EXPRESSION MECHANISMS UNDERLYING DIFFERENT FORMS OF HIPPOCAMPAL LONG-TERM POTENTIATION

A thesis submitted for the degree of Doctor of Philosophy
of the Australian National University

June 2011

by
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STATEMENT

All the work described in this thesis is original. These experiments were performed by me between February 2008 and June 2011under the supervision of Dr Clarke Raymond.

During this time a number of abstracts were published in conjunction with presentations made at scientific meetings:

Johnstone, V.P.A., Raymond, C.R. (2011) Requirement for nitric oxide and protein synthesis in presynaptic components of hippocampal LTP. *Proceedings of the Australian Neuroscience Society,* **21**: POS-TUE-038. Auckland, New Zealand.

Johnstone, V.P.A., Raymond, C.R. (2010) Nitric oxide and protein synthesis are required for a presynaptic component of persistent LTP in hippocampal area CA1. *FENS Abstracts,* **5**: 014.12. Amsterdam, The Netherlands.

Johnstone, V.P.A., Raymond, C.R. (2010) Protein synthesis dependent enhancement of transmitter release in persistent forms of hippocampal LTP. *Proceedings of the Australian Neuroscience Society,* **20**: POS-TUE-136. Sydney, Australia.

Johnstone, V.P.A., Raymond, C.R. (2009) Alterations to vesicle turnover rates associated with different forms of long-term potentiation in the hippocampus. *Proceedings of the 27th Australasian Winter Conference on Brain Research,* **27**: 7.4. Queenstown, New Zealand.

In addition, the following paper has been submitted for publication:

Johnstone, V.P.A., Raymond, C.R. (2011) Nitric oxide and protein synthesis are required for a presynaptic component of hippocampal LTP. *Learning & Memory*.

Victoria P.A. Johnstone

ACKNOWLEDGEMENTS

I would like to thank all those in the Department of Neuroscience who have helped me over the years. First and foremost my supervisor Dr Clarke Raymond, whose sound advice, encouragement and seemingly endless supply of patience created an environment in which I was enabled to flourish scientifically. I am also grateful to Peter Lohmann and Garry Rodda for experimental help and technical assistance and John Bekkers and Greg Stuart for critical appraisal of my research. There are of course many others who also deserve acknowledgement, yet unfortunately far too little space to do so.

Particular thanks must also go to my father Ray, my siblings Lizzy, Ceri and Bart, my parents-in-law Jeanette and Roger and my friends for their words of encouragement and support over the last three and a half years.

I dedicate this thesis to the memory of my mother Valerie Johnstone, who lost her long battle with ill health during the final year of my PhD. From her I learnt the value of dedication and perseverance in the face of adversity and the importance of remaining objective about ones own research. I also dedicate this manuscript to my husband Kyle, without whose support such perseverance would not be possible. Thank you for your understanding and for putting up with all of my sullen moments.

"Swiftly the brain becomes an enchanted loom, where millions of flashing shuttles weave a dissolving pattern – always a meaningful pattern – though never an abiding one."

– Sir Charles Sherrington (1941)

ABSTRACT

Long-term potentiation (LTP) in the hippocampus is a model system for investigating the mechanisms of synaptic plasticity and its role in learning and memory in the brain. Progress in understanding the mechanisms responsible for LTP has been somewhat hampered by the existence of multiple LTP variants, even at a single set of synapses. Three discrete forms of LTP (LTP1, 2 and 3) that differ in their persistence and the calcium signals required for their induction have recently been characterised at the CA3-CA1 synapse. While the different induction mechanisms of LTP1, 2 and 3 are well characterised, it is less clear whether they are also discrete in regard to their expression and maintenance mechanisms.

In this study the presynaptic expression mechanisms underlying LTP1, 2 and 3 were determined by measuring FM 1-43 destaining from CA3 terminals in hippocampal slices from male Wistar rats. No difference in vesicle turnover rate was observed for LTP1 up to 160 min following induction by 1 train of theta-burst stimulation (1TBS), indicating that this weak form of LTP is primarily postsynaptic. A presynaptic enhancement was found for LTP2 at 160 min after induction by 4TBS and for LTP3 at 80 min and 160 min after induction by 8TBS. This demonstrates that more robust forms of LTP recruit a presynaptic component to their expression and that perhaps the onset of this recruitment may be earlier for more persistent LTP.

Given that all three forms of LTP require unique postsynaptic signalling mechanisms for their induction and that LTP2 and LTP3 exhibit presynaptic changes, it is logical that there may be a retrograde signal (such as nitric oxide [NO]) released from postsynaptic spines. Disruption of NO signalling did not affect the persistence of LTP1, but blocked both LTP2 and LTP3 maintenance and the associated enhanced release. This finding supports a role for NO signalling in the establishment of presynaptic changes associated with more persistent forms of LTP.

The maintenance mechanisms associated with LTP1, 2 and 3 were next investigated by inhibiting translation and transcription. Data from these experiments established that LTP1 is independent of new gene transcription and protein synthesis. In contrast, LTP2 maintenance and its presynaptic expression were dependent on protein synthesis, but not gene transcription. LTP3 maintenance was dependent on both translation and transcription, but like LTP2 the enhanced release only required translation. Subsequent

experiments using a membrane impermeable translation inhibitor indicated that new protein synthesis within the postsynaptic compartment is required for the enhanced presynaptic release.

The present experiments provide a detailed examination of the expression and maintenance mechanisms underlying LTP1, 2 and 3. These results considerably strengthen the mechanistic separation of these forms of LTP, supporting a model of multiple discrete forms of LTP at CA3-CA1 synapses rather than different temporal phases. This is likely to explain some of the discrepancies in the LTP field and suggests that the pattern of afferent input to the hippocampus may dictate the mechanisms and duration of information encoding.

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List of Abbreviations

ACSF	artificial cerebrospinal fluid
Act D	Actinomycin D
AMPAR	α-amino-3-hydroxy-methyl-isoxazole-propionic acid receptor
ANI	anisomycin
BDNF	brain-derived neurotrophic factor
CaM	calmodulin
CAMK	CaM-kinase
CBP	CREB-binding protein
CICR	calcium-induced calcium-release
CNS	central nervous system
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CREB	cAMP response element binding protein
D-APV	D-(-)-2-amino-5-phosphonopentanoic acid
E-LTP	early-LTP
EPP	end-plate potential
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
Erk	extracellular signal-regulated kinase
fEPSP	field excitatory postsynaptic potential
GABA	γ-aminobutyric acid
HFS	high frequency stimulation
IEG	immediate early gene
IP_3R	inositol (1,4,5)-triphosphate receptor
IPSP	inhibitory postsynaptic potential
L-LTP	late-LTP
L-NAME	*N*-ω-nitro-L-arginine methyl ester
LFS	low frequency stimulation
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MEPP	miniature end-plate potential
mGluR	metabotropic glutamate receptor
NMDAR	*N*-methyl-*D*-aspartate receptor
NO	nitric oxide
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PPF	paired-pulse facilitation
PPR	paired-pulse ratio
PTK	protein tyrosine kinase
P_r	release probability
RRP	readily releasable pool
RyR	ryanodine receptor
spH	synaptopHlourin
TBS	theta-burst stimulation
TP	total pool
Trk	tyrosine kinase receptor
VGCC	voltage-gated calcium channel

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1. Preamble

The word synapse was first used in print in the late nineteenth century by Foster and Sherrington, who stated "a special connection of one nerve-cell with another might be called a *synapsis*" (Foster & Sherrington, 1897). The term has since taken on a broader meaning and is now used to describe non-neuronal as well as neuronal junctions. An area of synaptic physiology that has piqued particular interest over the past three decades is the change in synaptic strength that occurs in response to certain patterns of electrical activity– a phenomenon known as synaptic plasticity. Synaptic plasticity is referred to as either short-term (ranging from tens of milliseconds to several minutes) or long-term (lasting for hours, days or indefinitely, Abraham *et al*., 2002). In addition, plasticity can involve either facilitation or depression of the postsynaptic response. Such plasticity of a synapse must be initiated by changes in biochemical processes localised within the presynaptic cell, the postsynaptic cell, or some combination of the two.

The most intensely studied form of synaptic plasticity is a form of synaptic facilitation termed long-term potentiation (LTP). LTP is defined as an activity-dependent and persistent increase in the postsynaptic response to a given stimulus and is thought to be a cellular correlate of memory. LTP is most widely studied in the hippocampus, a structure that is known to be vital for learning and memory. A great deal of controversy exists with regard to the mechanisms underlying hippocampal LTP and in particular whether it is expressed presynaptically or postsynaptically. Recently three forms of hippocampal LTP (LTP1, 2 and 3) have been distinguished on the basis of their induction mechanisms (Raymond, 2007), allowing for clear comparisons between each form to be made. The experiments described here investigate whether LTP1, 2 and 3 can also be differentiated on the basis of their expression mechanisms, i.e. whether each form involves enhanced presynaptic release.

This chapter will provide a broad introduction to synaptic transmission at central synapses, hippocampal neuroanatomy and plasticity, as well as providing a more detailed discussion and evaluation of the literature pertinent to hippocampal LTP and in particular LTP1, 2 and 3. Finally, the aims of the project and experimental hypotheses will be considered.

2. Introduction

2.1. Synaptic Transmission

2.1.1. Overview of synaptic transmission

The majority of neuronal communication occurs via chemical synaptic transmission. In the central nervous system (CNS) this involves the presynaptic release of neurotransmitter that diffuses across the synaptic cleft and elicits an electrical response in the postsynaptic dendrite (Dale, 1934; Dale & Feldberg, 1934a, b). Active zones (specialised sites of release) along the axon are comprised of synaptic vesicles, voltage-gated Ca^{2+} channels and macromolecular complexes that are essential for vesicle exocytosis (Schoch & Gundelfinger, 2006). When an action potential reaches an axon terminal containing an active zone and depolarises the membrane, voltage-gated Ca^{2+} channels open (Katz & Miledi, 1968). The resulting influx of Ca^{2+} induces vesicle fusion at the active zone and transmitter release. Released transmitter enters the synaptic cleft and diffuses towards the postsynaptic cell to activate postsynaptic receptors (Clements *et al*., 1992; Südhof, 2004). The synaptic cleft spans only 20 nm (Schikorski & Stevens, 1997) and the diffusing transmitter reaches postsynaptic receptors in less than 10 μs (Clements *et al*., 1992). Binding of transmitter to postsynaptic receptors induces electrical changes that are either inhibitory or excitatory, depending on the characteristics of the bound receptors.

