 2 protein expression over 24 h post-LTP induction relative to control animals. Protein expression over time when measured between hemisphere which received LTP and control animals. **A,** HDAC1 protein expression was unchanged over time. **B,** HDAC2 protein was downregulated 20 min post-LTP induction. Representative images from western blots at each time-point, HDAC of interest in red channel, Tubulin in green channel. Mean + S.E.M. *p<0.05.

3.3.e. Temporal profile of HDAC protein expression in the contralateral, non-LTP hemisphere.

With the addition of the unstimulated control group, the hemisphere of the test animals in which no LTP had been induced was investigated. In this non-LTP hemisphere there was no change in HDAC1 protein expression. However, there was a significant decrease in HDAC2 expression 12 h post-LTP induction (0.47 ± 0.08, n = 5, one-sample t-test; p<0.00. Fig. 3.9) and significantly increased expression 24 h post-LTP induction (1.96 ± 0.57, n = 5, one-sample t-test; p=0.02. Fig. 3.9).

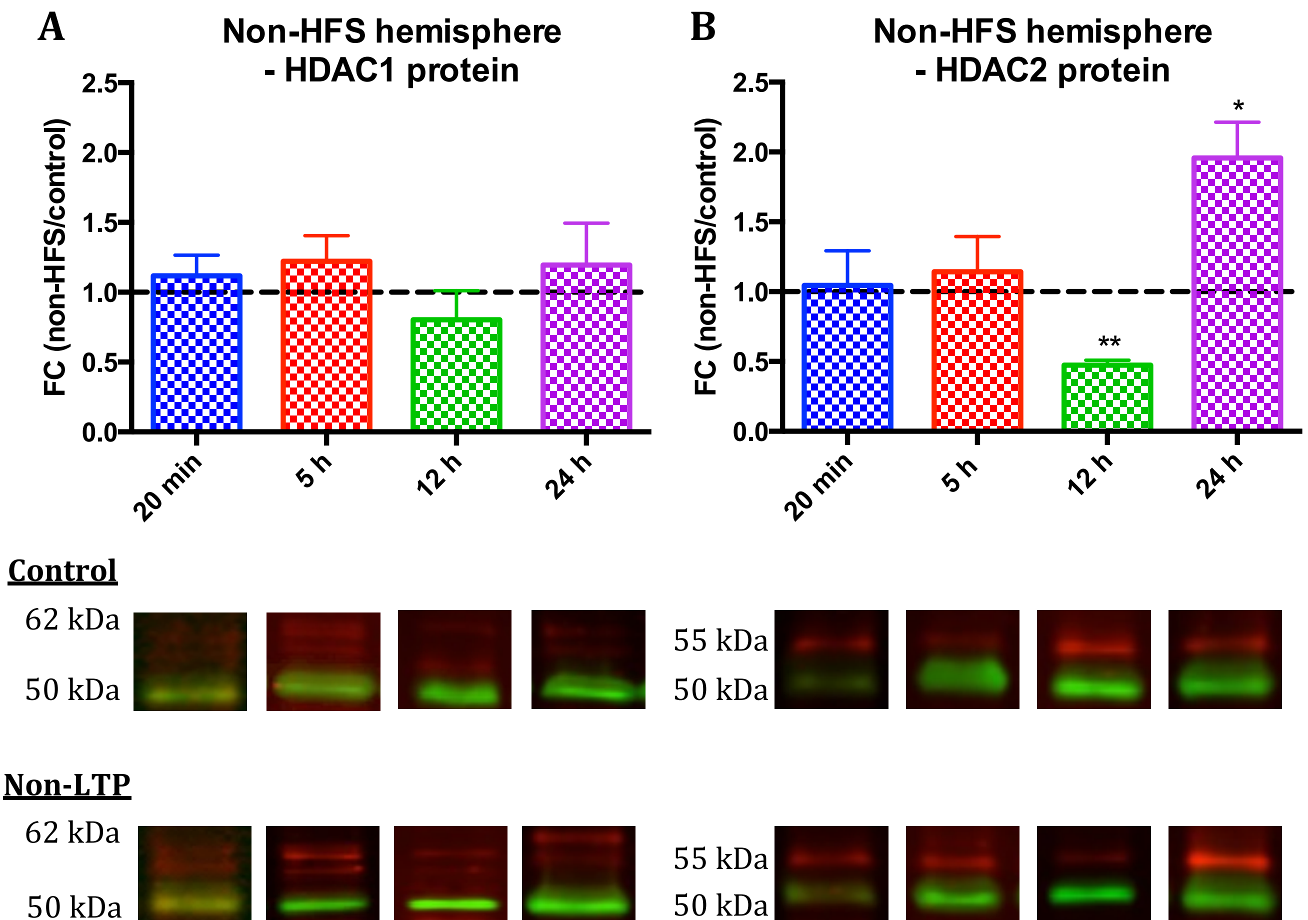

Fig. 3.9 HDAC1 and 2 protein expression over 24 h post-LTP induction in the non-LTP hemisphere, relative to control animals. Protein expression was modulated over time when measured as a fold change (FC) between the hemisphere in which no LTP was induced and control samples. **A,** HDAC1 protein expression was unchanged over time. **B,** HDAC2 protein was downregulated 12 h post-LTP induction and upregulated 24 h post-LTP induction. Representative images from western blots at each time-point, HDAC of interest in red channel, Tubulin in green channel. Mean + S.E.M. *p<0.05 **p<0.01

3.4. Brief discussion

There were two major findings from this set of experiments. The first was that the relative levels of activity and protein expression differed drastically depending on whether the measurement was between the HFS hemisphere and the contralateral, non-HFS hemisphere (within-animal comparison) or whether it was between the HFS hemisphere and control animals (between-animal comparison). When measured within animals, no change was found for either HDAC1 or 2 activity over the entire time course investigated (Fig. 3.3). However, if the HFS hemisphere was measured against a control group, the activity of both HDAC1 and 2 was found to be increased 20 min post-LTP induction. Additionally, HDAC2 activity was found to decrease below baseline by 5 h before returning to baseline (Fig. 3.4). HDAC1 activity was found to drop to baseline at 5 h before a second small increase at 12 h and recovering to baseline at 24 h (Fig. 3.4). The protein expression of both HDAC1 and 2 was also relatively stable over time if measured within test animals, with only a decrease in HDAC1 protein expression at 24 h and an increase in HDAC2 protein at 12 h. However, these changes were entirely lost when expressed as the hemisphere in which LTP was induced compared to control animals, where no statistically significant differences were identified. Accordingly, the decision was to proceed with analysis between test and control animals. Thus, two time-points of increased HDAC activity were identified in relation to LTP. The first was 20 min post-LTP induction, a time-point at which it was hypothesised that HDAC2 activity would be decreased and yet both HDAC1 and HDAC2 activity were significantly increased, while HDAC1 protein expression remained unchanged and HDAC2 expression decreased very

slightly. The second was 12 h post-LTP induction, where HDAC1 activity increased slightly but there was no change in HDAC2 activity, nor was there any change in the protein expression of HDAC1 or HDAC2. Though not at the time-points hypothesised, these exploratory experiments provided two time-points of increased HDAC activity to test the roles that HDAC1 and HDAC2 play in the persistence of LTP. Indeed, inhibiting these HDACs at these two time-points, and evaluating the effect on the persistence of LTP could enable further understanding of what HDAC1 and 2 are doing at each discrete stage (Chapter 5).

The second major finding of these experiments was that LTP in one hemisphere caused dynamic changes in HDAC activity and protein expression in the contralateral, non-HFS hemisphere. HDAC1 and HDAC2 activity was found to be increased 20 min post-LTP induction. HDAC1 activity then returned to baseline over the remaining time-points whereas HDAC2 activity dropped very slightly below baseline at 5 h before returning to baseline at 12 and 24 h (Fig. 3.5). HDAC1 protein expression remained relatively stable over the 4 time-points but HDAC2 protein dramatically decreased 12 h post-LTP induction and significantly increased 24 h post-LTP induction (Fig. 3.9). Had the results for the western blots been reported by comparison within animals, this final result would have been reported as a significant increase in the LTP hemisphere at 12 h. Indeed none of the results from the activity assays and western blots from the LTP and non-LTP hemispheres would have been identified without the addition of the control group. This resulted in the opportunity to extend the hypotheses tested and to question how LTP in one DG affects the contralateral DG. Thus, the

contralateral DG could be used to test the effect of heightened HDAC activity on the subsequent induction of LTP (Chapter 4).

4. Effect of enhanced HDAC1 and HDAC2 activity on LTP persistence.

4.1. Introduction

Routinely, molecular mechanisms investigated in relation to unilateral LTP *in vivo* have used the contralateral, non-LTP hemisphere as a within-animal control to try to reduce between animal variability. However, in Chapter 3, we identified changes in HDAC1 and 2 protein expression and activity in the contralateral hemisphere, despite there being no clear physiological change occurring in the fEPSP or population spike. In particular, our findings suggested that HDAC1 and HDAC2 activity were significantly increased in both the HFS hemisphere in which LTP was induced and the non-HFS hemisphere compared to control, 20 min post-LTP induction (c.f. Fig. 3.5).

There are at least two ways in which stimulation of the PP may lead to activation of the contralateral DG without evidence of LTP in the medial PP on that side. First, there is a direct but small cross-projection from the PP to the outer two thirds of the contralateral DG molecular layer (Goldowitz et al., 1975; Steward & Scoville, 1976). The density of these projections seems to have a dorsal to ventral gradient (Goldowitz et al., 1975; Steward & Scoville, 1976). The LTP induced in the animals used in Chapter 3 was from the dorsal region of the DG and samples used in the activity assays and western blot analysis of protein expression, were specifically from that region. The other potential mode of communication between hemispheres is the mossy cells of the DG region. Mossy

cell bodies, and the majority of their dendrites, are located deep in the hilus of the DG (Ribak et al., 1985) and are activated by granule cell mossy fibres (Scharfman, 2016). Axons from mossy cells project to the inner molecular layer of both the ipsilateral and contralateral DG but also to a number of inhibitory interneurons in the molecular layer (Ribak et al., 1985) (c.f. Fig. 1.2). Thus PP stimulation could indirectly excite contralateral DG cells via activation of mossy cells or directly cause excitation via the crossed PP.

The discovery of increased HDAC activity in the contralateral hemisphere provided a unique environment in which to the study enhanced HDAC activity, and thus presumed enhanced repression of transcription, *in vivo* in awake freely moving, healthy animals. Previous studies which have investigated environments of enhanced HDAC activity have done so using HDAC1 and 2 overexpressing animals (Guan et al., 2009), or using disease models which overexpress HDAC2 (Fischer et al., 2007; Gräff et al., 2012). Due to the chronic nature of these studies, significant changes to synaptic structure and function (Fischer et al., 2007; Gräff et al., 2012; Guan et al., 2009) may obscure any acute effects of the regulation of HDAC1 and 2 activity.

4.2. Hypothesis

The hypothesis tested was that increased HDAC activity in the contralateral hemisphere, 20 min post-LTP induction, would restrict the capacity for gene expression that is critical to the persistence of LTP and thus inhibit the induction of persistent LTP, without affecting the induction of E-LTP. This was tested by

inducing LTP in the first hemisphere (LTP_{hemi1}) followed, 20 min later, by LTP induction in the contralateral hemisphere ($LTP2_{hemi2}$). Additionally, a control group of animals were used in which LTP was only induced in one hemisphere so as to determine any interhemispheric effects of bilateral LTP induction.

4.3. Results

LTP of the expected magnitude (31.6% ± 8.9, n = 7; Fig. 4.1) was induced in the unilateral control group of animals (LTP_{unilat}). In bilaterally treated animals, LTP was induced in the first hemisphere (LTP_{hemi1}) (42.3% ± 12.1, n = 5; Fig. 4.1) and 20 min later this was followed by a second LTP induction in the contralateral hemisphere (LTP_{hemi2}) (23.2% ± 10.1, n = 5; Fig. 4.1). Upon visual inspection of these results, there was an apparent difference between the magnitude of the LTP induced by the second HFS (LTP_{hemi2}) compared to the first (LTP_{hemi1}) and indeed this difference was significant (paired t-test; p=0.003). When the persistence was followed for 21 days, while the decays of LTP_{hemi2} and LTP_{unilat} were similar, (both remained above baseline at the final time-point of 21 days), the LTP_{hemi1} however decayed more rapidly, dropping to baseline by day 9 (ANOVA with repeated measures: Quadratic Time*Treatment (Greenhouse-Geisser corrected); $F_{(2,522.679)} = 9.493$, p=0.002; Fig. 4.1).

By contrast, a similar increase in population spike amplitude was induced in all three conditions (LTP_{hemi1}; 235% ± 138.9, n = 5; LTP_{hemi2}; 219% ± 68.3, n = 5; LTP_{unilat}; 199% ± 55.4, n = 7; Fig. 4.1). All three groups decayed at the same rate over the 21 days in which plasticity was followed (ANOVA with repeated measures: p>0.05; Fig. 4.1).