2.1.2. The quantal hypothesis

The proposal and subsequent verification of the quantal hypothesis by Fatt and Katz (1952) and del Castillo and Katz (1954) was a fundamental event in the history of synaptic physiology and established that neurotransmitters are released from presynaptic nerve terminals in discrete packets, or 'quanta' (Augustine & Kasai, 2007). Single-cell electrophysiological recordings from the frog neuromuscular junction revealed that small depolarisations of muscle fibres occurred spontaneously, without specific nerve stimulation (Fatt & Katz, 1952). Subsequent examination revealed that these depolarisations were approximately 0.5 mV in amplitude and occurred in a probabilistic manner, at an average frequency of one per second (Figure 2.1). It was observed that these spontaneous depolarisations resembled nerve-evoked end-plate potentials (EPPs) in their shape, spatial spread and pharmacological sensitivity (Fatt & Katz, 1952). These spontaneous depolarisations were consequently termed miniature

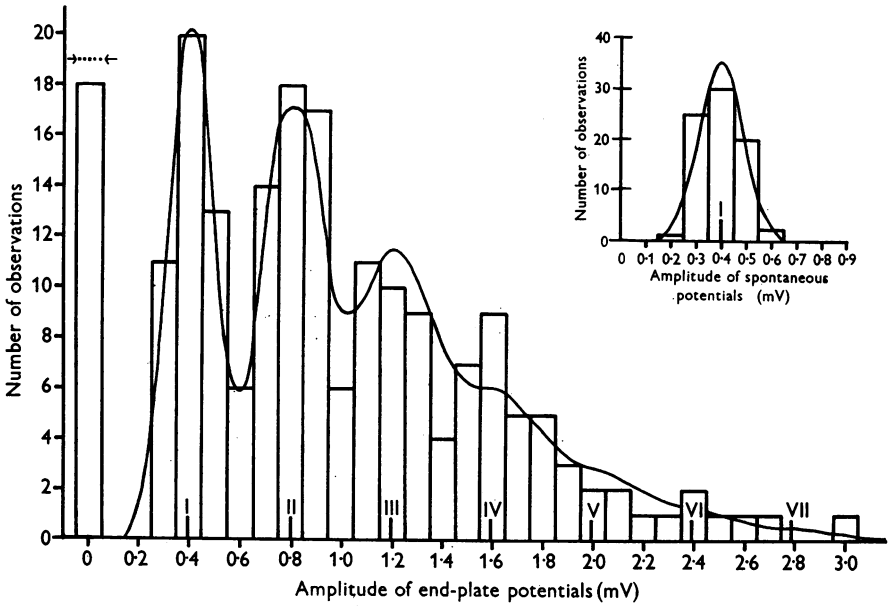

Figure 2.1. Histogram of EPP and MEPP amplitudes from a mammalian (cat) end-plate bathed in low Ca^{2+} solution. Peaks of EPP amplitude occur at 1, 2, 3 and 4 times the mean amplitude of the MEPP (see inset). A Gaussian curve has been fitted to the MEPP distribution and used to calculate the theoretical distribution of EPP amplitude (shown by the continuous line). The expected number of failures, in which no EPP was observed in response to stimulation, is indicated by arrows (zero amplitude). Failures occur due to the immersion of the preparation in a solution low in Ca^{2+}, which reduces the probability of transmitter release. Modified from Boyd & Martin (1956).

end-plate potentials (MEPPs) and it was concluded that MEPPs resulted from the spontaneous release of acetylcholine (the predominant motor neuron transmitter) from the nerve terminal (Fatt & Katz, 1952).

The next advance in the establishment of vesicular mode of release came when del Castillo and Katz (1954) examined the relationship between MEPPs and EPPs at the frog neuromuscular junction. With low Ca^{2+} levels in the bathing solution (reducing the probability of transmitter release) it was demonstrated that the size of the EPP became comparable to the size of the MEPP. Under these conditions, successive impulses in the motor nerve resulted in EPPs that varied in a stepwise manner, so that each EPP amplitude was an integer multiple of the MEPP (Figure 2.1). This led to the conclusion that transmitter is released in discrete quanta and that a single quantum generates a MEPP. Subsequent electron-microscopic studies performed by Palay and Palade (1955) revealed the presence of small spherical structures located within the presynaptic nerve terminal. These findings suggested that neurotransmitter was contained in spherical

synaptic vesicles within the motor nerve terminal and that spontaneous release of the contents of one vesicle resulted in a MEPP (Del Castillo & Katz, 1955). The verification of the quantal hypothesis was pivotal in establishing vesicular release as a mode of transmission and has provided valuable insight into the biophysical properties of transmitter release and receptor activation. The model also allows for certain characteristics of release to be determined, such as the probability of release at each release site (P_r), the quantal size at each release site (q) and the number of available quanta (n).

Although valid at the neuromuscular junction, it has proven difficult to extrapolate this model of transmission to hippocampal synapses, partly due to the differences in structure between central and peripheral nerves (Christie & Jahr, 2006; Ninio, 2007). Whilst the neuromuscular junction contains long terminals, with *multiple* vesicle-dense active zones spanning the terminal length (Desaki & Uehara, 1981), neurons in the hippocampus typically contain multiple terminals along an axon, each with a single small active zone with only a few vesicles closely apposed to the presynaptic membrane (Figure 2.2; Sik *et al*., 1993; Branco *et al*., 2009). Therefore postsynaptic signals often fluctuate between failures and an amplitude that reflects release of only a single quantum (Malinow, 1991). The use of quantal analysis at central synapses is further complicated by the indirect nature of recording somatic currents that potentially arise from multiple synaptic inputs. In these circumstances it is not possible to ensure that postsynaptic responses arise via release from a single presynaptic terminal. Nevertheless, application of quantal theory to central synapses has in certain circumstances yielded insight into the mechanisms underlying changes in synaptic strength (Walmsley *et al*., 1988; Malinow, 1991; Ikeda *et al*., 2008; Branco & Staras, 2009). For example, if it is assumed that failures of transmission reflect cases where the presynaptic action potential did not elicit adequate Ca^{2+} influx for exocytosis to occur, it is possible to use the frequency of such failures to indirectly demonstrate the state of the presynaptic terminal (Stricker *et al*., 1996; Chevaleyre & Siegelbaum, 2010). If particular stimulation protocols decrease the probability of a failure, it can be inferred that such protocols have increased release probability (Stricker *et al*., 1996; Biró *et al*., 2005).

Therefore whilst quantal analysis has been successfully applied to make inferences about central synapses, such approaches rely on making several assumptions about the number of synaptic inputs onto a given postsynaptic cell and must be used with caution (Walmsley *et al*., 1988).

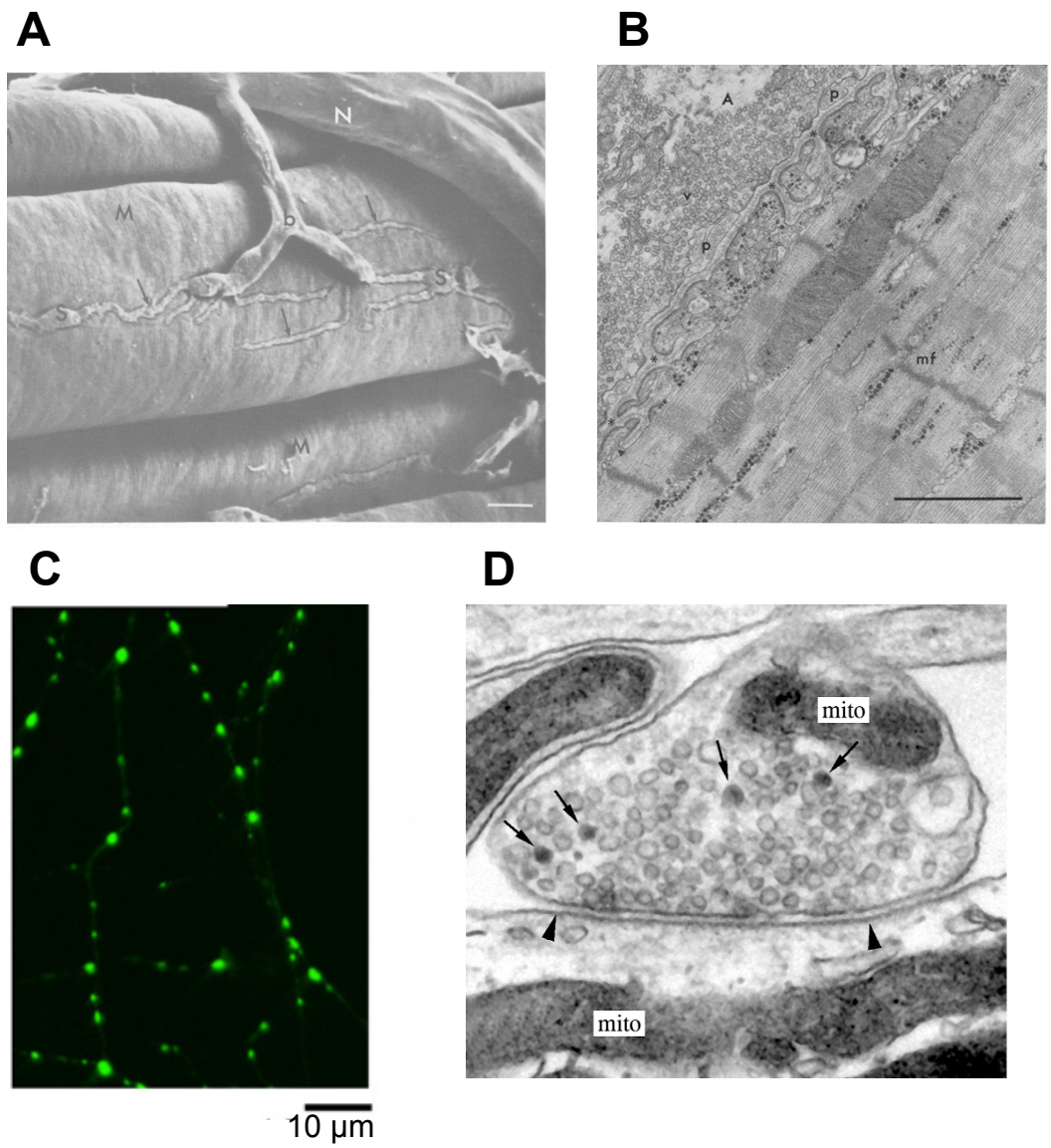

Figure 2.2. Differences in structure between the neuromuscular junction and central nerve terminals. A) Electron micrograph showing the surface features of a neuromuscular junction in the sartorius muscle of the frog. Three muscle fibres (M) can be seen running parallel to one another. The side branch (b) of a motor nerve (N) narrows into thin terminals that run along the muscle fibre (indicated by arrows). Scale bar indicates 10 µm. **B)** Electron micrograph showing a portion of a neuromuscular junction from the cutaneous pectoris muscle of the frog. The axonal ending (A) contains multiple synaptic vesicles (v). Active zones are visible opposite regions of high receptor density on the postsynaptic muscle fibre. Glial cell projections (p) are interposed between the terminal and the end plate region. Scale bar represents 1 µm. **C)** Two-photon image showing multiple fluorescent terminals along individual hippocampal axon branches. Scale bar represents 10 µm. **D)** Electron micrograph of a single rodent hippocampal terminal in culture. The postsynaptic density (region of high receptor density) and active zone are indicated by arrowheads and 2 mitochondria are labelled (mito). Vesicles are situated at various distances from the active zone, with very few directly docked. Images adapted from **A**) Desaki and Uehara (1981); **B)** Ceccarelli *et al*. (1972); **C)** Staras *et al*. (2010) and **D)** Schikorski and Stevens (2001).