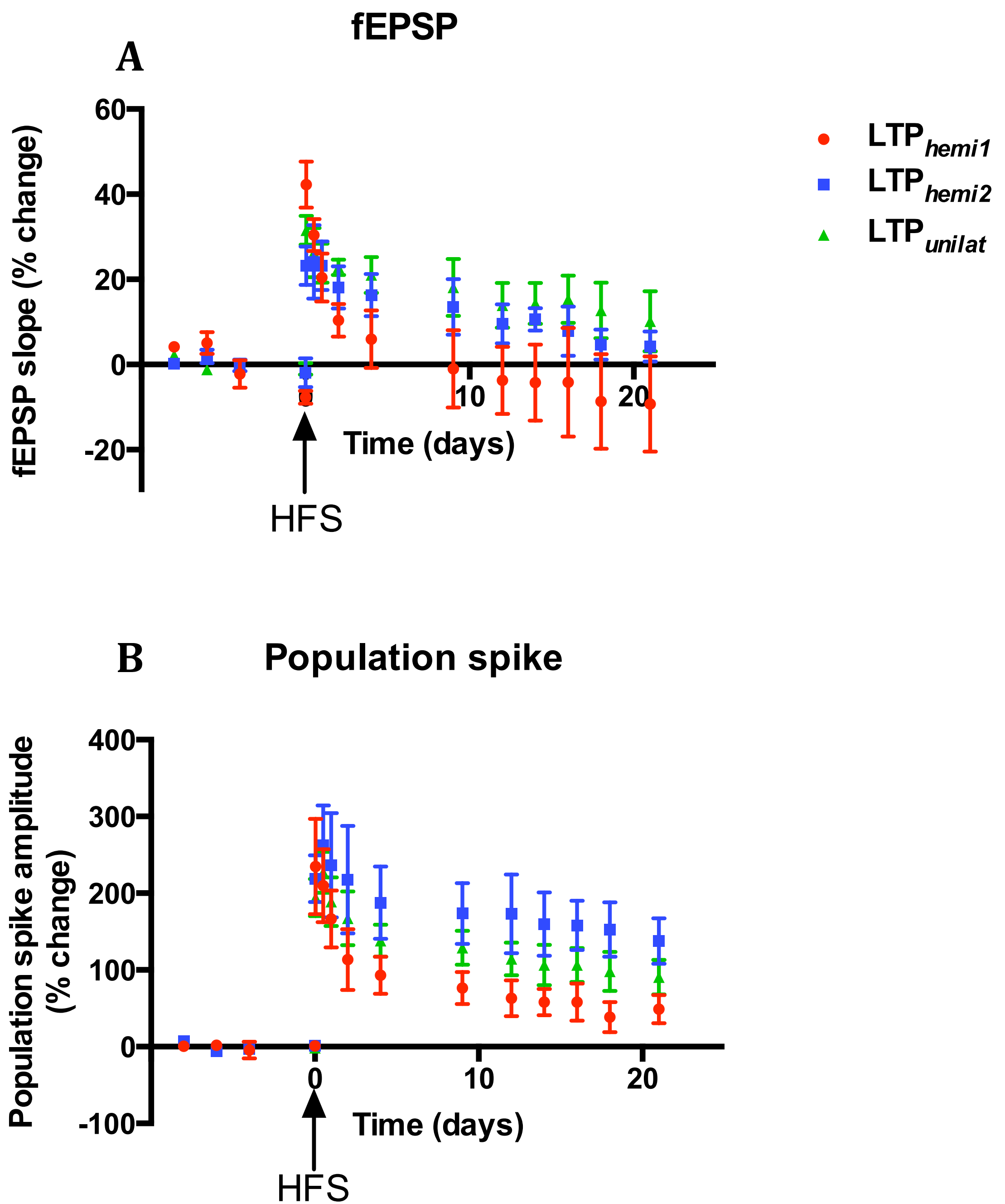

Fig. 4.1. The effect of bilateral (*n* = 5) and unilateral (*n* = 7) LTP induction. A - fEPSP slope percent change from baseline over 22 days following LTP induction. B – Population spike amplitude percent change from baseline, over 22 days following LTP induction. Mean ± S.E.M.

4.4. Brief discussion

HDAC1 and 2 function negatively regulate gene expression by closing down the chromatin around regions of DNA and limiting transcription factor access (Gräff et al., 2011). HDAC2 in particular has been shown to regulate a number of genes essential for LTM and persistent LTP (Gräff et al., 2012; Guan et al., 2009). Thus, an environment of enhanced HDAC activity, such as we identified in the non-LTP hemisphere 20 min post-LTP induction (Fig. 3.5), was hypothesised to inhibit the ability to induce persistent LTP. To test this, we induced unilateral LTP, as was done in Chapter 3, LTP_{hemi1}. Next, LTP was induced in the contralateral hemisphere of the same animals, LTP_{hemi2}, 20 min later. LTP was also induced in a control group of animals (LTP_{unilat}). Contrary to our hypothesis, though the magnitude of LTP_{hemi2} upon induction was almost half of LTP_{hemi1}, it persisted just as long as LTP in the control animals. Surprisingly however, LTP_{hemi1} was detrimentally affected by stimulation of the contralateral LTP_{hemi2} and decayed rapidly. As early as day 3, the LTP_{hemi1} had dropped lower than the other groups and was back to baseline by day 9. These data support the notion that there are interhemispheric effects between each DG after *in vivo* LTP induction however due to a slightly unstable baseline prior to LTP induction, these results are viewed with caution and additional *n*'s are needed to confirm these results. It was also interesting that the fEPSP was effected but that the population spike was not significantly. This suggests some divergence between the intrinsic plasticity and synaptic plasticity and that the depotentiation of LTP_{hemi1} is via a synaptic plasticity mechanism alone. Nevertheless, the fact that the environment in which LTP_{hemi2} was induced (high HDAC activity) only

affected the magnitude of LTP upon induction and had no effect on persistence suggest that the HDAC activity assays may be indicative of HDAC activity balancing high HAT activity and maintaining a relatively neutral state of acetylation overall.

5. Effect of HDACi on the persistence of LTP

5.1. Introduction

HDAC1 and 2 have been found to be independently and dynamically regulated over 24 h following LTP induction *in vivo*. The activity of both HDACs increased 20 min post-LTP induction and HDAC1 activity increased again 12 h post-induction (see Section 3.2). Though the previous findings of LTP-associated changes in HDAC1 and HDAC2 mRNA expression (Ryan et al., 2012) were found when comparing between HFS and non-HFS hemispheres, and we have identified changes in HDAC activity in both of these hemispheres, as well as HDAC1 and 2 protein expression found for the most part in the non-HFS hemisphere (see Section 3), there is sufficient evidence to suggest that both HDACs may play a role in the maintenance of LTP.

Inhibition of HDAC activity at the time of LTP induction, by either knockdown (Gräff et al., 2012), knockout (Guan et al., 2009; Morris et al., 2013) or by using the HDACis TSA or NaBut (Levenson et al., 2004; Vecsey et al., 2007), enhances the magnitude and maintenance of LTP in CA1 slices. These studies are limited by the loss of anatomical integrity in slice preparations and the very short duration (maximum 3 h in these experiments) of recording. However the changes in gene expression and HDAC regulation *in vivo* occur over at least a 24 h period post-induction (Ryan et al., 2012), and thus 3 h is too early to detect any effects of the HDAC expression at 5 h and 24 h on LTP persistence. Further,

HDACi's have been proposed to enhance LTM and are being investigated as treatment options for diseases of impaired memory formation such as Alzheimer's disease (Gräff et al., 2012; Gräff & Tsai, 2013). However, assessment of LTM has been completed for the most part by 24 h post-learning (Fischer, Sananbenesi, Wang, Dobbin, & Tsai, 2007; Gräff et al., 2012; Guan et al., 2009; Levenson et al., 2004; Morris, Mahgoub, Na, Pranav, & Monteggia, 2013; Vecsey et al., 2007). Much like the *in vivo* LTP experiments, changes in HDAC1 and HDAC2 gene expression is ongoing at 24 h and thus assessing LTM at that time may be premature when trying to establish the affects HDAC1 and HDAC2 have on the formation and maintenance of LTM. Utilizing *in vivo* LTP to follow its persistence over weeks, we can assess the effect of using an HDACi both at 20 min, when we found increased activity of both HDAC1 and HDAC2 and also at 12 h when we found increased activity of HDAC1. This will provide considerably more detail about the roles HDACs play in the persistence of LTP and by extension memory. If these HDACs do in fact play a role maintaining LTP, then the use of HDACi's as a treatment for neurodegenerative disorders may need to be reconsidered.

Even if HDAC activity alone has no effect on the persistence of LTP, we have hypothesised that it may still have a metaplastic effect on the ability for subsequent activity to induce plasticity. In accordance, we predict that an upward shift in the threshold for plasticity assists in the maintenance of the previously established connectivity. LTP of the PP-DG synapses can be induced *in vivo* using a variety of stimulation protocols, leading to LTP1 (with an average decay rate of ~2 h), 2 (with an average decay rate of ~3.5 days) and 3 (with an

average decay rate of ~20.5 days) (Abraham, 2003). Indeed, one such protocol can induce LTP lasting for months and even up to a year (Abraham et al., 2002). Stimulation of the major excitatory inputs to the DG, the medial PP or lateral PP fibres (White, Nadler, Hamberger, Cotman, & Cummins, 1977), or a mixture of both can induce LTP at the DG (McNaughton & Barnes, 1977). Arising from layer II of the entorhinal cortex, the medial PP projects to the middle molecular layer of the DG whereas the lateral PP project to the outer molecular layer (Hjorth-Simonsen, 1972; Hjorth-Simonsen & Jeune, 1972). When either the medial PP or lateral PP synapses are potentiated individually, the other pathway undergoes heterosynaptic depression (Abraham & Goddard, 1983). When heterosynaptic depression of the lateral PP is induced, it has generally been found to return to near baseline by 2 days post-induction (Abraham, Mason-Parker, Bear, Webb, & Tate, 2001) though it can last longer than 20 days depending on the protocols used (Abraham, Christie, Logan, Lawlor, & Dragunow, 1994). Nevertheless, even if the LTD of the lateral PP has decayed to baseline, or if it is de-depressed by HFS, subsequent LTP of the lateral PP is inhibited until 28-35 days post-heterosynaptic depression (Abraham et al., 2001). This suggests that there is a metaplastic shift in the plasticity of these synapses which prevents their expression of LTP (Abraham et al., 2001). This effect provides a model for which to test whether HDAC1 and 2 play a metaplastic role in the maintenance of LTP.

The HDACi TSA has been used for a number of LTP experiments *in vitro* but has also been used *in vivo* to inhibit HDAC activity for assessment of LTM, by either infusion into the hippocampus (Blank et al., 2014; Hawk, Florian, & Abel, 2011; Vecsey et al., 2007) or by intraperitoneal injection (Korzus et al., 2004;

Vargas-Lopez, Lamprea, & Munera, 2016). Our group has tried another common HDACi, NaBut, but found the animals to suffer from severe drowsiness and lethargy for a period of time, combined with a reduction in the field potential amplitude (Grattan and Abraham, unpublished observation). The use of an HDACi, as opposed to a short-hairpin RNA (shRNA) against one of the HDACs, allows timing of the administration of the drug to target the temporal window of interest which would be lost with a longer term or chronic inhibition. In previous studies in which the efficacy of the injection has been assessed, the level of H3 acetylation was measured in response to the inhibitor by immunohistochemistry or western blot analysis (Korzus et al., 2004; Vecsey et al., 2007). Acetylation of H3, and in particular lysine 14 (acH3K14), has been found to be regulated by learning and plasticity (Chwang, Arthur, Schumacher, & Sweatt, 2007; Chwang, O'Riordan, Levenson, & Sweatt, 2006; Korzus et al., 2004; Levenson et al., 2004). Further, acetylation of lysine 9 and 14 on H3 (acH3K9/K14) has previously been shown to be regulated by TSA (Lopez-Atalaya, Ito, Valor, Benito, & Barco, 2013). Injections of 1 – 2mg/kg, i.p., have been shown to increase acetylation between 2 (Sng, Taniura, & Yoneda, 2005) and at least 6 h (Korzus et al., 2004) post-injection.

5.2. Hypotheses

Based on prior *in vitro* LTP studies, we hypothesised that:

1. HDAC inhibition targeting the increased HDAC1 and 2 activity 20 min post-LTP induction would enhance the persistence of the LTP induced.

Due to our finding of increased HDAC1 activity 12 h post-LTP induction and our overall hypothesis that HDAC activity is critical to the persistence of LTP, we hypothesised that:

either

2. HDAC inhibition targeting the increased HDAC1 activity 12 h post-LTP induction would disrupt the maintenance of LTP, causing it to decay rapidly to baseline.

or

3. HDAC inhibition, targeting the increased HDAC1 activity 12 h post-LTP induction would render the cells more plastic and readily adaptable. Thus when challenged by subsequent plasticity induction at a different pathway, the established LTP would be susceptible to change because of this disruption and decay back to baseline, while the new LTP would be induced more strongly.