2.1.3. Vesicle cycle

Chemical neurotransmission is mediated by exocytosis of synaptic vesicles at the presynaptic active zone and diffusion of transmitter across the synaptic cleft to the postsynaptic receptor. To maintain rapid and repeated rounds of release, synaptic vesicles undergo a trafficking cycle within the nerve terminal that can be divided into sequential steps (Figure 2.3; Ceccarelli *et al*., 1973; Heuser & Reese, 1973; Bennett & Scheller, 1994; Südhof, 1995; De Camilli & Takei, 1996; Südhof, 2000, 2004). First, neurotransmitters are actively transported into synaptic vesicles (*step 1*) and synaptic vesicles cluster in front of the active zone (*step 2*) (Südhof, 2004). Synaptic vesicles then dock on the active zone (*step 3*), a process that is defined as the initial contact between the vesicle membrane and the presynaptic membrane (Südhof, 2004) and is only able to occur at the active zone (Südhof, 1995; Schweizer & Ryan, 2006). After docking the vesicles undergo a maturation process (*step 4*), in which they become

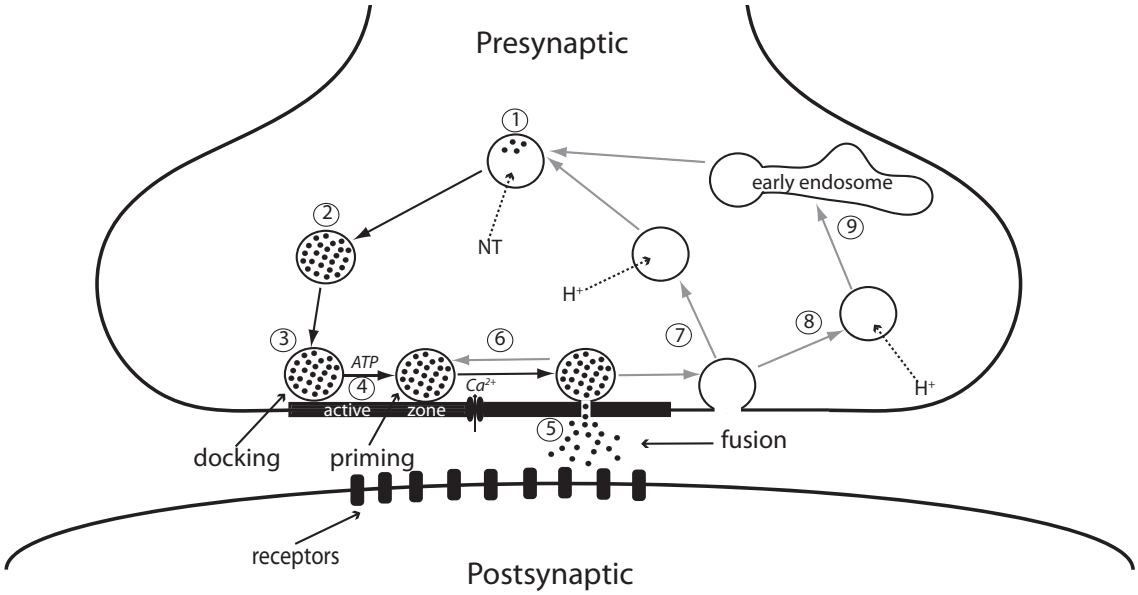

Figure 2.3. The synaptic vesicle cycle. Vesicles are filled with neurotransmitter (step 1) and join a vesicle pool within the terminal (step 2). Filled vesicles dock on the active zone (step 3), where they undergo a priming reaction (step 4) that makes them competent for Ca^{2+}-triggered fusion (step 5). After fusion and subsequent neurotransmitter release, vesicles undergo endocytosis and recycle via one of several routes: local reuse (step 6), fast recycling via a non-endosomal pathway (7), or clathrin-mediated endocytosis (step 8), via an endosomal intermediate (step 9). Steps in exocytosis are indicated by black arrows, Steps in endocytosis and recycling are indicated by grey arrows.

capable of undertaking fast Ca^{2+}-triggered fusion (*step 5*). This so called 'priming' of vesicles is poorly understood, however is likely to be driven by SNARE (soluble N-ethylamide-sensitive factor attachment protein receptor) proteins, which form a membrane-bridging assembly between the vesicle and presynaptic membrane enabling fusion (Borisovska *et al*., 2005; Sørensen *et al*., 2006; Guzman *et al*., 2010). After opening of the fusion pore and transmitter release, synaptic vesicles endocytose and are recycled via one of three potential pathways (Bennett & Scheller, 1994; Südhof, 2004):

(a) vesicles are re-acidified and refilled with transmitter without undocking (*step 6*, 'kiss-and-stay')

(b) vesicles undock and recycle locally (*step 7*, 'kiss-and-run') to be re-acidified and refilled with transmitter (back to steps 1 and 2) or

(c) vesicles endocytose via clathrin-coated pits (*step 8*) and re-acidify and refill with transmitter either directly, or after passing through an endosomal intermediate (*step 9*).

It is thought that fast recycling pathways such as 'kiss-and-run' and 'kiss-and-stay' allow for rapid transmission under conditions of high frequency stimulation (HFS; Smith *et al*., 2008).

2.1.4. Vesicle pools

Mammalian nerve terminals have distinct vesicle pools, each with different capacities for exocytosis. Early electrophysiological studies performed on rat neuromuscular junctions (Liley & North, 1953) and on human intercostal muscle (Elmqvist & Quastel, 1965) highlighted the existence of these pools and led to preliminary inferences that there are two distinct presynaptic stores of transmitter – a 'readily releasable' fraction, which is depleted during high frequency tetanic stimulation, and a 'non-readily releasable' fraction. Much work has been undertaken to distinguish between these pools, and although terminology varies, most models agree that central nerve terminals contain three pools. These pools are the readily releasable pool, the recycling pool and the reserve pool (Figure 2.4; Rizzoli & Betz, 2005).

The *readily releasable pool* (RRP) is defined as the synaptic vesicles that are immediately available on stimulation (Südhof, 2000). These vesicles are docked on the active zone and primed for release. The RRP is depleted rapidly by 5-15 stimuli at frequencies of approximately 30 Hz (Schneggenburger *et al*., 1999; Delgado *et al*., 2000; Richards *et al*., 2003), a few milliseconds of depolarisation (Mennerick & Matthews, 1996; Neves & Lagnado, 1999) or approximately 1 second of hypertonic

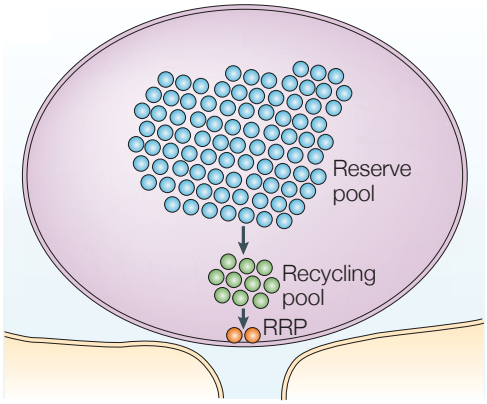

Figure 2.4. Illustration of the classically accepted distribution of the three vesicle pools at a typical central nerve terminal. The reserve pool makes up the largest fraction of the total pool and the recycling and readily releasable pools are substantially smaller. The readily releasable pool (RRP) consists of vesicles that are docked on the active zone and primed for release. Taken from Rizzoli and Betz (2005).

shock (via sucrose application) in hippocampal boutons (Rosenmund & Stevens, 1996). In cultured hippocampal neurons the total vesicle population in each bouton (the total pool, TP) comprises 100-200 vesicles (Schikorski & Stevens, 1997), although studies from hippocampal slices suggests the TP may be even larger than this (Harris & Sultan, 1995). The RRP is thought to consist of 5-9 vesicles (Murthy & Stevens, 1999), which equates to approximately 5% of the TP of vesicles.

The *recycling pool* is defined as the pool of vesicles that maintain release during moderate stimulation (Rizzoli & Betz, 2005). This pool is thought to contain 10-20% of the TP, which equates to approximately 20-30 vesicles (Murthy & Stevens, 1999). Normal physiological frequencies of stimulation (2-5 Hz) cause this pool to turnover continuously (Harata *et al*., 2001; de Lange *et al*., 2003; Richards *et al*., 2003).

The *reserve pool* is defined as the pool of vesicles from which release is only triggered during intense stimulation frequencies (30-100 Hz) and can be thought of as a depot of vesicles that is utilised during prolonged intense stimulation patterns (de Lange *et al*., 2003; Richards *et al*., 2003). These vesicles constitute the majority of the TP, typically 80-90% (Murthy & Stevens, 1999), and are less likely to undergo exocytosis in response to either electrical stimulation or K^+ depolarisation (Takei *et al*., 1996). During either short bursts of HFS or prolonged periods of low frequency stimulation (LFS), the RRP is depleted and transmission is probably sustained via RRP replenishment from the reserve pool (de Lange *et al*., 2003).

The schematic of the vesicle pools shown in Figure 2.4 is typical of those that have been reproduced for several decades showing the three pools morphologically segregated into discrete clusters. This proposed distribution has been challenged at motor nerve terminals where fluorescently labelled RRP vesicles were found at sites

remote from the active zone (Rizzoli & Betz, 2004). Whether the same is true for central nerve terminals remains unknown, however it is likely that the physical proximity of vesicles to the active zone is not the only determinant of release capacity.

More recently it has been postulated that an additional so-called 'superpool' of vesicles exists in hippocampal neurons acting as a highly mobile, functionally interchangeable source of vesicles that can be utilised by multiple terminals along an axon branch (Darcy *et al*., 2006; Chen *et al*., 2008; Fernandez-Alfonso & Ryan, 2008; Westphal *et al*., 2008; Staras *et al*., 2010). This common pool of vesicles has been observed in both hippocampal culture and slice preparations and appears to release and capture new vesicles whilst traversing intersynaptic regions (Staras *et al*., 2010). It is hypothesised that these vesicles may act as an extension of the recycling pool of vesicles within terminals, providing an extra source of transmitter that can be drawn upon during periods of intense stimulation (Fernandez-Alfonso & Ryan, 2008; Staras *et al*., 2010). The existence of this pool challenges the traditional notion that presynaptic efficacy is determined solely by the functional capacity of the vesicles residing within the terminal and suggests instead that presynaptic performance can be impacted by transmitter residing at extra-synaptic loci.

2.1.5. Excitatory and inhibitory Transmission

Synaptic excitatory transmission was first studied at the frog neuromuscular junction using intracellular recordings to measure depolarising electrical changes (Fatt & Katz, 1951). Later work on the synaptic mechanisms of spinal motor neurons gave fundamental insight into excitatory and inhibitory synaptic transmission in the central nervous system (Coombs *et al*., 1955a, b; Curtis & Eccles, 1959; Eccles, 1959). These studies provided direct electrophysiological evidence that transmission could be either excitatory or inhibitory, resulting in either an increase or a decrease in the probability of postsynaptic action potential firing. Excitatory inputs generate small depolarisations called excitatory postsynaptic potentials (EPSPs) and inhibitory inputs generate hyperpolarising potentials denoted inhibitory postsynaptic potentials (IPSPs). Central neurons receive both excitatory and inhibitory inputs and it is the summation and integration of the resulting EPSPs and IPSPs by a neuron that ultimately determines the nature of its output (Koch *et al*., 1983; Larkum *et al*., 2009). Also, in addition to IPSPs there exists another form of inhibition called 'presynaptic inhibition', in which the inhibitory action occurs in the presynaptic pathway and reduces the probability of neurotransmitter release (Eccles, 1961).

It is important to note that whether a synapse is excitatory or inhibitory is not determined by the type of transmitter released from the presynaptic neuron, but rather the type of postsynaptic ion channels gated by the transmitter and in particular the electrochemical gradient that exists across the membrane. Whilst it is common to refer to a particular transmitter as either excitatory or inhibitory, particular neurotransmitters exist (for example acetylcholine, [McCormick, 1992]) that can produce both excitatory and inhibitory effects, depending on the type of postsynaptic receptor activated or the electrochemical gradient (Kuhlenbeck, 1967).

2.2. Hippocampal Neuroanatomy and Neurotransmission

2.2.1. Hippocampal formation

In the rodent, the hippocampal formation is a C-shaped structure that is situated in the caudal part of the brain, with one terminal positioned dorsally near the septal nuclei and the other caudoventrally near the temporal lobe (Figure 2.5). Early studies using Golgi staining established the basic hippocampal circuitry (Ramón y Cajal, 1911; Lorente de No, 1934) and have since been elaborated upon using neuroanatomical tract-tracing techniques (Záborszky *et al*., 2006). Recent advances in viral tracers (Wickersham *et al*., 2007) and genetic analysis (Luo *et al*., 2008) provide further scope for the precise mapping of hippocampal circuits.

The hippocampal formation comprises four cortical areas: the entorhinal cortex, the dentate gyrus, the hippocampus proper and the subicular complex. The hippocampus proper is further divided into three sub-fields (cornu Ammonis fields CA1, CA2 and CA3) based upon the nomenclature of Lorente de No (1934). Circuitry in the hippocampal formation is unique, as all four cortical areas are linked by a largely unidirectional excitatory pathway called the *trisynaptic circuit* (Figure 2.5). Briefly, neurons of the entorhinal cortex excite dentate gyrus granule cells via the perforant path. Mossy fibres from the dentate granule cells then activate CA3 pyramidal neurons, which give rise to collateralised axons (Schaffer collaterals), which in turn excite CA1 pyramidal neurons. CA1 pyramidal neurons relay information back to the entorhinal cortex via the subiculum.