To test hypothesis 1 and 2:

TSA was injected at the appropriate time-point to target the increased HDAC activity and LTP was followed for at least 21 days.

To test hypotheses 3:

LTP was induced in the medial PP, thereby simultaneously inducing heterosynaptic depression in the lateral PP. This was followed by TSA injection 8 h later and lateral PP HFS 4 h after that (i.e. 12 h after the initial HFS to the

medial PP). The threshold for LTP induction is raised for ~ a month after heterosynaptic depression in vivo (Abraham et al., 2001). Thus, the hypothesis that TSA could enhance plasticity and overcome a metaplastic blockade of LTP induction would allow for LTP to be induced in the lateral PP. Moreover, this would also lead to destabilization of the medial PP LTP, causing it to decay rapidly to baseline.

5.3. Results

5.3.a. TSA increases acetylation in the DG

The effect of TSA on the level of acH3K9/K14 was assessed using immunohistochemistry. TSA injection, at a dose of 2 mg/kg, had no detectable effect on acetylation 20 min (n = 1, 3 slices per animal) or 1 h (n = 1, 3 slices per animal) post-injection but significantly increased the level of acetylation 4 h post-injection in the DG, relative to vehicle-treated controls (2.3 ± 0.7, n = 3, 3 slices per animal, unpaired t-test; p=0.03) (Fig. 5.1). Thus 2 mg/kg, 4 h prior to the intended target time point was used for the remainder of the experiments.

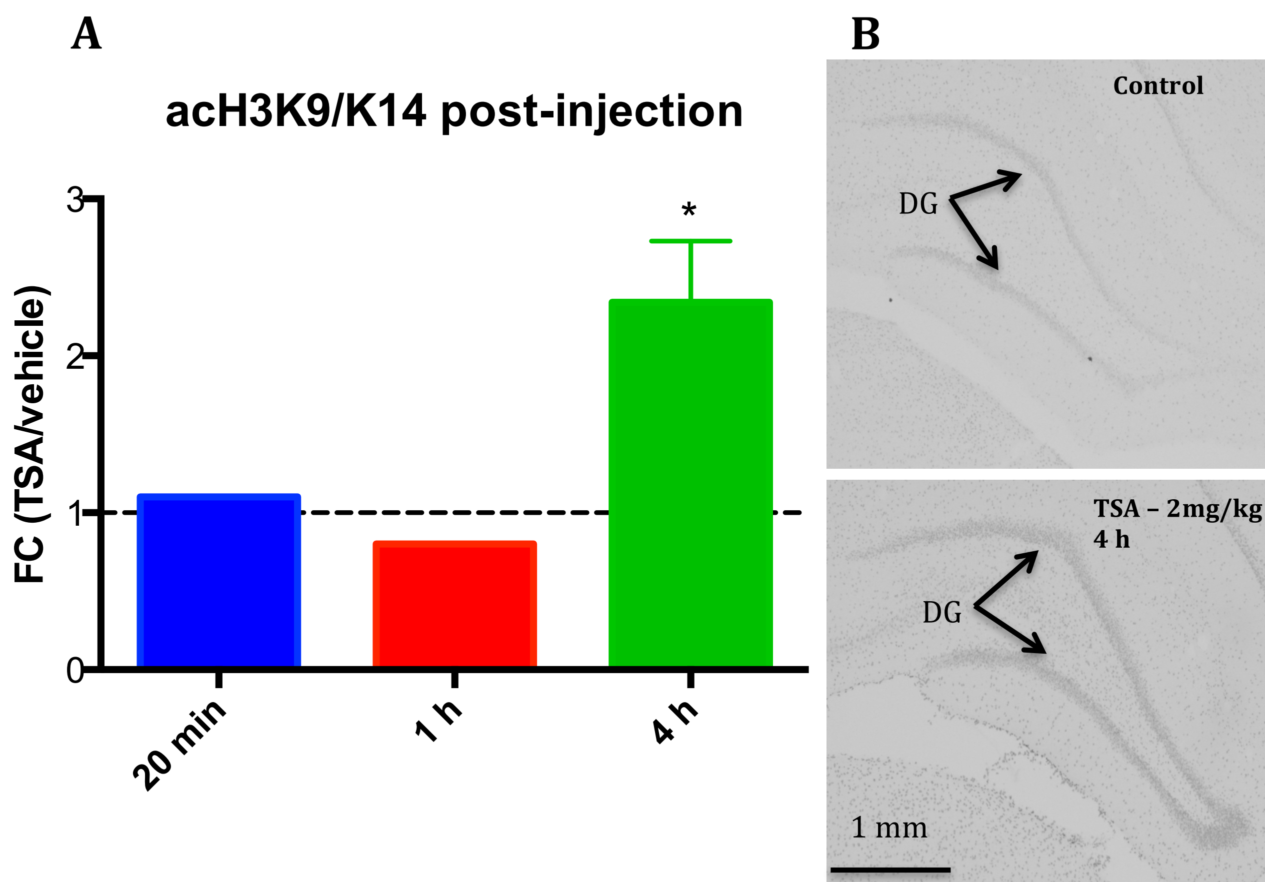

Fig. 5.1 Time course of the effect of TSA i.p. (2mg/kg) injection on the level of acetylation in the DG. **A,** TSA had no effect on the level of acetylation 20 min or 1 h post-injection, but significantly increased acetylation 4 h post-injection. Measured as a fold change (FC) from control animals treated with vehicle at the same time points, mean ± S.E.M. **B,** Representative images of the DG of control animal treated with vehicle and TSA treated animals. *$p<0.05$.

5.3.b. HDACi targeting the 20 min increase in HDAC activity, potentially enhances LTP2

To explore the effect of inhibition of HDAC activity 20 min post-LTP induction, TSA was injected 3 h 40 min prior to induction (refer Fig. 5.1). Due to the injection prior to LTP induction, we can not rule out there being effects of TSA before or after the 20 min time-point. Nonetheless, there was no significant difference between TSA and vehicle-treated control groups when measuring the

fEPSP (Fig. 5.2 A) and population spike (Fig. 5.2 B) over 22 days (ANOVA with repeated measures: $p>0.05$).

On visual inspection of the data, there was an apparent divergence between groups during the first days after LTP induction, where the TSA-injected group increased further over the 12 h post-LTP whereas the vehicle injected group decreased. Thus, the timeframe of LTP was split into previously described classifications based on decay rates (Abraham, 2003). LTP1 was defined as induction to 5 h, LTP2 was defined as 12 h to 7 days and LTP 3 was defined as 10 days to 22 days. Analysis of LTP2 times revealed an extremely strong trend towards a significant effect of treatment (ANOVA with repeated measures: between subject effects; $F_{(1,978.062)} = 5.401$, $p=0.037$; Fig. 5.2 C). There was no significant difference between groups, nor a significant group by time interaction during LTP1 (ANOVA with repeated measures: $p>0.05$) or LTP3 (ANOVA with repeated measures: $p>0.05$).

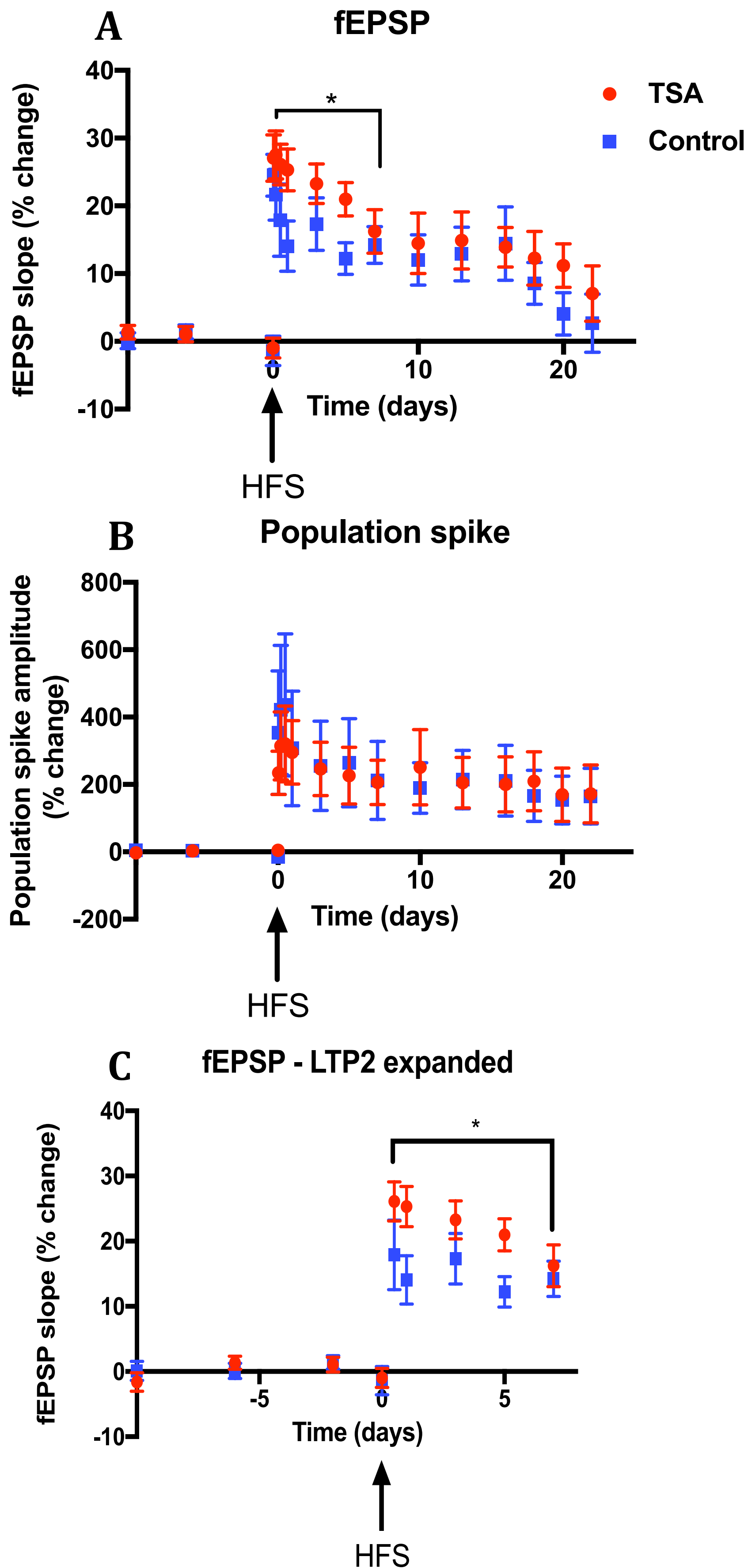

Fig. 5.2 The effect of TSA (*n* = 8) or vehicle (*n* = 7) injection 3 h 40 min, prior to LTP induction. **A,** fEPSP slope percent change from baseline over 22 days following LTP induction. **B,** Population spike amplitude percent change from baseline, over 22 days following LTP induction. **C,** fEPSP measurement, percent change from baseline, of data in **A,** during LTP2 timeframe only (12 h – 7 day post-LTP induction). Mean ± S.E.M.

5.3.c. HDACi targeting the 12 h increase in HDAC1 activity had no effect on LTP

Inhibiting the increased HDAC1 activity found 12 h post-LTP induction (see Fig. 3.4) by injecting TSA 8 h post-LTP induction, had no effect on the persistence of LTP (Fig. 5.3 A) (ANOVA with repeated measures: $p>0.05$), nor did it have any effect on the population spike potentiation (ANOVA with repeated measures: $p>0.05$) over 25 day post-LTP induction (Fig. 5.3 B).

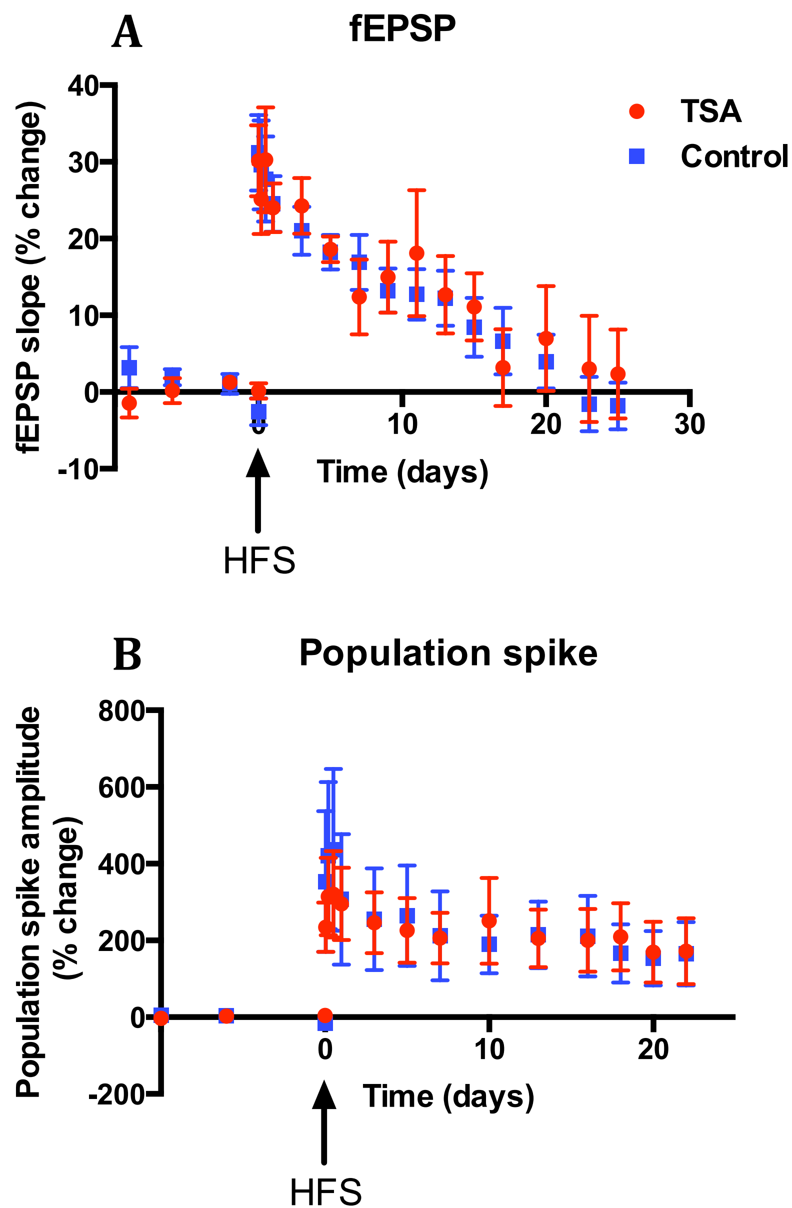

Fig. 5.3 The effect of TSA ($n = 7$) or vehicle ($n = 8$) injection 8 h post-LTP induction. **A,** fEPSP slope percent change from baseline over 25 days following LTP induction. **B,** Population spike amplitude percent change from baseline, over 25 days following LTP induction. Mean ± S.E.M.

5.3.d. TSA does not regulate the metaplastic inhibition of lateral PP LTP after medial PP LTP

While HDAC inhibition 12 h post-induction had no effect on LTP persistence alone, we hypothesised the injection may have made the LTP susceptible to disruption by activity at other inputs. Thus, the metaplastic inhibition of lateral PP LTP after medial PP LTP, as well as the stability of LTP at medial PP synapses, was tested using TSA (Fig. 5.4).

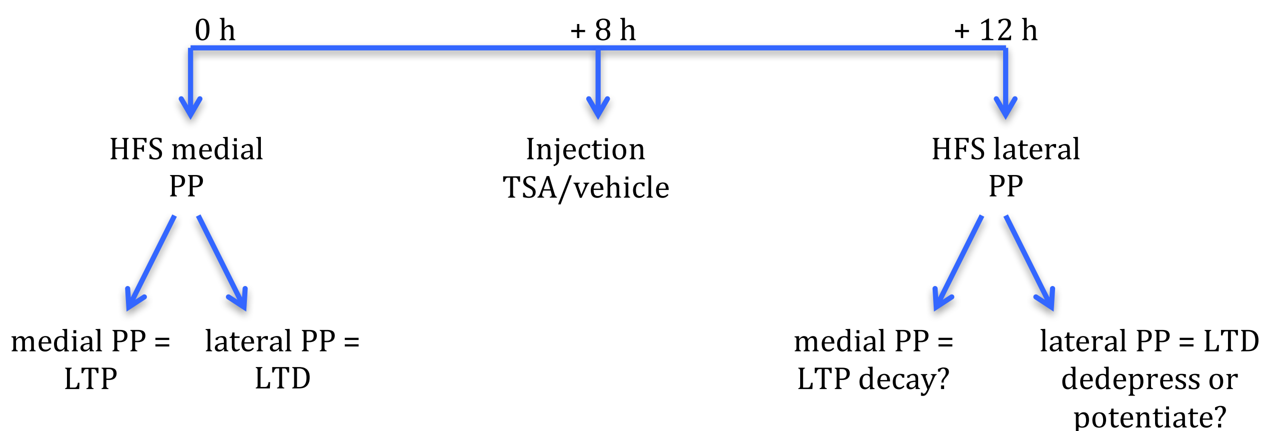

Fig. 5.4 Timeline of experiment testing metaplastic regulation of LTP maintenance. 1. HFS of the medial PP induces LTP at medial PP synapses, and heterosynaptic depression of lateral PP synapses. 2. Injection of TSA or vehicle, 8 h post-HFS of medial PP. 3. HFS of the lateral PP, 12 h post-HFS of medial PP. Decay of both medial PP and lateral PP LTP/LTD followed for 22 days.

LTP of the medial PP was induced to the same degree in both the TSA treated group (30.4% ± 5.4, n = 7) and vehicle treated group (32.4% ± 4.4, n = 5). Injections of TSA or vehicle were given 8 h post-medial PP LTP induction. Medial PP LTP measured immediately before HFS of the lateral PP was identical in both the TSA treated (20.0% ± 5.4) and vehicle treated (20.2% ± 4.8) groups. There was no significant difference in the effect of lateral PP HFS on the maintenance of

medial PP LTP between TSA and control groups over the 22 days of recording (ANOVA with repeated measures: $p>0.05$; Fig. 5.5 A).

LTP of the medial PP caused heterosynaptic depression of the lateral PP responses in both the TSA (-16.4% ± 2.5, $n = 5$) and vehicle (-21.4% ± 4.0, $n = 5$) treated groups (Fig. 5.5). Following the injections of TSA or vehicle 8 h post-HFS, the heterosynaptic LTD measured immediately before HFS of the lateral PP was not significantly different between the TSA treated (-13.2% ± 2.3) and vehicle treated (-17.3% ± 1.6) groups (ANOVA with repeated measures; $p>0.05$. Fig. 5.5). There was no significant difference in the effects of HFS on lateral PP responses between TSA and control groups (ANOVA with repeated measures: $p>0.05$; Fig. 5.5 B). However, there was a trend towards the maintenance of depression of the lateral PP, where there was a significant difference between the TSA and vehicle treated groups on the final day of testing (unapired t-tested: $p=0.03$; Fig. 5.5 B). Thus, the TSA did not alter the metaplastic blockade of LTP in the lateral PP but it may have consolidated the depression of the lateral PP, however this result needs further confirmation.

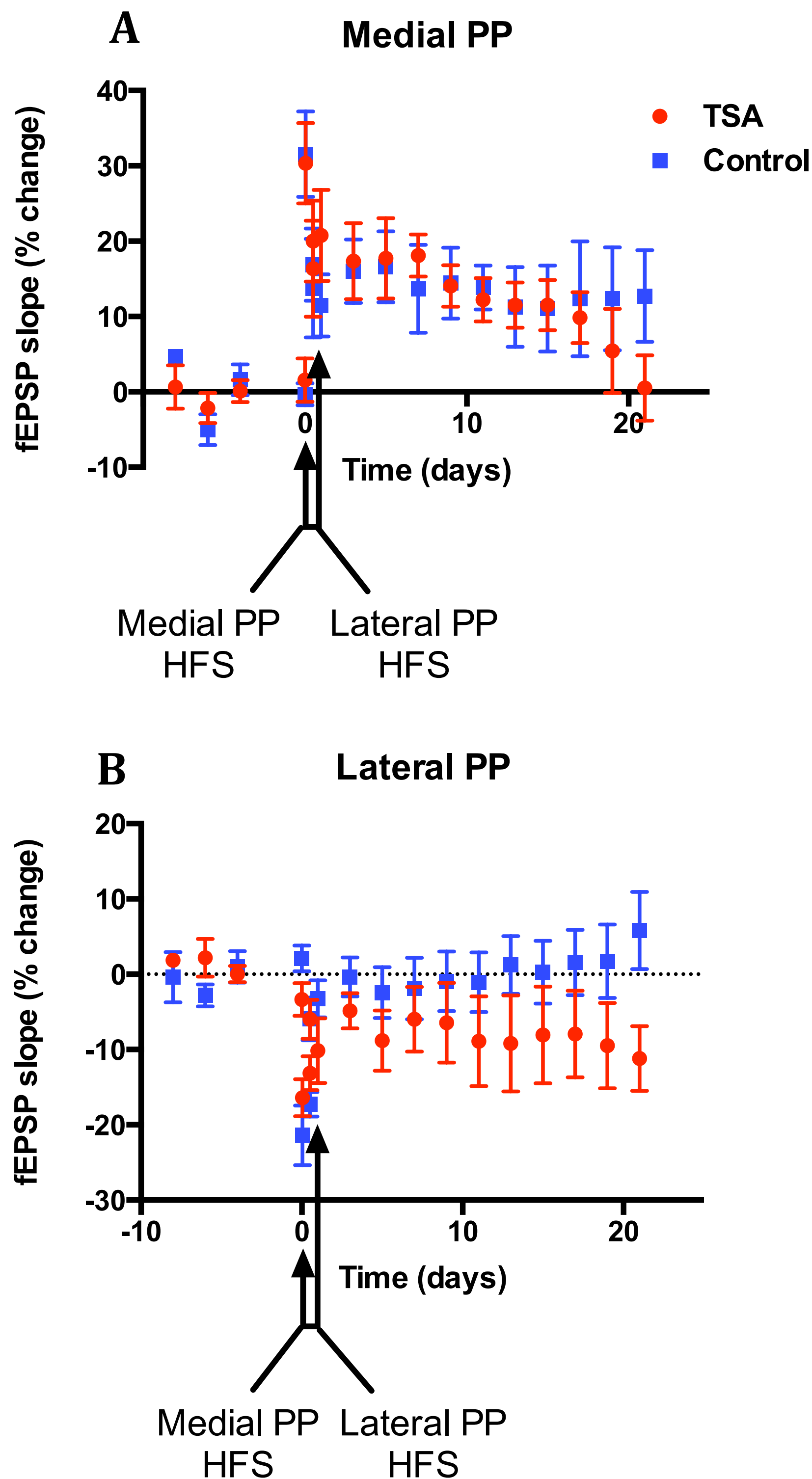

Fig. 5.5 The effect of TSA (*n* = 5) or vehicle (*n* = 5) injection on the ability to induce LTP at the lateral PP and for this to cause disruption to previously established LTP at the medial PP. **A,** fEPSP slope percent change from baseline of medial PP synapses over 25 days following LTP induction. **B,** fEPSP slope percent change from baseline of LPP synapses over 25 days following LTP induction. Data shown are mean ± S.E.M.

5.4. Brief discussion

To test how HDAC activity at distinct phases (20 min and 12 h) post-HFS might be contributing to LTP persistence, HDAC1 and 2 were inhibited using TSA. We first determined that it took 4 h for TSA to have a significant effect on the level of acetylation of H3K9/K14 in the DG after I.P. injection. Thus, in subsequent experiments TSA was administered 3 h 40 min pre-LTP induction, to target the observed 20 min increase in HDAC1 and 2 activity and 8 h post-LTP induction to target the observed 12 h increase in HDAC1 activity.

TSA injection pre-LTP induction did not significantly affect the magnitude of LTP induced, nor the persistence of LTP over 22 days. However, upon visual observation of the data, the magnitude of LTP seemed to be enhanced in the TSA treated group over the first week post-induction (Fig. 4.2 A). Thus, to investigate this more, we divided the LTP into periods of time representing the 3 different phases of LTP that have previously been identified LTP1, 2 and 3 (Abraham, 2003). In analysing the 3 phases separately, we found a very strong trend towards TSA significantly enhancing the magnitude of LTP2 (Fig. 4.2 C). TSA had no effect on the change in population spike amplitude after LTP induction (Fig. 4.2 B), suggesting it does not play a role in the regulation of excitability accompanying LTP. This finding broadens the previous LTP work, completed *in vitro*, as our experiments appear to have the ability to differentiate between LTP2 and 3. Indeed, if our strong trend holds true, it is not the persistence of LTP over weeks that has been enhanced by HDACis as previously reported (Levenson

et al., 2004; Vecsey et al., 2007) but the magnitude of an earlier component of LTP that has been enhanced which is an important distinction when trying to correlate effects on LTP with those on memory.

TSA had no effect on the magnitude or the persistence of LTP when injected 8 h post-LTP induction, nor did it have any effect on the change in population spike amplitude. This suggests that the HDAC1 activity at this time point is not critical for the persistence of LTP or the regulation of excitability at this time-point. Thus, to test whether TSA instead may increase the susceptibility for plasticity, thereby possibly decreasing LTP stability through depotentiation mechanisms, the previously established technique of individually inducing LTP at lateral PP and medial PP synapses was utilized. LTP induction at medial PP synapses (Fig. 4.5 A), as expected, caused an immediate heterosynaptic depression of lateral PP synapses (Fig. 4.5 B). Counter to our hypothesis, the administration of TSA and the subsequent delivery of HFS to the lateral PP had no effect on the maintenance of medial PP LTP, which remained potentiated over 22 days (Fig. 4.5 A). Similarly, the TSA did not significantly affect the ability to de-depress or potentiate the lateral PP synapses (Fig. 4.5 B).