2.2.1.1. The entorhinal cortex

The entorhinal cortex receives inputs from a number of different cortical regions and has its major output via the perforant path fibres, which ultimately interconnect the neocortex and the hippocampal formation (Bartesaghi *et al*., 2006). The entorhinal cortex therefore plays an important role in information flow through the hippocampal formation, acting as both the entry point for sensory information into the hippocampus and the exit point for processed information to be relayed back to the neocortex (Insausti & Amaral, 2008). Cortical inputs to the entorhinal cortex are divided into two groups: those that terminate in the superficial layers of the entorhinal cortex and are a direct source of input to the hippocampal formation and those distributed to the deep layers that influence entorhinal cortical output.

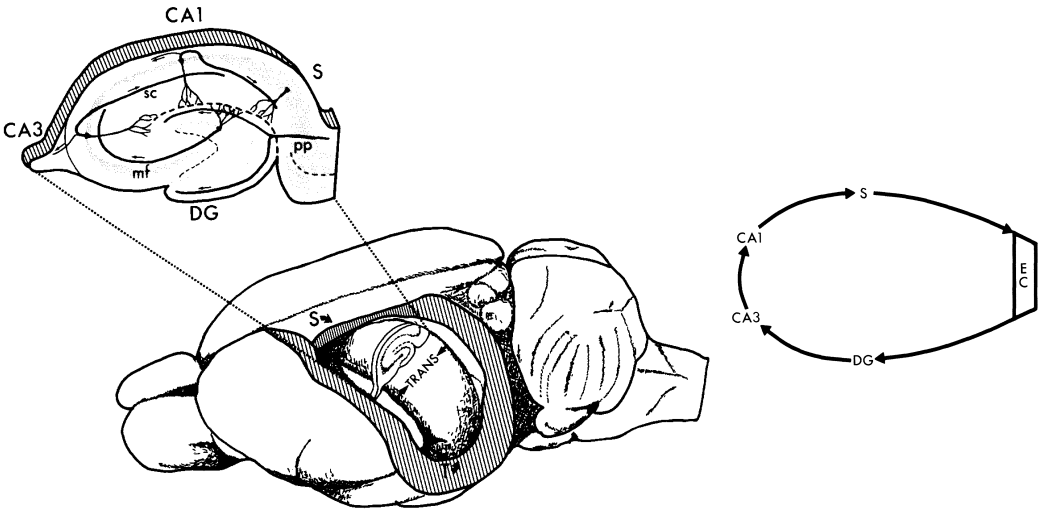

Figure 2.5. The hippocampal formation in the rodent brain. With the overlying cortex removed the hippocampus can be seen as a C-shaped structure. A transverse slice depicts the major fields of the hippocampal formation (excluding the entorhinal cortex). A schematic of the trisynaptic circuit is shown on the right, with information flow directed by arrows. Information flows from the entorhinal cortex, through the hippocampus proper and back to the entorhinal cortex. DG, dentate gyrus; mf, mossy fibres; pp, perforant path; sc, Schaffer collaterals; EC, entorhinal cortex; S, subiculum. Modified from Amaral & Witter (1989).

Substantial cortical inputs to the entorhinal cortex include those from olfactory structures (for example the olfactory bulb, the anterior olfactory nucleus and the piriform cortex; Krettek & Price, 1977; Luskin & Price, 1983; Boeijinga & van Groen, 1984; Biella & de Curtis, 2000) and the laterally adjacent perirhinal and postrhinal cortices (Naber *et al*., 1997; Burwell & Amaral, 1998a, b). The entorhinal cortex also receives significant input from subcortical structures such as the amygdaloid complex, the basal forebrain, the hypothalamus, the thalamus and from the ventral tegmental area of the brain stem (Witter *et al*., 1989; Izquierdo *et al*., 1997). The major output from the entorhinal cortex is via the perforant path fibres (Dolorfo & Amaral, 1998; van Strien *et al*., 2009) which distribute selectively to different regions of the hippocampal formation (Witter, 1993). A particular subset of fibres project almost exclusively to the dentate gyrus and CA3/CA2 (however see also Tamamaki & Nojyo, 1993), whereas another subset projects to CA1 and the subiculum (van Strien *et al*., 2009).

2.2.1.2. The dentate gyrus

Having received the perforant path projection from the entorhinal cortex, neurons in the dentate gyrus in turn provide a prominent input to hippocampal sub-field CA3. The dentate gyrus is divided into three layers: the molecular layer (in which the perforant path fibres terminate), the granule cell layer (which is populated by the principle cell type, the granule cell) and the polymorphic layer (which is populated by several neuronal cell types; Amaral & Witter, 1989). The granule cells give rise to axons called 'mossy fibres' that are so named because of their abundance of large terminal boutons along the axon length, giving them a mossy appearance. These fibres collateralise in the polymorphic layer before entering the CA3 field where they form *en passant* synapses on the proximal dendrites of the pyramidal cells (Blackstad *et al*., 1970; Gaarskjaer, 1978; Claiborne *et al*., 1986; Gaarskjaer, 1986).

2.2.1.3. The hippocampus proper

The hippocampus proper is classically subdivided into three fields (CA3, CA2 and CA1), based upon the terminology of Lorente de No (1934). The hippocampus has a particularly well-characterised laminar organisation, which is generally similar for all fields (Figure 2.6). The principal cell layer is called *stratum pyramidale* and is tightly packed in CA1 and more loosely packed in CA2 and CA3 (Ishizuka *et al*., 1995). The basal dendrites of pyramidal cells descend into *stratum oriens*, a relatively acellular layer, containing different classes of interneurons. It is in this layer in which some of the CA3 to CA3 associational connections and CA3 to CA1 connections are located (Ishizuka *et al*., 1995). Superficial to the pyramidal cell layer is a narrow acellular region, the *stratum lucidum*, containing the mossy fibres from the dentate gyrus. This layer is only present in area CA3 (Swanson & Cowan, 1975). The *stratum radiatum* is located superficially to the *stratum lucidum* in CA3 and immediately above the pyramidal cell layer in CA2 and CA1. This suprapyramidal region is where the majority of CA3 to CA3 associational connections and CA3 to CA1 Schaffer collateral connections are located (Swanson & Cowan, 1975). The most superficial layer of the hippocampus is the *stratum lacunosum-moleculare* and is the site of termination of entorhinal cortical fibres (Hjorth-Simonsen, 1971), as well as afferents from other regions such as the nucleus reuniens of the midline thalamus (Bokor *et al*., 2002). The pyramidal cells of CA3 have highly collateralised axons that diverge to form two major pathways. The first is comprised of associational projections that terminate within CA3 and is termed the 'longitudinal association bundle'(Swanson *et al*., 1978). Schaffer

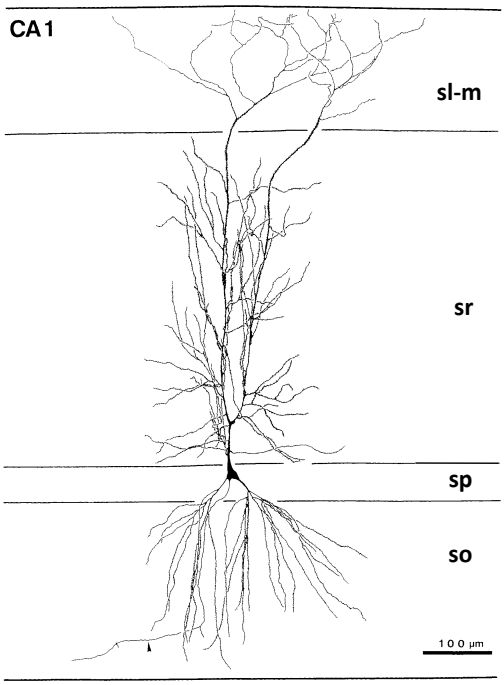

Figure 2.6. Camera lucida drawing of a CA1 pyramidal cell located in the midportion of the field showing the cellular morphology in the different layers. The axon of this neuron is indicated by an arrowhead. so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; sl-m, stratum lacunosum-moleculare. Modified from Ishizuka *et al*. (1995)

collaterals make up the second pathway and form the major projection to CA1. In contrast to CA3 pyramidal cells, cells in CA1 do not communicate in an associational manner, but rather project to the subiculum via large spreading longitudinal fibres (Swanson & Cowan, 1977).

The CA2 hippocampal field has often been the subject of controversy and its existence as an area distinguishable from CA3 and CA1 the subject of some debate. Based upon a number of criteria, including biochemical markers and electrophysiological characteristics (Zhao *et al*., 2001; Lein *et al*., 2004; Lein *et al*., 2005; Zhao *et al*., 2007), it is now generally agreed that area CA2 is distinct from area CA3 and CA1 (Zhao *et al*., 2007). Indeed, recent work demonstrates a unique functional role for area CA2 and suggests that CA2 pyramidal cells may form the nexus of a highly plastic circuit linking the neocortical input to the hippocampal CA1 output (Chevaleyre & Siegelbaum, 2010).

2.2.1.4. The subiculum

The cytoarchitecture of the subiculum is the least studied of all regions of the hippocampal formation (O'Mara et al 2001). The boundary between CA1 and the subiculum is characterised by widening of the pyramidal cell layer and the termination of *stratum radiatum* and *stratum oriens* of CA1 (Witter *et al*., 1989). The subiculum is a predominant source of efferent projections from the hippocampal formation and is the major origin of subcortical connections to the diencephalon and brain stem (via the

postcommissural fornix; Swanson & Cowan, 1975; Donovan & Wyss, 1983; Groenewegen *et al*., 1987; Witter & Groenewegen, 1990; Canteras & Swanson, 1992; Ishizuka, 2001; Kloosterman *et al*., 2003). Fibres from the subiculum project to the presubiculum (Brodmann's area 27, a region adjacent to the subiculum), which then project to the entorhinal cortex, completing the trisynaptic loop (Swanson & Cowan, 1977).

2.2.2. Neurotransmission in the hippocampus

2.2.2.1. Glutamatergic

The main excitatory transmitter in the hippocampus is glutamate (Biscoe & Straughan, 1966; Dolphin *et al*., 1982; Walker *et al*., 1995). Receptors responsive to glutamate can be divided into two broad categories: the ionotropic receptors that directly gate ion channels and the metabotropic receptors that indirectly gate channels through second messengers (Ozawa *et al*., 1998).

2.2.2.1.1. Ionotropic receptors

There are three principle types of receptors that mediate ionotropic excitatory transmission in the hippocampus: α-amino-3-hydroxy-methyl-isoxazole-propionic acid (AMPA), *N*-methyl-*D*-aspartate (NMDA) and kainate receptors, all of which take their names from agonists that activate them in a selective fashion (Watkins & Evans, 1981; Ozawa *et al*., 1998). AMPA and kainate receptors are responsible for fast excitatory transmission at most synapses in the central nervous system, whereas NMDA receptors are involved in slower excitatory synaptic responses and in certain types of synaptic plasticity (Ozawa *et al*., 1998). At most excitatory hippocampal synapses excitatory postsynaptic currents (EPSCs) are mediated by AMPA and NMDA receptors, which exhibit different biophysical and pharmacological properties.

AMPA receptors (AMPARs)

AMPARs are cation-selective channels that are predominantly permeable to Na^+ and K^+ and can be permeable to Ca^{2+}, depending on the subunit composition (Ozawa *et al*., 1998). These receptors mediate fast excitatory transmission in the hippocampus and activate and subsequently deactivate rapidly (Colquhoun *et al*., 1992; Jonas *et al*., 1993). AMPARs are either homomeric or heteromeric oligomers composed of multiple subunits.

NDMA receptors (NMDARs)

NMDARs are cation-selective channels that mediate excitatory transmission in the hippocampus, but exhibit much slower opening and closing kinetics than AMPARs due to their slow unbinding rate (Lester *et al*., 1990). In addition to their slow kinetics, there are three other properties of NMDARs that are important to note. First, they are highly permeable to Ca^{2+} ions (Ascher & Nowak, 1988) and Ca^{2+} influx via NMDARs plays a central role in initiating intracellular signalling pathways important for long-term plasticity (Cavazzini *et al*., 2005). Second, allosteric modulatory agonist-binding sites exist for glycine (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988; Thomson, 1989; Xu & Gong, 2010), protons (Vyklický *et al*., 1990) and Zn^{2+} (Choi & Lipton, 1999). Third, Mg^{2+} ions block the channel in a voltage-dependent manner (Nowak *et al*., 1984), therefore at resting membrane potentials (more negative than approximately -50 mV), NMDARs are unable to mediate ion flux. These receptors therefore act as 'coincidence detectors' (Cooke & Bliss, 2006), whereby Ca^{2+} influx only occurs upon simultaneous presynaptic glutamate release and postsynaptic depolarisation. The significant implications of this phenomenon for long-term plasticity will be discussed in greater detail in *Section 2.4.2. Long-Term Potentiation – LTP properties*.

Kainate receptors

Although kainate receptors share many biophysical characteristics with AMPA receptors, the physiological role of kainate receptors remains uncertain (Feldmeyer & Cull-Candy, 1994; Contractor *et al*., 2011). Despite the abundance of kainate receptors on hippocampal dendrites (Petralia *et al*., 1994; Siegel *et al*., 1995), their inability to participate in fast synaptic transmission has led to debate over their function. Recent advances speculate that kainate receptors may be key modulators of network activity by regulating inward current (Melyan *et al*., 2002; Fisahn *et al*., 2005; Ruiz *et al*., 2005) and excitatory and inhibitory neurotransmitter release (Huettner, 2003; Lerma, 2003). Despite such advances, the exact role of these receptors remains speculative.

2.2.2.1.2. Metabotropic receptors

Metabotropic receptors contain seven transmembrane segments and are coupled to G-proteins that mediate the majority of their actions (Schoepp & Conn, 1993). There are three classes of glutamatergic metabotropic receptors, denoted Group I – III. Group I receptors (mGluR1 and mGluR5) generally localise to postsynaptic membranes and

exert their actions via activation of phospholipase C (Niswender & Conn, 2010). Group II receptors (mGluR2 and mGluR 3) and Group III receptors (mGluR4, mGluR6-8) are typically located on presynaptic membranes and operate via modulation of adenylyl cyclase pathways (Niswender & Conn, 2010).

2.2.2.2. GABAergic

Inhibitory transmission in the hippocampus is mediated predominantly by γ-aminobutyric acid (GABA), which acts on two receptor types, $GABA_A$ and $GABA_B$.

$GABA_A$ receptors are ionotropic receptors that are selectively permeable to anions, with the majority of current being carried by Cl^- ions (Kaila, 1994). As neurons are normally depolarised relative to the Cl^- reversal potential, $GABA_A$ receptor-mediated postsynaptic currents are generally hyperpolarising (Gonzalez-Burgos & Lewis, 2008).

$GABA_B$ receptors are metabotropic receptors that exist at postsynaptic and presynaptic membranes, a well as at extrasynaptic sites (Fritschy *et al*., 1999). Postsynaptic activation of these receptors leads to the opening of G-protein gated inward-rectifying K^+ (GIRK) channels (Andrade *et al*., 1986; Misgeld *et al*., 1995). GIRK channel activation results in a slow IPSP that is easily distinguished from the $GABA_A$-mediated IPSP both by its slow kinetics and its independence of the Cl^- reversal potential. Presynaptically, $GABA_B$ receptors are coupled to voltage-gated Ca^{2+} channels via a G protein cascade (Anwyl, 1991; Mintz & Bean, 1993) and therefore play a role in recurrent modulation of transmitter release.

2.2.2.3. Other transmitters

Other transmitters are also involved in transmission in the hippocampus, although are present at far fewer synapses in comparison with glutamate and GABA. Substances such as ATP, noradrenaline, dopamine, serotonin, histamine, acetylcholine, neuropeptides and somatostatin are known to be released from presynaptic terminals from various regions in the hippocampal formation (Cole & Nicoll, 1983; Hopkins & Johnston, 1984; Gall *et al*., 1990; Markram & Segal, 1990; Pankratov *et al*., 1998), however relatively less is known about their mode of release and postsynaptic actions. Nevertheless, these substances are undoubtedly important for modulation of hippocampal function by the various extrahippocampal circuits.

2.2.3. Variation between rodent and human hippocampal neuroanatomy

As most work on hippocampal neurophysiology is undertaken in rodents with the aim of understanding more about the human brain, it is pertinent to note the subtle

differences between primate and rodent hippocampi. The pyramidal cell layer in the CA1 region of the rat is thicker and more heterogeneous than in the human (Seress, 2007). Some differences in the connectivity are also apparent. In the rodent there exists a large network of commissural connections in the dentate gyrus that accounts for nearly one sixth of its excitatory drive (Gottlieb & Cowan, 1973). However in the macaque monkey (and perhaps in humans), the dentate commissural connections are almost entirely absent (Amaral *et al*., 1984). Despite these differences, the overwhelming similarities suggest the rodent hippocampus is an appropriate model to aid further understanding of primate hippocampal function.

2.2.4. Hippocampal firing rhythms

Studies in the late 1930s identified regular large amplitude waves of electrical activity in the rat hippocampus (Jung & Kornmuller, 1938). Further investigation of this so-called theta activity in anaesthetised cats, rabbits and monkeys demonstrated its inverse correlation with neocortical desynchronisation, suggesting that it represented the hippocampal arousal pattern (Green & Arduini, 1954). The theta rhythm is observed as a prominent 4-10 Hz rhythm in the hippocampal local field potential of all mammals studied to date (Green & Arduini, 1954; Vanderwolf, 1969; Winson, 1972; Arnolds *et al*., 1980) and occurs as a travelling wave through the hippocampal formation (Buzsaki, 2002; Lubenov & Siapas, 2009; Düzel *et al*., 2010). Theta oscillations are essential for normal hippocampal function and manipulations that disrupt them produce behavioural impairments that mimic hippocampal lesions (Winson, 1978; Mitchell *et al*., 1982).

Theta oscillations depend on ongoing behaviour and are most consistently present during various modes of locomotor activities and exploration (Vanderwolf, 1969; Benchenane *et al*., 2010). Theta activity is associated with mnemonic processes (Lisman & Idiart, 1995; Raghavachari *et al*., 2001) and is important for the induction of plasticity during encoding, as well as the consolidation and retrieval of stored memories (Buzsaki, 2002; Rutishauser *et al*., 2010). This is supported by findings that theta-frequency stimulation is optimal for inducing long-term changes in synaptic efficacy and that bidirectional modifications of synaptic strength can be induced by brief periods of theta stimulation (Pavlides *et al*., 1988; Huerta & Lisman, 1993; Martin *et al*., 2000).

2.3. Hippocampal Synaptic Plasticity

2.3.1. Overview of synaptic plasticity

The dynamic nature of neuronal networks was first demonstrated in cat motor neurons, in which it was shown that the efficacy of synaptic connections could be modulated (Lloyd, 1949; Eccles & Rall, 1950; Eccles & McIntyre, 1953). Such plasticity has been the subject of much research in the ensuing years, particularly as a result of its potential involvement in learning and memory. Due to the emergence of the hippocampus as a putative memory-encoding structure, the majority of work on synaptic plasticity has focused on hippocampal long-term plasticity. Plastic changes can involve either a strengthening or weakening of transmission and the persistence of these changes is either short- or long-term in duration. A weakening of transmission is referred to as *depression* and is characterised by a reduction of synaptic efficacy in either the short- or long-term. In contrast, a strengthening of transmission is referred to as either *facilitation* or *potentiation*, depending on whether the changes persist in the short- or long-term respectively. While most synapses display a combination of both facilitation and depression, one response dominates to produce a notable change in individual synaptic strength (Zucker, 1989).

2.3.2. Short-term plasticity

Short-term plasticity is important in the information processing function of synapses, allowing them to act as either high-pass or low-pass filters, depending on their initial P_r (Abbott & Regehr, 2004). To date at least three types of short-term plasticity are known to exist, each distinguishable by degree of persistence. Paired-pulse facilitation (PPF) is one example of short-term plasticity and is elicited by delivering a pair of stimuli to the presynaptic cell at short inter-stimulus intervals (tens to hundreds of milliseconds; Zucker & Regehr, 2002). When two pulses are delivered to a terminal in rapid succession, the second pulse can result in an enhancement of transmitter release and hence an increased postsynaptic response (Figure 2.7). The classical explanation for this facilitation is the so-called 'residual Ca^{2+} hypothesis', which posits that residual Ca^{2+} in the terminal from the first pulse summates with additional Ca^{2+} entering during the second pulse to elicit an increase in the amount of transmitter released (Santschi & Stanton, 2003). Alternatives to this hypothesis include the suggestion that PPF arises from the partial saturation of a Ca^{2+} buffer following the first pulse, leaving less available to buffer Ca^{2+} after the second (Rozov *et al*., 2001). Despite differing

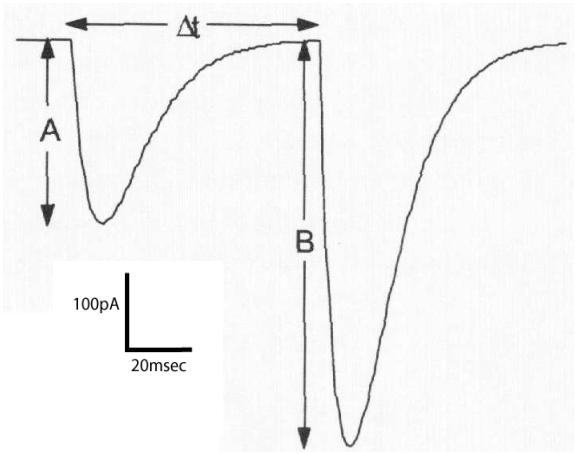

Figure 2.7. Paired-pulse facilitation. Delivery of a pair of stimuli separated by an interval of Δt results in the postsynaptic current in response to the second pulse (B) being larger than the current in response to the first pulse (A). Modified from Zucker & Regehr (2002).

opinions on the underlying mechanisms it is generally agreed that PPF is purely presynaptic and it has therefore proved useful in measurements of changes in presynaptic efficacy at central hippocampal synapses (Schulz *et al*., 1994, 1995; Sokolov *et al*., 1998; Sokolov *et al*., 2002).

Augmentation is another form of facilitation that is Ca^{2+}-dependent (Swandulla *et al*., 1991) and usually persists for 1-7 seconds (Magleby & Zengel, 1975; Thomson, 2000). Post-tetanic potentiation is a third form of facilitation that requires relatively high frequency or tetanic firing for its induction and decays more slowly than the other forms of facilitation mentioned (over a period of tens of seconds or minutes; Liley, 1956; Hubbard, 1963). Post-tetanic potentiation is thought to result from the Ca^{2+}-dependent mobilisation of the RP of vesicles, increasing the number of vesicles available for release (Humeau *et al*., 2001).

2.3.3. Long-term plasticity

Several forms of long-term synaptic plasticity have been identified, including long-term potentiation (LTP; Blundon & Zakharenko, 2008), long-term depression (LTD; Collingridge *et al*., 2010), depotentiation (Collingridge *et al*., 2010), homeostatic plasticity (Turrigiano & Nelson, 2004; Pozo & Goda, 2011) and metaplasticity (Abraham, 2008; Mockett & Hulme, 2008). In this discussion the most widely studied forms of long-term plasticity, LTP and LTD, will be summarised.

2.3.3.1. LTP

LTP is defined as an activity dependent and persistent increase in the postsynaptic response to a given stimulus and can persist for hours, days or weeks (Bliss, 2003) and has been recorded for as long as a year in behaving rats (Abraham *et al*., 2002). LTP in region CA1 of the hippocampus is a model paradigm for investigating the mechanisms

of long-term plasticity and its role in learning and memory in the brain. As the issues surrounding LTP form the crux of this manuscript, a separate chapter has been devoted to the discussion of the mechanisms and characteristics of different forms of LTP (See *Section 2.4 – Long-Term Potentiation*).