6. Discussion

Considerable evidence supports the notion that LTP enhances connectivity of specific synapses forming neuronal networks, or engrams, and thus memories. However L-LTP induction, like learning, also triggers a number of other synaptic plasticity mechanisms such as heterosynaptic depression and depotentiation as well as changes in excitability mechanisms which regulate cell firing (W. Zhang & Linden, 2003). This coordinated response to activity suggests that a central integration point may control the functional outcomes of plasticity induction. The genome is a prime candidate for this role as gene expression is critical for LTM and persistent LTP. Epigenetic mechanisms can make long term changes to gene expression and thus have been proposed to be master regulators of gene expression that may control the maintenance of memory (Gräff & Tsai, 2013).

The focus of this thesis has been on the negative regulation of gene expression by HDACs, as HDAC2 in particular has been purported to restrain the formation of L-LTP and LTM, since HDAC inhibition has been suggested to promote the formation of L-LTP and LTM (Fischer et al., 2007; Gräff et al., 2012; Guan et al., 2009; Levenson et al., 2004; Morris et al., 2013; Vecsey et al., 2007) and interpretation of LTP-regulated gene networks suggested that HDAC1 and HDAC2 play central regulatory roles (Ryan et al., 2012). From this, I developed the hypothesis that HDAC1 and 2 activity after LTP induction play a role in regulating its maintenance. I proposed that at distinct time-points post-LTP induction, HDAC1 and 2 regulate specific networks of genes needed for the maintenance of plasticity. Further, I proposed that the ability of HDAC1 and 2 to

negatively regulate plasticity related genes rendered them top candidates to maintain LTP because they can inhibit the ability for subsequent activity within the same cells to make changes and disrupt the established network. This hypothesis was tested using *in vivo* LTP as a model for neuronal network formation and maintenance. HDAC activity assays and western blot analysis formed the foundation to establish if and when HDAC1 and 2 were regulated after LTP induction. A bonus to this work came in the form of finding increased HDAC activity in the contralateral hemisphere, leading to an experiment testing the effects of LTP induction in an environment of increased HDAC activity. From there, the necessity of their activity for the persistence of LTP was tested using an HDACi at the specific time points identified in the first experiments.

6.1. HDAC1 and 2 are dynamically regulated after LTP

Though gene expression is initiated immediately upon L-LTP induction (Abraham et al., 1991; Cole et al., 1989; Dragunow et al., 1989; Link et al., 1995; Lyford et al., 1995), the time periods when it is critical for persistent LTP are somewhat dependent upon the preparation (U. Frey, Frey, Schollmeier, & Krug, 1996; Nguyen et al., 1994; Otani, Marshall, Tate, Goddard, & Abraham, 1989). Nevertheless, there is consensus that gene expression is critical around the time of induction (Goelet et al., 1986). Similarly, gene expression has been shown in behavioural experiments to be critical at the time of learning and at subsequent time points over the following 24 h (Bekinschtein et al., 2007; Bekinschtein et al., 2010; Katche et al., 2010). The gene expression profiles initiated upon LTP induction are considerably different to the gene expression profiles 24 h later,

and are predicted to regulate distinct cellular and molecular mechanisms (Ryan et al., 2012). This suggests that over this time course, higher level regulatory mechanisms may determine the expression of specific networks of genes. One such mechanism is the regulation of histone acetylation which controls the ease by which gene expression can occur. HDAC1 and HDAC2 have been predicted by bioinformatics to be central hubs of networks of genes changing their expression at 5 h and 24 h post-LTP induction respectively (Ryan et al., 2012). Due to their ability to make widespread changes to the accessibility of DNA, they appeal as higher-level regulators of gene expression profiles.

To establish whether it was feasible that HDAC1 and 2 could play a role in the regulation of gene expression and LTP persistence, their activity and expression needed to be established over the same time course as our investigations into gene expression. The hypothesis tested was that HDAC2 would decrease immediately after LTP induction, when gene expression is increased and that its activity would gradually increase above baseline by 24 h, when the mRNA expression of HDAC2 is increased but overall net gene expression is decreased (Ryan et al., 2012). Additionally, HDAC1 activity was hypothesised to increase earlier, at the 5 h time-point in accordance with the previously established mRNA expression, and maintain a level of activity that was significantly higher than baseline throughout the subsequent time-points. I hypothesised that the protein expression of both HDAC1 and 2 would follow the increase in activity. To test these hypotheses, HDAC1 and 2 activity and protein expression was measured 20 min, 5 h and 24 h post-LTP induction, in accordance with the mRNA analysis (Ryan et al., 2012). Further, activity and protein expression was

measured 12 h post-LTP induction as subsequent waves of transcription and translation, occurring between 12 and 24 h after learning, have been shown to be necessary for LTM (Bekinschtein et al., 2007; Bekinschtein et al., 2010; Katche et al., 2010; Katche et al., 2012) and this is a time-point that has not been well studied in relation to LTP. An important consideration with regards to these results is that the entire dorsal DG was used to extract protein. This means there is no way of knowing exactly what type of cell from the DG region these changes are occurring in. Indeed, as will be discussed in more detail below, evidence of interhemispheric communication suggests that not only are changes occurring in the granule cells but also mossy cells. Thus, changes measured may indeed be indicative of the net result in changes throughout the region.

All hypotheses tested were false. Instead of decreased HDAC2 activity immediately following LTP induction, a substantial and significant increase in the activity of both HDAC1 and 2 was measured 20 min post-induction. Further, a small but significant increase in HDAC1 activity was found 12 h post-induction, which in part supports the hypothesis of a longer latency increase in HDAC1 activity. However, if this was indicative of a metaplastic shift, it appeared to be short-lived because this increased activity was not sustained at 24 h as was hypothesised. There was no increase in activity of either HDAC1 or HDAC2 at 24 h. There are two ways that these data can be interpreted. The first interpretation is that there is in fact increased HDAC activity at the chromatin, regulating actively transcribed genes. While most of the information regarding HDAC1 and HDAC2 suggest that they negatively regulate gene expression, they have both been found to be specifically recruited to actively transcribed genes (Wang et al.,

2009). Further, the effects of the HDACi TSA are restricted to regions of the genome that are already active such as regions found to be enriched in acH4K9/K14 and trimethylated H3K4 (H3K4me3) at basal levels and indeed these marks were found to be a prerequisite for H4 hyperacetylation in response to TSA (Lopez-Atalaya et al., 2013). Together, these results suggest that HATs and HDACs must be working at the same promoter regions to regulate the balance of acetylation (Lopez-Atalaya et al., 2013; Wang et al., 2009).

The second is that the activity assays are representing potential HDAC activity but not the actual level of transcription inhibition. HDAC1 and HDAC2 are class I HDACs located in the nucleus as part of a number of co-repressor complexes and can be posttranslationally modified in ways that de-repress gene expression (Seto & Yoshida, 2014). There is very little evidence to indicate how, or which, upstream signalling pathways regulate HDAC activity *in vivo* in the brain (Morris & Monteggia, 2013; Seto & Yoshida, 2014). However, *in vitro* nitrosylation of cysteine residues (s-nitrosylation) by nitric oxide is one mechanism that can control HDAC2 in particular (Nott, Watson, Robinson, Crepaldi, & Riccio, 2008). BDNF and Ca^{2+} signalling can lead to the s-nitrosylation of HDAC2, which dissociates it from chromatin leading to increased acetylation at the promoter region of a number genes previously shown to be regulated by HDAC2 such as *fos* and *egr1* and a concomitant increase in the expression of these genes (Nott et al., 2008).

HDAC1 can be phosphorylated by casein kinase 2 (CK2) and PKA at S421 and S423 on its carboxyl-terminal tail (R. Cai et al., 2001; Pflum, Tong, Lane, &

Schreiber, 2001; Tsai & Seto, 2002). HDAC2 is also phosphorylated by CK2 but not PKA, at S394, S422 and S424 (Tsai & Seto, 2002). CK2 activity is regulated by LTP (Charriaut-Marlangue, Otani, Creuzet, Ben-Ari, & Loeb, 1991) and neurotrophins such as BDNF (Blanquet, 1998) and thus it is possible that it may regulate HDAC1 and HDAC2 phosphorylation after LTP induction. However, a challenge in understanding the role phosphorylation plays in HDAC1 and HDAC2 activity stems from debate about whether HDAC1 and HDAC2 are more or less enzymatically active when they are part of their co-repressor complexes (R. Cai et al., 2001; Pflum et al., 2001; Seto & Yoshida, 2014; Tsai & Seto, 2002). Indeed, some evidence suggests that phosphorylation of HDAC2 increases its enzymatic activity, but this phosphorylation has no effect on HDAC2's ability to repress transcription (Tsai & Seto, 2002). An explanation for this separation between HDAC activity and gene repression could be that hyperphosphorylation of HDAC1 and HDAC2 may actually disrupt their corepressor complexes (Galasinski, Resing, Goodrich, & Ahn, 2002). Inhibiting phosphatase activity, most likely protein phosphatase I (PP1), led to increased HDAC1 and HDAC2 enzymatic activity but changed the composition of the corepressor complexes (Galasinski et al., 2002). This led the authors to hypothesise that when HDAC1 and HDAC2 are phosphorylated they become more enzymatically active but this also disrupts corepressor complexes and thus moves the HDACs away from the chromatin, thereby releasing inhibition of gene transcription (Galasinski et al., 2002). However, a more recent study of PP1 *in vivo* found that PP1 knock-out or inhibition decreases HDAC1 activity and increases acetylation at the promoter region of *creb* which leads to an increase in *creb* mRNA expression (Koshibu et al., 2009). This suggests that HDAC1 activity is increased when dephosphorylated by

PP1 and this negatively regulates gene expression. Thus, these data support the hypothesis that phosphorylation may lead to the movement of HDACs away from DNA and release the negative regulation of gene expression but it may not increase its activity. Conflicting evidence however has shown that the inhibition of PP1 also decreases acetylation at the promoter region of *nf-κb* and decreases mRNA expression (Koshibu et al., 2009) suggesting that PP1 may regulate acetylation at different regions, via regulation of different HDACs or other effector proteins, or indeed by regulating phosphorylation of histones themselves. Thus, s-nitrosylation and phosphorylation may, as a result of LTP induced signalling pathways, move HDACs away from the chromatin and, as a by-product of this, increase enzymatic activity. HDAC1 and HDAC2 can regulate other, non-histone proteins such as p53 and NF-κB and the movement of HDAC1 and HDAC2 away from the chromatin may be a mechanism by which this could be achieved. However, the separation of the enzymatic activity and the gene repression function of HDAC1 and HDAC2 has not been experimentally validated. Our measurement of enzymatic activity therefore may, or may not, be indicative of the role HDAC1 and HDAC2 play in the negative regulation of transcription. Further, the contradictory nature of the phosphorylation literature, especially considering none of these actions have been confirmed *in vivo* in relation to LTP induction or learning, renders this a very tentative interpretation and examination of transcriptional regulation by HDAC1 and HDAC2 is needed to confirm these results.

Our activity assay results have added to the growing understanding of how epigenetic mechanisms are dynamically regulated after LTP. Though the

activator or HDAC1 and HDAC2 activity is unclear in this circumstance, and whether the activity is indicative of increased or decreased regulation of gene expression, it seems most probable that the activity is indicative of increased HDAC regulation of gene expression. Indeed, being able to enhance LTP with inhibition of HDAC activity 20 min post-LTP and by having reduced magnitude of the LTP induced in LTP_{hemi2}, it would suggest that HDAC activity is negatively regulating the expression of LTP related genes. However, this does need to be confirmed. Nevertheless, the results did provide two time-points of enhanced activity for which to test specific roles they may play by inhibiting their actions at these times. Whether the activity is indicative of gene repression or indicative of decreased gene repression, the role that either of these actions play in the persistence of LTP is critical to our understanding of the role HDAC1 and 2 play in LTP and memory. Further, these results have added to the understanding of acetylation dynamics *in vivo*, particularly that HDACs are not a straightforward on-off switch and they are rapidly regulated in response to stimulation. This is of particular importance when considering widespread, chronic HDACi treatments as an option for neurodegenerative diseases such as Alzheimer's disease (Gräff et al., 2012). Detailed analysis of the regulation of specific genes by HDAC1 and HDAC2, such as by chromatin immunoprecipitation of DNA, with antibodies against HDAC1 and 2, followed by DNA sequencing would greatly add to the interpretation of these results by indicating which genes are more, or less, regulated by each HDAC at each time-point.