2.3.3.2. LTD

LTD is defined as an activity-dependent, long lasting decrease in synaptic strength (Collingridge *et al*., 2010). There are many different forms of LTD, with the most frequently studied being an NMDAR-dependent form. NMDAR-dependent LTD in area CA1 is typically induced by prolonged, repetitive LFS (approximately 900 stimuli at 1 Hz; Dudek & Bear, 1992; Mulkey & Malenka, 1992), although the number of stimuli can be reduced and the frequency changed if the postsynaptic neuron is moderately depolarised (to approximately -50 mV), partially relieving the Mg^{2+} block of the NMDAR (Selig *et al*., 1995). Calcium influx into the postsynaptic spine during the LFS binds a Ca^{2+}-binding protein called calmodulin (CaM), leading to the activation of protein phosphatase signalling pathways (Mulkey *et al*., 1993). These signalling pathways trigger predominantly postsynaptic changes (such as removal of AMPARs from the synapse; Collingridge *et al*., 2004), although presynaptic alterations such as reduced probability of glutamate release have also been noted (Stanton *et al*., 2003).

2.3.4. Polarity of plasticity

An important advance in the study of long-term synaptic plasticity was the establishment of an experimentally reproducible form of NMDAR-dependent LTD at excitatory synapses on hippocampal CA1 pyramidal cells at which LTP had already been studied (Dudek & Bear, 1992). This demonstrated for the first time that synaptic strength could be controlled in a bidirectional manner, supporting the notion that memories are encoded by changes in synaptic weighting not simply by LTP. This bidirectionality of neuronal strength has lead to the idea that LTP and LTD are essentially 'flip sides of the same coin' (Philpot & Bear, 2002), with each phenomenon combining to induce either a reduction or an increase in the strength of transmission at a particular synapse. Whilst it has been suggested in the past that LTP and LTD are simply the reversal of one another (Bear & Malenka, 1994), it is now apparent that the situation is more complex than this and that LTD plays important roles in learning and memory that extend beyond acting solely as a means of reversing LTP (Ge *et al*., 2010).

An important question raised from studies on LTP and LTD is how can both phenomena be induced from the same signal of NMDAR-dependent Ca^{2+} influx? The simplest model used to explain this is called the *minimum threshold hypothesis*, which is based upon the greater affinity for Ca^{2+} of protein phosphatases relative to protein kinases (Cavazzini *et al*., 2005). In this model low levels of Ca^{2+} lead to the dephosphorylation of key proteins and LTD and high levels of Ca^{2+} result in the phosphorylation of key proteins and LTP (Lisman, 1994). Consistent with this hypothesis, controlled iontophoresis of glutamate onto CA1 dendrites demonstrates that LTD induction has a threshold Ca^{2+} increase of approximately 180 nM, whilst LTP induction has a higher threshold of approximately 540 nM (Cormier *et al*., 2001). The validity of this hypothesis remains the subject of some dispute (Yang *et al*., 1999) and it seems that the determinant of plasticity polarity is perhaps more intricate than suggested.

2.4. Long-Term Potentiation

2.4.1. Overview

The first description of LTP arose in the early 1970s and documented the potentiation of EPSPs recorded from populations of granule cells in the dentate area of rabbit hippocampi in both anaesthetised and behaving animals following repetitive stimulation of the perforant path (Bliss & Gardner-Medwin, 1973; Bliss & Lomo, 1973). This work in conjunction with Hebb's theory of learning and surgical hippocampal lesion studies led to the conclusion that LTP in the hippocampus is vital for the formation of certain types of memory.

2.4.2. LTP properties

In the late 1940s Donald O. Hebb presented a clearly defined theory for the neural basis of learning and memory. He proposed that memories are formed by a process of synaptic modification that results in a strengthening of connections when presynaptic activity correlates with postsynaptic firing. His proposal is now called 'Hebb's postulate of learning' and can be summarised by one sentence:

"When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased"

(Hebb, 1949)

This postulate was famously reinforced by electrophysiological data that reported a Hebb-like synaptic enhancement following repeated bouts of HFS (Bliss & Gardner-Medwin, 1973; Bliss & Lomo, 1973) and has since been supported by a multitude of electrophysiological, pharmacological and behavioural studies. LTP is described as a Hebbian learning mechanism because it exhibits four critical Hebbian properties: cooperativity, associativity, input specificity and persistence.

2.4.2.1. Cooperativity

Cooperativity refers to the requirement that a threshold level of input must be met for successful LTP induction (Bliss & Gardner-Medwin, 1973). This threshold can be reached through intense stimulation of a single afferent fibre, or cooperatively via lower intensity stimulation of many afferent fibres (McNaughton *et al*., 1978). The basis for cooperativity is accounted for by the postsynaptic NMDAR and its biophysical

properties that only allow for the conduction of Ca^{2+} when the membrane is sufficiently depolarised (Citri & Malenka, 2008).

2.4.2.2. Associativity

Associativity is a consequence of cooperativity and refers to the observation that stimulation of two anatomically distinct pathways converging on the same cell can be sufficient for the induction of LTP, yet stimulation of each pathway individually is insufficient (Levy & Steward, 1979; Barrionuevo & Brown, 1983; Levy & Steward, 1983; Bliss & Collingridge, 1993). The two inputs therefore act associatively to surpass the cooperativity threshold and induce LTP. Both cooperativity and associativity are explained by the necessity for a sufficient level of postsynaptic activity and presumably reflect the summation of multiple postsynaptic Ca^{2+} signals (Shors & Matzel, 1997). The existence of cooperativity and associativity are particularly important as they indicate that physiologically relevant levels of stimulation exist that can induce LTP (Shors & Matzel, 1997).

2.4.2.3. Input specificity

Input specificity refers to the observation that LTP is elicited only at active synapses and that afferent input does not induce LTP at adjacent inactive synapses on the same postsynaptic cell (Andersen *et al*., 1977). This finding conforms to Hebb's model of learning in which synaptic enhancement is restricted to those synapses that participate in firing the postsynaptic cell. However this specificity may break down for synapses that are spatially close (on the order of tens of microns apart), most likely due to the lateral spread of an LTP-inducing messenger molecule (Engert & Bonhoeffer, 1997).

2.4.2.4. Persistence

If LTP is indeed the physiological engram of memory, then it must be persistent. Persistence is therefore often thought of as the most crucial requirement of all of the Hebbian properties of learning. Hippocampal LTP has been recorded for as long as a year in behaving rats (Abraham *et al*., 2002) and is known to persist for many hours in the *in vitro* setting (Frey *et al*., 1988). Research into the mechanisms by which LTP is able to persist in the long-term is crucial for the understanding of synaptic plasticity and memory and is discussed in greater detail in *Section 2.4.5. – Long-term potentiation – LTP maintenance*.

2.4.3. LTP induction

Induction refers to both the stimulation parameters that are required to initiate LTP, as well as the initial physiological processes that trigger the molecular changes underlying LTP.

2.4.3.1. Stimulation parameters

A variety of different stimulation protocols have been used to induce LTP in the hippocampus, ranging from brief (20-40 ms) 400 Hz trains (Douglas & Goddard, 1975), to 1 second trains at 100 Hz (Cavus & Teyler, 1996). Other protocols involve pairing presynaptic stimuli with postsynaptic depolarisation (Chen *et al*., 1999) or using chemically induced depolarisation (Zakharenko *et al*., 2001). However the physiological significance of these protocols is somewhat questionable. A more physiologically relevant protocol that has gained acceptance in recent years is theta-burst stimulation (TBS), which involves mimicking the high frequency burst firing normally exhibited by hippocampal neurons in synchronisation with the theta rhythm (Thomas *et al*., 1998). Protocols such as these are known to be optimal for the induction of LTP in area CA1 (Larson *et al*., 1986; Staubli & Lynch, 1987; Otto *et al*., 1991; Hernandez *et al*., 2005).

An important feature of LTP induced by TBS is the dependence of LTP magnitude on the number of TBS repetitions (Figure 2.8). Successive increases in the number of TBS trains induce LTP of increasing magnitude until a certain point, after which further repetitions result in progressively less LTP. This so-called 'over-stimulation' of LTP is thought to result from depotentiation processes (LTP reversal) mediated by adenosine and highlights the sensitivity of LTP to previous synaptic stimulation (Abraham & Huggett, 1997).

2.4.3.2. Physiological processes

LTP induction refers to the early physiological processes that trigger the molecular changes underlying LTP. The canonical view of LTP induction at glutamatergic synapses is that it requires an increase in postsynaptic Ca^{2+} concentration via the NMDAR (Cavazzini *et al*., 2005). However Ca^{2+} influx from other sources is also important for induction of particular types of LTP (Raymond, 2007). For example, non-NMDAR-dependent LTP can be induced in area CA1 provided that the tetanus (or postsynaptic depolarisation) is of sufficient intensity to activate voltage-gated Ca^{2+} channels (VGCCs; Grover & Teyler, 1990; Kullmann *et al*., 1992). Also, LTP induced

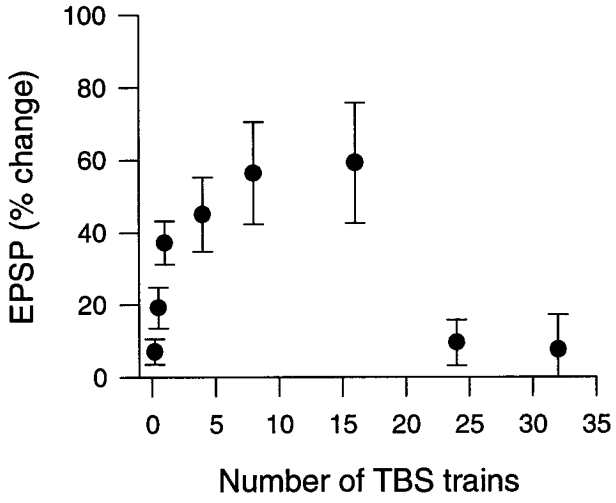

Figure 2.8. LTP induction function. The magnitude of LTP (as evidenced by the EPSP magnitude 1 hr post-TBS) is maximal after induction by 8-16 trains and becomes progressively less with increasing trains due to the effect of 'over-stimulation'. Taken from Abraham and Huggett (1997).

at the mossy fibre-CA3 pathway is independent of NMDARs and instead requires Ca^{2+} influx via VGCCs – although some debate exists regarding whether the critical Ca^{2+} signal occurs presynaptically (Castillo *et al*., 1994; Weisskopf *et al*., 1994) or postsynaptically (Williams & Johnston, 1989; Johnston *et al*., 1992). Whilst NMDARs may not be essential for all forms of LTP, it seems that Ca^{2+} is a critical player, consistent with its role in many cellular modifications believed to underlie conditioned behaviour responses (Walters & Byrne, 1985; Abrams & Kandel, 1988; Falk-Vairant & Crow, 1992; Matzel & Rogers, 1993). The increased Ca^{2+} concentration as a result of LTP induction results in the initiation of many different signalling pathways, the majority of which have the common end effect of modulating protein synthesis (Benito & Barco, 2010).

2.4.3.2.1. NMDAR-dependent Ca^{2+} influx

A defining feature of LTP is its dependence on postsynaptic Ca^{2+} influx. Early experiments showed that LTP induction is prevented by a pretetanus injection of Ca^{2+} chelators (Lynch *et al*., 1983; Malenka *et al*., 1988) and that induction occurs when the postsynaptic cell is artificially loaded with Ca^{2+}, elevating its concentration (Malenka *et al*., 1988; Malenka *et al*., 1989b). For many forms of LTP the primary source of Ca^{2+} influx during induction is via the NMDAR.

Many of the features of LTP that make it attractive as a memory and learning mechanism can be attributed to the properties of the NMDAR. For example, the basis of cooperativity (that sufficient presynaptic input and thereby postsynaptic activity) is explained by the proviso that the NMDAR is only active when the postsynaptic membrane is sufficiently depolarised and presynaptically released glutamate is bound. The property of associativity can also be explained by the NMDAR and the ability of

local changes in membrane potential to spread to neighbouring dendritic regions. Therefore EPSPs originating locally and distally can combine to sufficiently depolarise the membrane to activate the NMDAR. This allows for LTP to be induced at synapses receiving weak stimulation that is concurrent with the delivery of higher frequency stimulation to other inputs. Input specificity is also explained by the NMDAR, as glutamate release in conjunction with postsynaptic depolarisation is required for LTP and therefore only active inputs will be potentiated. Although activation of the NMDAR is crucial for many forms of LTP, it is not essential for all.