6.2. If HDAC activity is increased 20 min post-LTP induction, and negatively regulating LTP related gene expression, inhibiting the activity should lead to increased LTP.

To test the role of increased HDAC activity 20 min and 12 h post-LTP induction, the HDACi TSA was administered via I.P. injection. TSA is a non-specific class I HDACi which means it inhibits not only HDAC1 and 2 but also HDAC3 and 8. Importantly HDAC1 and 2, like other HDACs, have non-histone targets such as p53, and NF-κB, in particular the p65 subunit (Kelly & Cowley, 2013). Further, TSA can potentially modify transcription factors activity such as by inhibiting HDAC corepressor complex interaction with NF-κB, rather than through histone acetylation per se (Ashburner, Westerheide, & Baldwin, 2001). Acetylation at the promoter regions containing binding sites for the transcription factors NF-κB and EBF1 were found to be the most affected by TSA injection and genes which were upregulated in response to TSA treatment alone were components of the Sin3-HDAC complex (Lopez-Atalaya et al., 2013). Nevertheless, the decision to proceed with TSA, instead of designing an inhibitor specific to each HDAC under investigation (such as an siRNA against HDAC1 and 2) was taken because the temporal specificity of HDAC activity was more pertinent to this investigation than the specificity of the HDAC inhibited. Using viral delivery of a siRNA we would not have been able to specifically target the 20 min and 12 h time-points.

The hypotheses tested with these experiments were that HDAC activity at 20 min and 12 h played an important role in the persistence of LTP. Specifically, we hypothesised that the increased HDAC1 and 2 activity, 20 min post-LTP induction, identified in Chapter 3 was perhaps as a homeostatic mechanism to return gene expression stimulated by LTP induction back to basal levels. This we proposed to be essential to the persistence of LTP so as not to continually express genes involved in the restructuring of the cell which would lead to a lack of specificity at the stimulated synapses. This hypothesis proved to be too simplistic for the interpretation of the results. Injection of the HDACi TSA to target the 20 min increase in HDAC1 and HDAC2 activity led to the very specific enhancement of what appears to be LTP2. The use of *in vivo* LTP has, in this circumstance, enabled this more detailed investigation into the enhancement of LTP by HDAC inhibition. Previous *in vitro* LTP experiments have shown that inhibition of HDAC activity enhanced the magnitude and duration of LTP (Gräff et al., 2012; Guan et al., 2009; Levenson et al., 2004; Morris et al., 2013; Vecsey et al., 2007). However, when investigating LTP *in vitro*, the more refined classifications of LTP1, LTP2 and LTP3 are often not been identified and thus much of the mechanistic discussion of LTP maintenance is limited to whether LTP decays over a matter of hours. Nevertheless, the three categories have been teased apart *in vitro* and shown to depend upon 3 different sources of Ca^{2+} with location specific actions (Raymond & Redman, 2006). Of particular interest is that LTP1 and LTP2 are both dependent upon NMDAR activation at synapses where LTP3 has a significant NMDAR independent component at the cell body, dependent instead upon L-type voltage-gated calcium channels (L-type VGCC) (Raymond & Redman, 2006). Thus, it is possible for L-LTP to be divided into

LTP2 and LTP3. Indeed, STC, which has been discussed as a mechanism of L-LTP for example, may actually be mechanisms of either LTP2 or LTP3 and thus these components could, and should, be manipulated individually to further refine our understanding.

Upon LTP induction, STC mechanism are induced which includes CaMKII setting a tag at activated synapses (Malenka, 2003; Opazo et al., 2010; Redondo et al., 2010), PKMζ acting as a PRP being captured (Sajikumar & Korte, 2011; Sajikumar et al., 2005; Tsokas et al., 2016) to promote the clustering of PSD-95 and aiding in the increase in spine size and the movement of the AMPAR subunit into the potentiated synapse (Shao et al., 2012). Of these proteins involved in STC, all have already been shown to be regulated by HDAC2 (Gräff et al., 2012; Guan et al., 2009). Further, there is strong evidence for HDAC2's involvement in this restructuring of synapses, where HDAC2OE, HDACKO and Ck-p25 mice which overexpress HDAC2 as a by-product, all have large-scale morphological changes of synaptic structure and density (Gräff et al., 2012; Guan et al., 2009; Morris et al., 2013). The vast majority of the STC work has been completed *in vitro* and, therefore, there is little evidence to suggest that STC is a mechanism which maintain LTP for weeks to months and thus is a mechanism of LTP3. Together with our data which suggests that HDAC inhibition may have only enhanced LTP2, we propose that the previously described enhancement of LTP, and the promotion of E-LTP to L-LTP, is actually and enhancement of LTP1 to LTP2, potentially via the addition of the STC mechanism regulated by the 'plasticity' transcriptome, which has been readily induced by inhibition of HDAC activity. This may not, however, promote E-LTP to the longest lasting form of

LTP, LTP3, which appears to be unaffected by the inhibition of HDAC activity and thus regulated by the expression of the 'maintenance' transcriptome or at least not regulated by the 'plasticity' transcriptome. This interpretation calls for a more fluid definition of LTP1, 2 and 3 where mechanisms which have been specifically associated with one classification can be enhanced by another. For example, though LTP2 is independent of gene expression, the expression of genes related to LTP2 can enhance that process. Going forwards, this calls for reconsideration of whether STC, the expression or PRPs and the 'plasticity' transcriptome are one mechanism which can be enhanced by HDAC inhibition and whether an entirely different mechanism underlies LTP3. To address this question, and to identify the maintenance mechanism, work must be complete *in vivo* over a much longer timeframe i.e. later than the 12 h time-point we covered.

6.3. Do the present results fit with the current memory literature?

It has previously been postulated that long-term epigenetic modifications are candidates for LTM storage (Gräff & Tsai, 2013). For the most part, studies have shown that inhibiting HDAC activity, by a range of HDACis including TSA, NaBut, vorinostat and SAHA either via I.P. injection or via intraventricular or intrahippocampal injection, around the time of learning enhances long-term declarative memories when tested at the relatively short time interval of 24 h (Alarcón et al., 2004; Fujita et al., 2012; Levenson et al., 2004; Stefanko et al., 2009; Vecsey et al., 2007). These results have been reported to support the hypothesis that long-term epigenetic modifications, induced by having inhibitors present at the time of learning, are a maintenance mechanism for memories.

However, whether 24 h post-learning should be considered a LTM and if HDAC inhibition at the time of learning does lead to a long-term epigenetic modification (at 24 h or a longer timeframe) is not well substantiated by these experiments. To the best of my knowledge, only one study has investigated the effect HDAC inhibition on memory at longer time intervals (Blank et al., 2014). The HDACi TSA was infused immediately, 1.5, 3 or 6 h post inhibitory avoidance training and memory followed for 21 days (Blank et al., 2014). Intrahippocampal infusion immediately after learning, enhanced memory over 11 days but no longer and infusion 3 h post-learning enhanced memory over 4 days but, again, no longer (Blank et al., 2014). Infusion at neither 1.5 nor 6 h post-learning had any effect on memory (Blank et al., 2014). Another group has found that HDAC2KO animals have enhanced memory at 24 h but no later when followed out to 5 days (Morris et al., 2013). Thus assessing the effect of HDACi on memory at 24 h appears to be too early if trying to establish the effect on LTM (> 24 h), which our LTP results support. Further, no experiments have investigated whether inhabiting HDAC activity at later time-points, such as when they may play a role in establishing a long-term epigenetic marks, have an effect on LTM. Our results, in conjunction with the previous literature, suggest that the long-term maintenance mechanisms of LTP and LTM are still to be elucidated and do not appear to be determined by the level of acetylation or HDAC activity at the time of learning.

6.4. Significance of HDAC activity at 12 h post-LTP

HFS caused a small but significant increase in HDAC1 activity, 12 h post-induction in our experiments. A second wave of protein and gene expression between 12 and 24 h post-learning has previously been shown to be important for LTM (Bekinschtein et al., 2007; Bekinschtein et al., 2010; Katche et al., 2010; Katche et al., 2012; Nakayama et al., 2015). The hypothesis tested here was that the increase in HDAC1 activity managed the appropriate gene expression profiles at this time-point. This hypothesis was not supported, since inhibition of HDAC activity at this time-point had no effect on LTP persistence. The second hypothesis tested was that inhibition of HDAC activity at 12 h would render the LTP susceptible to disruption from other inputs, by allowing the 'plasticity' transcriptome to be induced, therefore causing LTP to decay more rapidly or depotentiate more readily. This was tested by inducing LTP at the medial PP-DG synapses and thereby inducing heterosynaptic depression at the lateral PP-DG synapses. This was followed by injection of TSA targeting the 12 h increase in HDAC activity and the subsequent attempt to induce LTP at the lateral PP-DG synapses. Though the HDAC activity assays had been performed on tissue taken from animals which had received mixed path stimulation, rather than medial PP alone, the LTP induction and persistence was no different in response to the medial PP-alone stimulation than in response to the mixed pathway. TSA had no effect on the persistence of the LTP at the medial PP-DG synapses. Further, it had no effect on the ability to induce LTP or cause de-depression of the lateral PP synapses. Thus HDAC1 and 2 appear to regulate mechanisms involved in plasticity at the time of LTP induction but not at 12 h post-LTP. Therefore, though the exact role of enhanced HDAC1 activity plays at 12 h remains unclear,

our results provide impetus to investigate the role activity at this time-point has in LTP maintenance. Further, the mechanisms which separate LTP2 and 3 may also be temporally dynamic since inhibition at induction seemed to effect LTP2 (from 12 h – 7 days) but inhibition at 12 h did not.

6.5. Interhemispheric communication

A striking finding from the activity assays and western blot experiments was that in addition to HDAC activity being regulated in the DG ipsilateral to the HFS, where LTP was induced, it was also regulated in the contralateral, non-LTP DG. Both HDAC1 and HDAC2 activity was upregulated 20 min post-LTP induction and HDAC2 activity was downregulated 5 h post-LTP in the contralateral DG (Fig. 3.5). Further, HDAC2 protein expression was downregulated 12 h post-LTP and upregulated 24 h post-LTP in the contralateral hemisphere (Fig. 3.9). Neither the fEPSP slope nor the population spike amplitude was changed in the contralateral hemisphere, seemingly indicating that there was no change in the function of dentate cells in the contralateral hemisphere, at least in response to the PP input within that hemisphere (Fig. 3.1). Due to a number of early studies that found IEGs to be upregulated in the LTP hemisphere alone (Dragunow et al., 1989; Dragunow et al., 1993; Jeffery, Abraham, Dragunow, & Mason, 1990), the contralateral hemisphere has since routinely been used as a within-animal control to try to reduce between-animal variability. The microarray analysis from which my study stemmed is an example of this, where the changes in HDAC mRNA expression were found when measuring changes in the LTP hemisphere as a fold change difference relative to the non-HFS hemisphere (Ryan et al.,

2012). However, there have been instances where changes have been observed in the non-LTP hemisphere as well as the LTP hemisphere. Increases in pCREB after LTP induction have been found in both the LTP and non-LTP DG (Schulz et al., 1999) as has the expression of BDNF and trk receptors (Bramham et al., 1996) though this is not always the case. Moreover the mRNA expression of enzymes which regulate the function of neural cell adhesion molecule, a protein important for synaptic plasticity, *stx* and *pst* have been shown to increase bilaterally after unilateral LTP induction (Guiraudie-Capraz et al., 2011). Thus, there have been examples of evidence to supporting a role for the regulation of gene expression in the contralateral hemisphere after LTP induction.

Pathways crossing between hemispheres provide an anatomical basis for how unilateral stimulation may cause bilateral changes post-LTP. The first is a direct cross-projection by the PP to the outer two thirds of the contralateral DG molecular layer (Goldowitz et al., 1975; Steward & Scoville, 1976; Witter, 2007). This projection is not believed to play a major role in the cross-hemisphere effects, however, because the projection is generally considered weak. Further, though this cross-projection is a monosynaptic pathway, it does not seem to potentiate as readily as the ipsilateral pathway does (Levy & Steward, 1979). The second projection, with substantially more evidence to support a possible role in cross-hemisphere effects of LTP, are the mossy cell projections between hemispheres. The mossy cell bodies are located deep in the hilus of the DG, along with the majority of their dendrites. Axons of mossy cells project to the inner molecular layer, innervating granule cell dendrites and inhibitory interneurons

such as basket cells (Fig 1.2). Their projections are both ipsilateral and contralateral.