2.4.3.2.2. Voltage-gated Ca^{2+} influx

LTP can be induced in area CA1 following tetanic stimulation in the presence of D-APV (an NMDAR blocker; Grover & Teyler, 1990) and VGCCs are critical for persistent forms of LTP induced by repetitive bouts of TBS-patterned stimulation (Raymond & Redman, 2002, 2006; Raymond, 2007).

The identity of the VGCC type remains the topic of some debate and is likely to vary depending on the stimulation patters and type of preparation used. There is evidence for roles of both L-type (Grover & Teyler, 1990; Morgan & Teyler, 2001; Raymond & Redman, 2002, 2006) and R-type VGCCs (Yasuda *et al*., 2003b). Activation of VGCCs may allow for the influx of sufficient Ca^{2+} to activate the plasticity pathways that are necessary for more persistent forms of LTP. Indeed there is evidence suggesting that Ca^{2+} influx via VGCCs is essential for the recruitment of CREB-binding protein (CBP), a protein essential for the long-term maintenance of certain forms of LTP (Impey *et al*., 1996; Hardingham *et al*., 1999).

2.4.3.2.3. Intracellular Ca^{2+} stores

An additional source of Ca^{2+} in neurons is the endoplasmic reticulum (ER). Activity-dependent Ca^{2+} release from the ER occurs via ryanodine receptors (RyRs) and inositol (1,4,5)-triphosphate receptors (IP_3Rs) (Berridge, 1993; Pozzan *et al*., 1994; Banerjee & Hasan, 2005). RyRs are activated by an increase in intracellular Ca^{2+} and are largely responsible for Ca^{2+}-induced Ca^{2+} release (CICR; Bezprozvanny *et al*., 1991; Fill & Copello, 2002). IP_3Rs are predominantly activated by IP_3 produced by phospholipase C (PLC)-linked GPCRs, for example the Group I mGluRs (Banerjee & Hasan, 2005). In area CA1, IP_3Rs are concentrated on dendritic ER, whereas RyRs are mainly found on the ER of spines (Sharp *et al*., 1993). The two modes of intracellular store-dependent Ca^{2+} release also differ in the speed in which they mobilise Ca^{2+} - IP_3 mediated release is

generally slow compared to CICR (due to the required metabolic step) and therefore higher frequencies of stimulation are required to generate IP_3- mediated Ca^{2+} signals in CA1 neurons (Zhou & Ross, 2002).

Several experiments have demonstrated the importance of intracellular Ca^{2+} stores in the induction of different forms of LTP. Application of thapsigargin (an inhibitor of the ATPase pump required for store Ca^{2+} loading) blocks electrically-induced LTP at CA1 synapses (Harvey & Collingridge, 1992), dentate gyrus (Wang *et al*., 1996) and in various forms of chemically induced LTP (Auerbach & Segal, 1994; Tekkök & Krnjević, 1996). Of particular importance are studies by Raymond and Redman (2002, 2006) showing that different numbers of repetitive TBS trains induced forms of LTP that were mediated by different modes of Ca^{2+} mobilisation from the intracellular store, with one form being dependent on RyR-mediated release, another more persistent form on IP_3R-mediated release and the most persistent form being independent of store-mediated release and dependent on L-type VGCCs (Raymond & Redman, 2002, 2006; Raymond, 2007). These results demonstrate that multiple forms of LTP can coexist at the same set of synapses, with each form dependent on a unique source of Ca^{2+} for its induction.

2.4.3.2.4. Signalling cascades initiated by Ca^{2+}

The most well studied postsynaptic Ca^{2+} cascade in LTP is initiated by CaM, which is activated by the transient rise in intracellular Ca^{2+} during the LTP induction period (Chin & Means, 2000). This induces a conformational change in CaM, exposing hydrophobic residues that promote interaction of the Ca^{2+}/CaM complex with numerous target proteins, thereby regulating their activity (Hook & Means, 2001). A particular family of proteins regulated by Ca^{2+}/CaM are a group of serine/threonine protein kinases known as CaM-kinases (CaMKs). Of these, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has been shown to play a particularly important role in LTP induction (Lisman, 1994; Lucchesi *et al*., 2011). CaMKII is highly abundant in the brain where it is responsible for the phosphorylation of numerous protein substrates, including the GluR1 subunit of the AMPAR (Rongo, 2002; Hédou & Mansuy, 2003). Importantly, CaMKII constitutes the major protein of the postsynaptic density in dendritic spines of excitatory neurons, where it interacts with the NR2 subunit of the NMDAR and is readily activated upon Ca^{2+} influx (Strack *et al*., 1997a; Strack *et al*., 1997b; Colbran, 2004).

Another important signalling cascade initiated by LTP induction is the cAMP messenging pathway. Ca^{2+}/CaM acts on a calmodulin-sensitive adenylate cyclase, stimulating its production of cAMP. cAMP then activates cAMP-dependent protein kinase (PKA), which seems to be important for later phases of LTP involving gene expression, as PKA inhibitors do not disrupt LTP at earlier time points (Frey *et al*., 1993; Matthies & Reymann, 1993). PKA may also indirectly facilitate the effects of CaMKII by decreasing competing protein phosphatase activity (Lisman, 1989; Blitzer *et al*., 1998; Makhinson *et al*., 1999).

The extracellular signal-regulated kinase (Erk)/mitogen-activated protein kinase (MAPK) pathway is also thought to be important for LTP, as well as some forms of learning and memory (Xiong & Ferrell, 2003; Yasuda *et al*., 2003a; Kelleher *et al*., 2004a; Sweatt, 2004; Thomas & Huganir, 2004). The Erk/MAPK pathway is reported to be critical for the initiation of gene transcription that is required for long-term persistence of LTP (Sweatt, 2001) and may also be a point of convergence integrating signals from other kinase cascades (Impey *et al*., 1998; Roberson *et al*., 1999; Vanhoutte *et al*., 1999).

In addition, the protein tyrosine kinases (PTKs) are another family of enzymes that have been strongly implicated in LTP. The PTKs include two families of kinases – one is a family of transmembrane receptor kinases (Trk), activated by growth factors such as brain-derived neurotrophic factor (BDNF), the other is a family of cytosolic kinases with the best characterised example being Src kinase. Src kinase has been implicated in the enhanced NMDAR function during LTP induction (Kalia *et al*., 2004). NMDAR currents are governed by a balance between tyrosine phosphorylation and depohosphorylation and Src enhances these currents by inhibiting endogenous phosphatase activity and activating kinase activity (Wang & Salter, 1994; Kalia *et al*., 2004).

Finally protein kinase C (PKC) and in particular the PKC isozyme PKMζ has received attention because of its rapid expression upon LTP induction and potential maintenance of the late phase of LTP in both *in vitro* and *in vivo* preparations (Hrabetova & Sacktor, 1996; Ling *et al*., 2002; Serrano *et al*., 2005; Pastalkova *et al*., 2006; Sacktor, 2011). PKMζ is constitutively active and may be important for long-term maintenance of LTP via its role in AMPAR trafficking to the postsynaptic membrane (Ling *et al*., 2002; Ling *et al*., 2006; Yao *et al*., 2008).

The task of identifying key cascades in the induction of LTP remains a difficult one. Recent technical advances in mass spectrometric techniques for the profiling of

posttranscriptional modifications of key groups of proteins (Witze *et al*., 2007), as well as RNA interference approaches for the knockdown of putative proteins should help characterise the key LTP molecules and their interplay with one another (Foster *et al*., 2010).

2.4.4. LTP expression

LTP is frequently considered in terms of the molecular mechanisms that directly underlie the increase in synaptic efficacy, namely its expression. The locus of expression could theoretically be presynaptic, postsynaptic, or some combination of the two. The site of expression of LTP remains a matter of intense debate, with evidence for both postsynaptic (reviewed by Yuste & Bonhoeffer, 2001; Malinow & Malenka, 2002; Derkach *et al*., 2007) and presynaptic alterations (reviewed by Lisman & Raghavachari, 2006).

2.4.4.1. Presynaptic vs. postsynaptic expression

2.4.4.1.1. Electrophysiological methods

Classically, electrophysiological approaches have been applied to solve the problem of LTP expression. Two methods used to identify presynaptic changes in expression are quantal analysis and PPF.

Early quantal analysis studies lent support to a presynaptic locus of expression, however subsequent studies disputed these findings and were suggestive of a postsynaptic locus. Initial studies using either minimal stimulation or paired recordings from single CA3 or CA1 neurons showed that the number of failures of the AMPAR-mediated postsynaptic response decreased significantly following LTP induction (Bekkers & Stevens, 1990; Malinow & Tsien, 1990; Bolshakov & Siegelbaum, 1995). According to the assumptions of quantal analysis, this finding is supportive of an increase in the probability of neurotransmitter release. These results were later challenged by the observation that the number of NMDAR-mediated synaptic failures was much lower than that of AMPAR responses and induction of LTP caused little decrease in the number of NMDAR failures (Nicoll & Malenka, 1999; Malinow *et al*., 2000). Moreover, the induction of LTP in some preparations resulted in the rapid appearance of synaptic responses that could be blocked by AMPAR antagonists (Isaac *et al*., 1995; Liao *et al*., 1995).

These observations were explained by the 'silent synapse' hypothesis, which proposed that a significant number of postsynaptic sites lack functional AMPARs, but

contain functional NMDARs (Isaac *et al*., 1995; Kullmann & Siegelbaum, 1995; Liao *et al*., 1995). At the normal resting potential NMDARs are blocked by Mg^{2+}, therefore synapses lacking AMPARs are postsynaptically silent, as they are unable to be sufficiently depolarised to relieve the Mg^{2+} block. Extensive research has shown that induction of LTP leads to the insertion of new AMPARs in the membrane (Lissin *et al*., 1999; Shi *et al*., 1999; Yang *et al*., 2008) and it is argued that this process decreases the number of silent synapses. Therefore, according to the silent synapse hypothesis, LTP is purely a postsynaptic phenomenon.

However several discrepancies exist between this hypothesis and other published data. The presence of AMPARs in apparently silent synapses has been confirmed (Kimura *et al*., 1997; Choi *et al*., 2000; Gasparini *et al*., 2000; Renger *et al*., 2001; Maggi *et al*., 2003) and similar LTP of NMDAR- and AMPAR-mediated responses have been reported in some studies (Bashir *et al*., 1991; Berretta *et al*., 1991; Bayazitov & Kleschevnikov, 2000; Bayazitov *et al*., 2002). Other work has led to the suggestion that silent synapses result from low transmitter release, rather than lack of functional AMPARs and that the majority of synapses are presynaptically, rather than postsynaptically silent (reviewed by Voronin & Cherubini, 2003; Voronin *et al*., 2004).

Results from investigations into PPF changes have also proven inconsistent, with some studies reporting no change in the level of PPF (McNaughton, 1982; Muller & Lynch, 1989; Zalutsky & Nicoll, 1990; Foster & McNaughton, 1991; Manabe *et al*., 1993), supporting postsynaptic expression, and others reporting a decrease in the level of PPF (Kuhnt & Voronin, 1994; Schulz *et al*., 1994, 1995; Sokolov *et al*., 1998), supporting presynaptic LTP expression.

2.4.4.1.2. Optical methods

The advancement of imaging techniques has allowed for more direct measurements of presynaptic function during LTP to be conducted. Fluorescent markers can be specifically expressed in either a presynaptic terminal or a postsynaptic dendritic spine and are therefore less biased towards the postsynaptic cell than classical electrophysiological techniques. These techniques are also advantageous as they do not rely on somatic recordings that are influenced by multiple synaptic inputs.