Of the three of the molecular layers of the DG, the inner molecular layer has been shown to have the most GluN1 expression, indicative of the number of NMDARs, and MAP2 expression (Adams et al., 2001). This has been proposed to enhance plasticity at the inner molecular layer. Indeed in mice, mossy cell-granule cell synapses can be potentiated either by stimulation of the PP alone, or by stimulation of the mossy cell axons at the same time as PP stimulation (Kleschevnikov & Routtenberg, 2003). However, LTP of the mossy cell-granule cells synapses could not be induced by stimulation of the mossy cell axons alone (Kleschevnikov & Routtenberg, 2003). Further, an *in vivo* LTP study, using fMRI and electrophysiology has shown that the mossy cell cross-projections to the contralateral granule cells undergo potentiation after ipsilateral PP-DG LTP (Alvarez-Salvado et al., 2014). Thus, both ipsilateral PP-granule cell synapses and the mossy cell – granule cell synapses (both contralateral and ipsilateral) can undergo potentiation after PP stimulation (Alvarez-Salvado et al., 2014). The use of rats enabled this group to also assess the effects at the PP – granule cell cross-projections which are absent in mice. At these synapses they identified heterosynaptic depression in the hemisphere in which LTP was induced as well as at the mossy cell cross-projections onto the synapses of the granule cells that were potentiated by unilateral stimulation (Alvarez-Salvado et al., 2014). Thus, it is possible that 2 sets of synapses are potentiated and 2 depressed in the ipsilateral DG after LTP induction. The two potentiated are the PP – granule cell and the mossy cell – granule cell synapses. The two depressed are the

contralateral PP and the contralateral mossy cell projections to that DG. Further, in the contralateral DG, the cross-projecting mossy cells can potentiate the granule cells (Fig. 6.1).

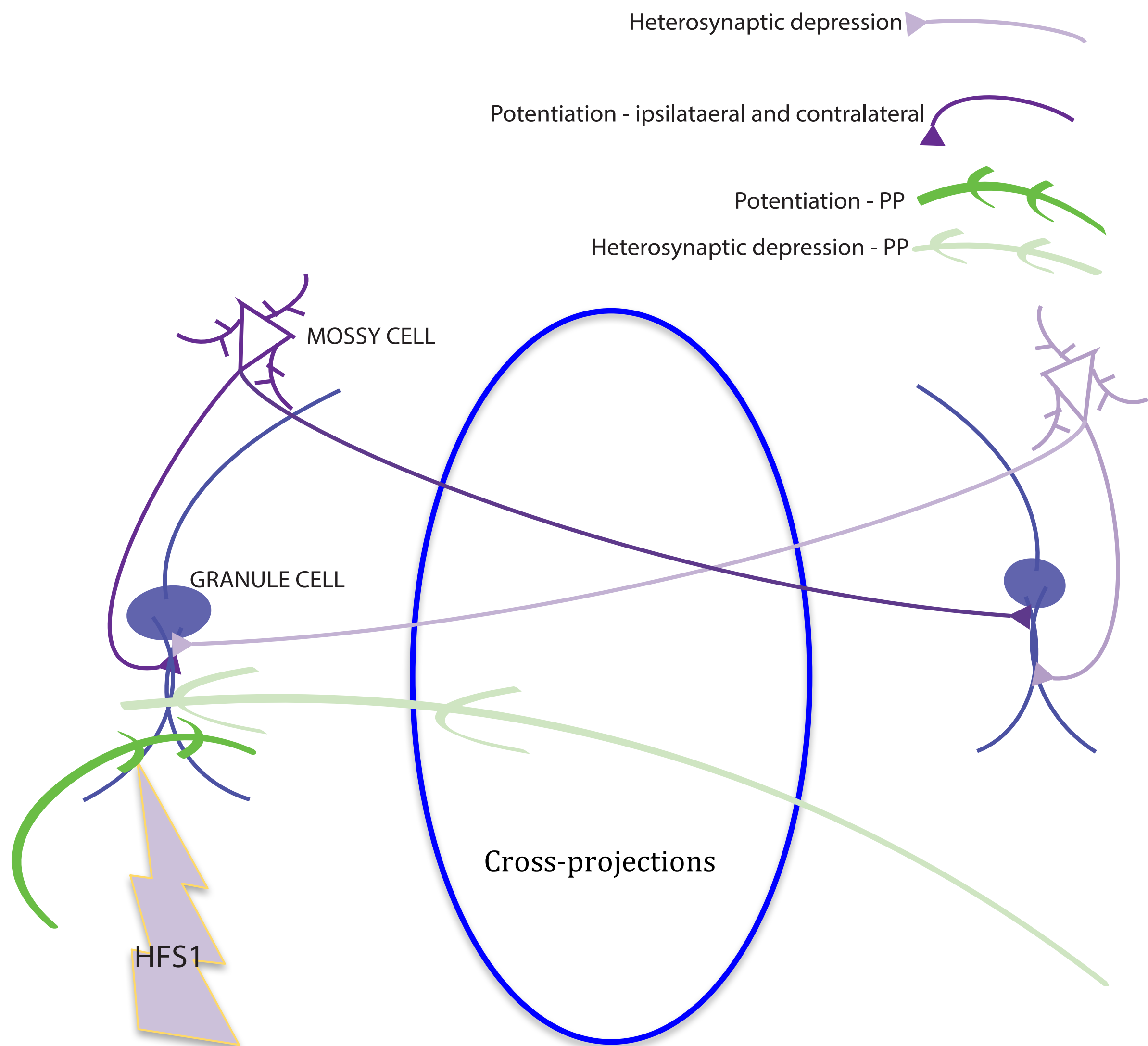

Fig. 6.1 The previously described multi-input plasticity after LTP induction at ipsilateral PP-DG granule cell synapses. HFS delivered to the PP causes LTP of those PP-DG granule cell synapses, as well as potentiation of the ipsilateral and contralateral projections of mossy cells. Further, heterosynaptic depression of both the incoming contralateral projections from the PP and mossy cells is induced. Alvarez-Salvado, Pallares, Moreno, and Canals (2014).

In our experiments, we induced LTP in the contralateral hemisphere 20 min after the first LTP induction. This caused the first LTP to rapidly decay back to baseline. Given that there were possibly 2 sets of potentiated synapses and 2 sets of depressed synapse in the DG in which LTP was induced first, the second LTP has the potential to de-depress and depotentiate any of these. Given that synapses on the granule cell readily undergo heterosynaptic depression along with LTP (Abraham & Goddard, 1983; Levy & Steward, 1983) and that recent computational models have suggested that the timing of cell firing, in combination with previously established plasticity mechanisms can regulate hetero- and homosynaptic plasticity mechanisms (Jedlicka, Benuskova, & Abraham, 2015), depotentiation may be an explanation as to why the first LTP is lost (Fig. 6.2).

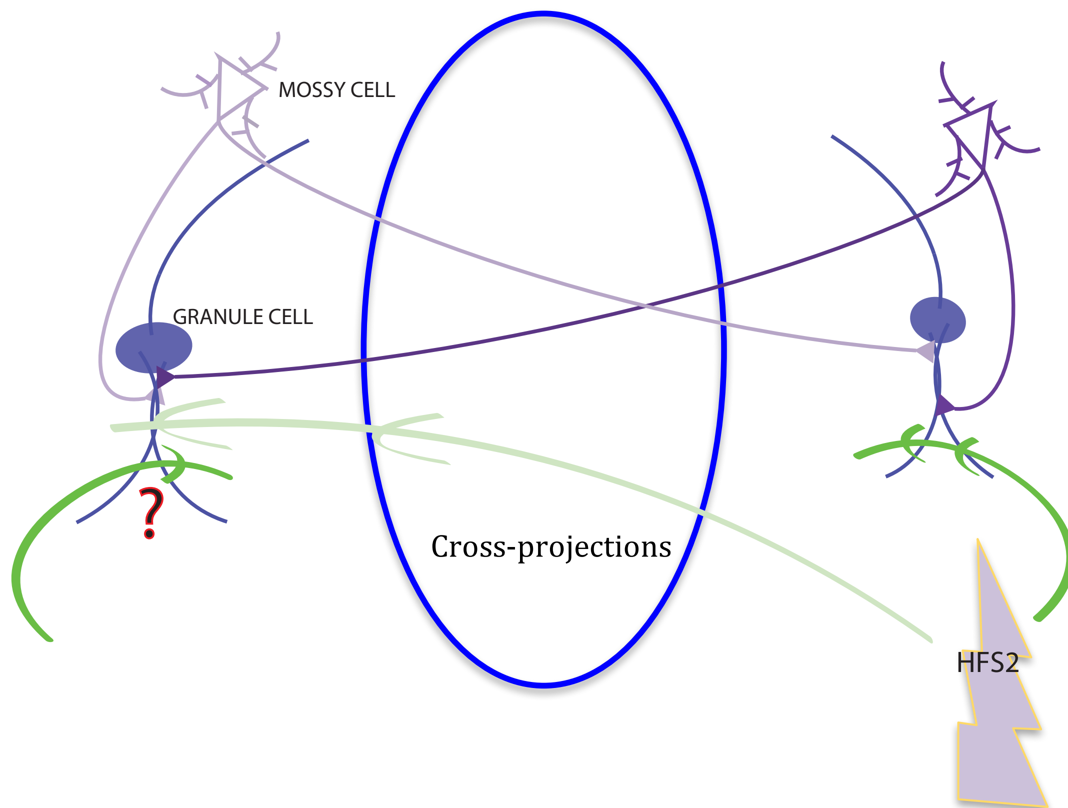

Fig. 6.2 Stimulation of the hemisphere 2, 20 min after stimulation of hemisphere 1 (as seen in Fig. 6.1) induced normal LTP in that hemisphere. However, it caused a slow decay of the PP – granule LTP in the contralateral hemisphere by some, as yet undetermined mechanism. However, if following what occurred in both hemispheres after LTP in hemisphere 1, LTP in hemisphere 2 may have de-depressed the contralateral mossy cell – granule cell projections and depotentiated the mossy cell projections coming into hemisphere 2.

If the decay of the first LTP represented heterosynaptic depotentiation, it would normally be expected to happen immediately upon stimulation, much like heterosynaptic depression (Abraham & Goddard, 1983; Levy & Steward, 1983). This was not the case, as the LTP did not decay below baseline until day 9, thus suggesting a slower form of depotentiation or alternatively, inhibition of the processes needed to turn LTP1 and 2 into LTP3. The mechanisms underlying this process are unfortunately unclear from these experiments and the interaction with HDAC activity is, at this stage, only correlational. Potentially, these results

may suggest that interruption of the gene expression processes with subsequent input during the early stages after LTP induction (20 min) may be detrimental to the expression of LTP3 but considerably more evidence is needed to confirm or refute this interpretation.

The biological relevance of LTP in one hemisphere leading to more rapid decay of LTP in the other hemisphere is somewhat unclear. Moreover, whether LTP reversal is a time-dependent characteristic and whether LTP becomes resistant to this type of interruption as it becomes more consolidated is also unknown. There are a number of suggested roles that mossy cells may play in memory formation and maintenance (Scharfman, 2016). Mossy cells – granule cell synapses may potentiate after PP – granule cell LTP to provide a mechanism to associate groups of granule cells that are not spatially close (Scharfman, 2016). This may assist in the pattern separation function of the DG by associating and separating inputs to granule cells by causing depolarisation of the granule cell either at the same time, or not, as PP inputs (Scharfman, 2016). Because mossy cells directly innervate both granule cells directly and inhibitory interneurons they may regulate this coincidence detection via either mechanism (Scharfman, 2016). In our experiments, LTP of the second hemisphere was out of time with the LTP of the first hemisphere, and thus perhaps to separate the pattern of activity and 'memory' the first LTP was lost. However, memory formation activates spares networks of neurons within the DG (Ramirez et al., 2013), whereas our LTP induction paradigm is considerably more blunt and widespread. Thus, the fine precision of pattern separation and network formation may be lost in the LTP preparation.

6.6. Metaplasticity as the maintenance mechanism of LTP

With the proposal that the genome is a central integrator which can control multiple components of plasticity throughout the cell, it is important to consider that a major focus of gene expression analysis in the literature is almost exclusively related to restructuring of potentiated synapses, though it has been discussed in relation to LTD (Abraham et al., 1994). However, when LTP is induced in the DG, measures of both the synaptic drive (fEPSP) and action potential firing (population spike) both increase, leading to some authors measuring each, or both, as LTP. However, these measures are of distinct components and indeed measuring them as such provide interesting insight into changes in synaptic strength and excitability (Abraham, Bliss, & Goddard, 1985). In fact, there are at least 3 mechanisms induced by LTP in PP-DG synapses; LTP at the synapses stimulated, LTD at heterosynaptic synapses and E-S potentiation (Abraham et al., 1985; Andersen et al., 1980). Intrinsic excitability can be regulated by transcription factors such as c-fos, sp-1, zif/268 which are known to be regulated after LTP induction and learning (Mucha et al., 2010; van Loo et al., 2012; J. Zhang et al., 2002). Further, there is strong evidence that CREB increases excitability as well as LTP maintenance and LTM (Lopez de Armentia et al., 2007). One of the core interpretations of the current literature, which provided a basis for testing the central role of HDAC1 and HDAC2 in the regulation of LTP, is that maintenance mechanisms of LTM and LTP are not at each specific point of adaptation i.e. every synapse that has potentiated, depressed, depotentiated and dedepressed, every ion channel that has changed, and every dendrite and spine

that has been altered. Instead, we proposed that metaplastic control of plasticity via the centralised regulation of gene expression, balances the adaptability of each cell and thus enable the maintenance of its position within an engram, which is maintained by a basal level of gene expression and protein production.

There is evidence supporting the notion that stability and plasticity can be centrally regulated by histone acetylation. Ocular dominance columns of the visual cortex are maintained by suppressed gene expression through either increased HDAC activity or decreased HAT activity (Baroncelli et al., 2016; Putignano et al., 2007). Plasticity can be restored, and restructuring of networks of neurons in these circuits can occur when in the presence of an HDACi (Baroncelli et al., 2016; Lennartsson et al., 2015; Putignano et al., 2007). Similarly, the ability of fear extinction learning to alter an established conditioned fear can only be achieved in the presence of an HDACi which drives expression of plasticity related genes such as *arc* and *c-fos* (Gräff et al., 2014). While my experiments aimed to add to this body of evidence, the timeframe of investigation over only 24 h was most probably too short to add any great detail as these previous experiments had investigated very well consolidated memories. Nevertheless, while this thesis does not provide supporting evidence for epigenetic control of the maintenance of LTP, it does highlight the dynamic nature of epigenetic regulation of gene expression and certainly supports the notion that there are early stages of structural remodelling of neurons and networks that are not the same as the maintenance mechanisms. While, from these experiments we have been able to provide support for HDAC1 and HDAC2 as the epigenetic regulators of LTP and LTM maintenance, there are a vast

number of other epigenetic mechanisms to explore which may play this role such as DNA methylation, as well as other histone modifications.

6.6.a. Are there reason to be cautious when proposing the use of broad-acting inhibitors of epigenetic mechanisms in the treatment of disease?

Research in the field of epigenetics is expanding as aberrant epigenetic modifications are being identified in numerous neurodegenerative diseases such as Alzheimer's disease (Fischer et al., 2010; Gräff et al., 2012), psychiatric disorders such as schizophrenia (Fischer et al., 2010) and neurological conditions such as epilepsy (Huang et al., 2012). Epigenetic research provides a platform whereby the environmental impact on the expression of genes can be investigated (Jaenisch & Bird, 2003; Kouzarides, 2007; Tammen et al., 2013). Further, epigenetic modifications can be modulated by drugs, some of which are showing promise as treatment options for the above-mentioned conditions (Adwan & Zawia, 2013; Esteller, 2008; Fass et al., 2013; Fujita et al., 2012; Gray, 2011).

Alterations to histone acetylation have been identified in a number of disease states (Fischer et al., 2010; Gräff et al., 2011). Rubenstein-Taybi syndrome, a disorder causing severe cognitive impairment, is mostly caused by a mutation in the CBP gene, which leads to decreased acetylation (Gräff et al., 2011). Decreased acetylation has also been reported in Huntington's disease, Parkinson's disease and Amyotrophic lateral sclerosis (Gräff et al., 2011). Increased HDAC2 expression has been shown to be increased in human tissue

from patients who had Alzheimer's disease (Gräff et al., 2012). This led Gräff et al (2012) to suggest that increased HDAC2 activity in response to stress decreased histone acetylation and therefore transcription (Gräff et al., 2012). However, it has also been found that in Alzheimer's brains the level of acH3 and acH4 as well as the total level of histone protein was increased (Narayan, Lill, Faull, Curtis, & Dragunow, 2015). Narayan et al (2015) suggested that there was in fact dysregulation of protein degradation pathways leading to the accumulation of histone proteins and thus acetylation of histone proteins (Narayan et al., 2015). Thus perhaps the increased regulation of acetylation seen in diseased brains may be a protective mechanism to limit the demands placed on cells by dysfunctional cellular processes and therefore overriding that protective mechanism may cause more harm than good. Further, it is important to note that any broad acting drug such as TSA has the ability to act on any cell type and thus, in our experiments as well as others and indeed as a consideration for treatment, TSA may not only be acting upon the intended target cells.

6.7. Going forward

Though for the most part it has been found that HDAC1 and HDAC2 negatively regulate gene expression, acetylation, L-LTP and LTM, there have been caveats where, for example, acetylation is positively regulated by HDAC inhibition (Guan et al., 2009). This suggests that the regulation of gene expression by acetylation alone is unable to answer all the questions about distinct gene expression profiles needed for particular functions within the brain. While this thesis has shown dynamic regulation of both HDAC1 and HDAC2 in

both temporally and spatially distinct ways, the results do not appear to support the hypothesis that HDAC1 and HDAC2 regulate metaplasticity and the maintenance of LTP over its lifetime. This does not exclude the hypothesis entirely. There are a number of epigenetic modifications which may act alone or together with the regulation of acetylation which could perhaps play this role. One such mechanism could be DNA CpG methylation at the promoter region of genes, which blocking transcription (Tammen et al., 2013). This can either occur by the methylation directly blocking the TF's access to the promoter region of the gene or by the recruitment of other epigenetic modifying agents that are associated with decreased transcription, particularly the recruitment of protein complexes containing HDACs (Curradi, Izzo, Badaracco, & Landsberger, 2002). As more CpGs are methylated, the necessity of HDAC-containing complexes diminishes and it has been suggested that larger conformational changes of the chromatin are then what contributes mostly to the inhibition of transcription (Curradi et al., 2002). Indeed, methylation has been proposed as a long-lasting mark of memory and metaplasticity (Baker-Andresen, Ratnu, & Bredy, 2013). Thus, investigations over the lifetime of a memory, or LTP, and the study of a wider breadth of epigenetic mechanisms is needed to answer the overriding question of an epigenetic metaplasticity maintenance mechanism.

Our results imply that plasticity and by extension learning and memory is not one process, occurring in discrete brain regions but instead a dynamic array of changes occurring in multiple regions, even across hemispheres, to create the cellular basis of these events in the brain. There are a number of questions driven by this thesis, such as can LTP1 and 2 be extended with the expression of

PRPs (via gene expression and protein synthesis) but still be distinct from LTP3? What regulates the waves of gene expression between 12 and 24 h after LTP and does TSA have an effect on them, if so why does it not affect LTP persistence? Most importantly, what is the elusive maintenance mechanism of memory? Do we need to re-examine our classifications of LTP1, 2 and 3? Is LTP3 really not regulated by enhancing the traditional synaptic plasticity related genes, since it was not enhanced by TSA? Our experiments can be expanded to look at different induction protocol, which induce different types of LTP, in the presence of TSA to begin to tease apart the LTP classification process. Further, in depth gene regulation analysis, such as chromatin immunoprecipitation in conjunction with DNA sequencing will help to identify which genes are being regulated by HDAC1 and HDAC2 at these specific time points. Finally, investigations during the LTP3 timeframe (>10 day post-LTP) need to be completed investigating the many epigenetic modifications that may regulate the persistence of LTP. These questions do not support the use of the gross application of an HDACi for any kind of treatment to do with learning and memory. Indeed the study of learning, memory and plasticity mechanisms in general perhaps needs a finer approach so as not to obscure the fine points and intricacies of this carefully crafted process that is so precious to who we are.

7. Conclusions

The overriding question of this thesis was whether central regulators of gene expression, such as HDAC1 and 2, maintain engrams. The hypothesis was that HDAC1 and 2, by negatively regulating plasticity related genes, would inhibit the ability to restructure a neuron and its connectivity and thus allow for the maintenance of the connectivity that is already in place. *In vivo* LTP was used as a model of memory, with the induction of LTP being equivalent to learning and the maintenance of LTP equivalent to LTM. HDAC1 and 2 were found to be dynamically regulated post-LTP induction, though not at the 24 h time-point where it had been hypothesised they would be playing a stabilizing role. Nevertheless, two time-points of enhanced HDAC activity, 20 min and 12 h post-LTP induction, were identified and thus the role they played in the persistence of LTP was tested. The increased HDAC1 and 2 activity 20 min post-induction appeared to dampen LTP over the first 5 – 7 days such that inhibiting this activity enhanced the magnitude of LTP over this time period. This coincides with what has previously been described as LTP2 and thus we conclude that what has been previously described as enhanced LTP persistence by HDAC inhibition is actually an enhanced magnitude of this intermediary stage. Inhibiting the increased HDAC1 activity 12 h post-LTP induction had no effect on the magnitude or the persistence of the LTP. From this we conclude that HDAC activity at 12 h plays a different role to that at 20 min and does not, on its own, appear to regulate the persistence of LTP. Nevertheless, inhibiting HDAC activity at 12 h may affect long-term alterations to a neuronal network by way of inhibiting de-depression. Thus, in the present experiments, HFS to the lateral PP

caused the previously established heterosynaptic LTD in that pathway to de-depress over the ensuring 5 days. This effect appeared to be blocked by the inhibition of HDAC activity, with a trend for the lateral PP LTD to persist over 21 days. Thus, the heterosynaptic depression seemingly became resistant to de-depression in the presence of TSA, although these are very preliminary results at this stage which need considerably more vigorous testing. Finally, a novel enhancement of HDAC activity was found in the contralateral hemisphere, 20 min post-LTP induction. This enabled us to test the opposing hypothesis, that a prior increase in HDAC activity would inhibit the subsequent induction of persistent LTP. This was not found to be the case and in fact the first LTP was depotentiated rapidly upon subsequent LTP induction in the contralateral hemisphere. LTP of the PP – DG granule cells does appear to dynamically regulate various synapses throughout the molecular layer causing heterosynaptic depression of a number of incoming synapses from the contralateral hemisphere but also potentiation of the contralateral mossy cell – granule cell synapses. By subsequent potentiation of the contralateral hemisphere 20 min after all of these synaptic changes, potentially induces a further array of potentiation, depotentiation, depression and de-depression mechanisms throughout the granule cells in both hemispheres, that can be exceedingly complex to interpret. However, our findings indicate that within-animal controls, using the contralateral hemisphere, should be very strongly reconsidered as an experimental approach. The question still remains, however, whether enduring changes in HDAC1 and 2 activity regulate LTP maintenance.

From the evidence gathered in these experiments the major interpretation is that inhibiting HDAC activity has effects when generated during the early stages of LTP induction but not during LTP maintenance. However, future studies should investigate roles of HDAC1 and 2 during the timeframe that has previously been attributed to LTP3, rather than LTP2, in order to confirm this interpretation. These results have not provided insight into the elusive maintenance mechanisms of LTP and by extension LTM. Nevertheless, they have not ruled out the hypothesis that negative regulation of plasticity related genes helps maintain memory. There are a myriad of other mechanisms which have the potential to play this role and the timeframe of investigation should be broadened to encapsulate the more consolidation mechanisms. Indeed, this thesis strongly supports the notion that changes in gene expression and its epigenetic regulation are ongoing for at least 24 h post-induction. Thus interpretation of LTM at 24 h and of L-LTP, particularly in slice preparations, should be perhaps limited to early consolidation mechanisms rather than concluding that any long-term changes or effects have taken place. The direction that learning and plasticity research has taken towards studying awake, freely moving animals with real-time imaging and manipulations will enable research that can identify these long-term consolidation and maintenance mechanisms. Only once long-term maintenance mechanisms have been identified can disease states affecting these maintenance mechanisms be fully understood. Hopefully, this will lead to the identification of treatment options with temporal and cell type or brain region specificity for disease states.

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9. Appendices

9.1. Appendix A: Western blot

Resolving gel (1 x gel, 15%):

MilliQ – H_20:	2.45 ml
4 x lower buffer*:	2.5 ml
Bis/Acrylamide:	5 ml
10% Amonium persulfate:	50 μl
Tetramethylethylenediamine:	5 μl

** 4 x lower buffer*

1.5 M tris(hydroxymethyl)aminomethane - Chloride pH 8.8
0.4% SDS

Stacking gel (1 x gel, 15%):

MilliQ – H_20:	5.76 ml
4 x upper buffer**:	2.5 ml
Bis/Acrylamide:	1.68 ml
10% Amonium persulfate:	50 μl
Tetramethylethylenediamine:	10 μl

*** 4 x upper buffer*

0.5 M tris(hydroxymethyl)aminomethane - Chloride pH 6.8
0.4% SDS

Running buffer:

25 mM tris(hydroxymethyl)aminomethane
192mM glycine
0.1% SDS

Loading buffer (per sample):

10 x sample buffer***:	1.5 μl
bromophenol blue:	3.5 μl
Bmercaptoethanol:	0.175 μl

*** 10 x sample buffer*

0.625 M tris(hydroxymethyl)aminomethane - Chloride pH 6.8
10% SDS

Transfer buffer:

MilliQ – H_2O:	760 ml
25 x NOVEX buffer****:	40 ml
Methanol:	200 ml

**** 25 x NOVEX buffer (1 L MilliQ – H_2O)*

tris(hydroxymethyl)aminomethane:	36.25 g
glycine :	180 g

9.2. Appendix B: Immunohistochemistry

Coated slides:

H_2O:	500 ml
Gelatine:	2.5 g
chromium potassium sulfate:	0.25 g