The first optical investigation into exocytotic/endocytic cycling during LTP in hippocampal culture measured the differential uptake of fluorescently tagged antibodies to the intraluminal domain of the synaptic vesicle protein synaptotagmin (Malgaroli *et al*., 1995). This study demonstrated that there was a heterogeneous presynaptic

component to LTP, with a greater degree of potentiation at synapses at which the initial rate of vesicle turnover was low. A subsequent culture study was the first to assay presynaptic function during LTP using the styryl dye FM 1-43 and opposed results from the previous optical study demonstrating that brief tetanic stimuli induced a potentiation that occurred equally at boutons of low and high initial release probabilities (Ryan *et al*., 1996).

The first FM 1-43 studies of presynaptic function in hippocampal slices came from Zakharenko *et al*. (2001) and Stanton *et al*. (2001). They reported an enhancement of transmitter release during LTP (Zakharenko *et al*., 2001) and a reduction during LTD (Stanton *et al*., 2001). A particularly interesting observation from the study by Zakharenko *et al*. (2001) was that the presynaptic enhancement was shown only to occur when LTP was induced with either 200 Hz or TBS protocols, but not with lower frequency (50 Hz) stimulation. Strong protocols such as 200 Hz stimulation are known to induce a form of 'compound' LTP, which consists of both NMDAR-dependent and independent components (Grover & Teyler, 1990). Antagonists of L-type VGCCs only partially inhibited the compound LTP, but completely eliminated the enhanced transmitter release (Zakharenko *et al*., 2001). These were the first results demonstrating that LTP can consist of both presynaptic and postsynaptic components, with the presynaptic component depending on L-type VGCCs and the postsynaptic component dependent on NMDARS.

Further advances in the optical monitoring of presynaptic LTP came with the emergence of pHlourin-based indicators. A disadvantage of FM dyes is that they cannot be used for continuous monitoring of presynaptic function, as the dye is lost to the extracellular medium upon exocytosis (see *Methods Chapter 3.5.1. for a discussion of FM 1-43 kinetics*). This problem has been overcome with the advent of pHlourins – pH sensitive GFP molecules linked to synaptic vesicle proteins (Sankaranarayanan *et al*., 2000). A pHlourin probe of particular relevance is synaptopHlourin (spH; pHlourin linked to the synaptic vesicle protein VAMP-2) which changes its fluorescence from low, when inside the acidic lumen of the synaptic vesicle, to high when it becomes exposed to the less acidic extracellular medium during exocytosis. The probe is then re-internalised during endocytosis and fluorescence is quenched upon re-acidification of the vesicle lumen. In this way, changes in fluorescence responding to changes in release can be monitored over repeated bouts of exocytosis and endocytosis. Bayazitov *et al*. (2007) recently monitored changes in presynaptic function over several hours before and after LTP induction in mouse models expressing spH in a subset of

hippocampal pyramidal neurons. Results from this study demonstrated that changes in presynaptic function were slow in onset (taking approximately 35 min to develop), yet persistent in the long-term (lasting for as long as 3 hours post induction). These experiments were the first to demonstrate that changes in presynaptic and postsynaptic function during LTP are temporally separated. This result may help explain some of the discrepancies that exist in the literature with regard to LTP expression as presynaptic components seem to exist only at late time-points when induced by 'strong' stimulation protocols.

2.4.4.2. Role of retrograde messengers in LTP expression

The finding that LTP induction requires a postsynaptic increase in Ca^{2+} and that some forms of LTP involve a presynaptic expression component suggests a role for a 'retrograde messenger', conveying the changes in postsynaptic signalling in the dendrite to the presynaptic terminal (reviewed by Regehr *et al*., 2009). Various diffusible molecules have been suggested to subserve this role, including BDNF and nitric oxide (NO).

An alternative possibility is that retrograde signalling is achieved by adhesive trans-synaptic interaction between post- and pre-synaptic membrane proteins across the synaptic cleft (Yamagata *et al*., 2003; Dalva *et al*., 2007). A number of synaptic adhesion molecules have been implicated in trans-synaptic signalling in development and plasticity, including N-cadherin (Bozdagi *et al*., 2000), neuroligins and neurexins (Missler *et al*., 2003; Graf *et al*., 2004), NCAM and L1 (Lüthl *et al*., 1994), netrin-G and netrin-G ligand (Kim *et al*., 2006), Eph receptors and ephrins (Gao *et al*., 1998; Contractor *et al*., 2002; Grunwald *et al*., 2004; Kayser *et al*., 2006). Bidirectional communication at synapses via direct contact is known to play an important role in synaptogenesis (McAllister, 2007), however the role of such contacts in LTP remains the matter of some debate.

2.4.4.2.1. Brain-derived neurotrophic factor (BDNF)

Several lines of evidence support a role for BDNF as a retrograde messenger in the induction of presynaptic LTP. Activity in postsynaptic cells can cause fusion of BDNF-containing secretory granules and retrograde signalling to the presynaptic TrkB receptors (Magby *et al*., 2006) where it can act very rapidly (Kafitz *et al*., 1999). BDNF then initiates cascades that enhance neurotransmitter release (Zhang & Poo, 2002; Du & Poo, 2004). In conjunction with these results, genetic and pharmacological studies suggest that BDNF is necessary for persistent forms of LTP. In heterozygous BDNF

(+/-) knockout mice, there is a significant deficit in LTP induced by several different patterns of stimulation, including TBS protocols (Griesbeck *et al*., 1996; Patterson *et al*., 2001; Pang & Lu, 2004). Also, BDNF is important for presynaptic components of persistent LTP (Zakharenko *et al*., 2003).

2.4.4.2.2. Nitric oxide (NO)

NO is one of the most prominent candidates for a retrograde messenger in the CNS and a number of studies have reported a dependence of LTP persistence on NO signalling (Schuman & Madison, 1991; Haley *et al*., 1992; Arancio *et al*., 1996a; Arancio *et al*., 1996b; Ko & Kelly, 1999; Bon & Garthwaite, 2001a). The general scheme for NO action in hippocampal LTP is that Ca^{2+} influx via NMDARs activates nitric oxide synthase (NOS), which synthesizes NO from L-arginine (Boehning & Snyder, 2003; Garthwaite, 2008). Strict control of NOS by NMDARs is ensured by their close physical proximity (Christopherson *et al*., 1999; Sattler *et al*., 1999; Mungrue & Bredt, 2004). Once synthesized, NO diffuses out of the postsynaptic cell and acts on soluble guanylyl cyclase in the presynaptic neuron (Figure 2.9), stimulating cGMP production which in turn switches on cGMP-dependent protein kinases that modulate presynaptic proteins (Garthwaite *et al*., 1989). NO can also act directly on a wide variety of presynaptic proteins via S-nitrosylation of cysteine residues (Meffert *et al*., 1996; Hess *et al*., 2001; Jaffrey *et al*., 2001; Stamler *et al*., 2001; Huang *et al*., 2005a; Wang *et al*., 2006; Janssen-Heininger *et al*., 2008).

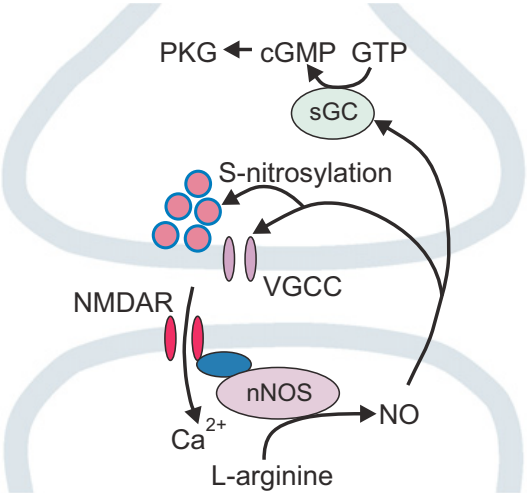

Figure 2.9. NO-mediated retrograde signalling. Activation of NMDARs leads to Ca^{2+} entry, which activates neuronal nitric oxide synthase (nNOS) to produce NO. NO affects release from presynaptic boutons either by activating soluble guanylyl cyclase (sGC), which ultimately activates PKG, or by S-nitrosylating presynaptic proteins. (Modified from Regehr *et al*., 2009)

2.4.4.3. Morphological alterations during LTP

Plasticity in the hippocampus extends beyond changes in synaptic strength and can also result in the formation or breakdown of synapses, changes in motility of spines and re-routing of axonal branches (reviewed by Butz *et al*., 2009). Although the predominant view is that such morphological changes occur following LTP induction, there is substantial inconsistency in the literature with regards to the features that change, or whether there is any change at all. In area CA1 some studies report no (or very small) changes in spine morphology or number (Hosokawa *et al*., 1995), whereas others demonstrate considerable numbers of new spines (Engert & Bonhoeffer, 1999; Toni *et al*., 1999) or filopodia (Maletic-Savatic *et al*., 1999). One reason for this discrepancy may be that changes that occur after tetanus-induced LTP can only be expected in a subset of spines and therefore the method used to identify potential sites of change is critical. Moreover, the majority of studies have utilised electron micrographs for analysis and therefore draw conclusions from fixed tissue. The advent of two-photon microscopic techniques has allowed for 'real time' analysis of the morphological changes that occur and perhaps give more accurate representations of the post-LTP spine dynamics (Bozdagi *et al*., 2010). Nevertheless, it seems that morphological changes do occur after LTP, perhaps as early as 30 mins after induction (Toni *et al*., 2001).

2.4.5. LTP maintenance

Maintenance of LTP refers to the mechanisms responsible for maintaining the potentiation effect in the absence of ongoing induction signals. The maintenance of LTP in the long-term is of particular importance in order to understand its role in memory formation and consolidation. One commonly accepted view is that LTP persistence involves a progression through two distinct biochemical phases. There is first an early, protein synthesis-independent phase, which is followed by a late, protein synthesis-dependent phase.

2.4.5.1. Early phase of LTP maintenance

The early phase of LTP maintenance is short lasting, involves posttranslational modifications of pre-existing proteins, glutamate receptor trafficking and is frequently referred to as 'early LTP' (E-LTP; Malinow *et al*., 1988; Malenka *et al*., 1989a; Sweatt, 1999; Malinow & Malenka, 2002). This early phase is typically induced by less intense stimulation than that required for the late phase and lasts only 1-2 hours. This is primarily because E-LTP is mediated only by posttranslational modifications of key

plasticity proteins, the identity of which has been the subject of considerable investigation. An example of one candidate is the GluR1 subunit of the AMPAR, which is phosphorylated by CaMKII at Serine831 during LTP (Barria *et al*., 1997a; Barria *et al*., 1997b; Mammen *et al*., 1997b). Such phosphorylation is thought to underlie the increased AMPAR conductance observed during LTP (Benke *et al*., 1998).

Another process involved in the early phase of LTP maintenance is the trafficking and delivery of AMPARs to the postsynaptic membrane. This can activate previously postsynaptically silent synapses (discussed in *Chapter 2.4.4. – Long-Term Potentiation – LTP expression*), or enhance the response of non-silent synapses by increasing AMPAR density. The mechanism by which AMPARs are delivered to synapses during LTP has not been fully elucidated, but is likely to be dependent upon a variety of kinases activated during the induction process that phosphorylate the GluR1 subunit c-tail (Esteban *et al*., 2003). These include phosphorylation by PKA at Serine845 (Roche *et al*., 1996), CaMKII at Serine831 (Barria *et al*., 1997a; Mammen *et al*., 1997b) and PKC at Serine818 and Serine831 (Roche *et al*., 1996; Boehm *et al*., 2006).

2.4.5.2. Late phase of LTP maintenance

The early phase of LTP is followed by a protein synthesis-dependent late phase that lasts many hours or perhaps even permanently (Abraham *et al*., 2002) and is frequently referred to as 'late-LTP' (L-LTP; Frey *et al*., 1988; Nguyen *et al*., 1994; Lu *et al*., 1999). It is proposed that the newly synthesized proteins during the late phase consolidate the synaptic changes initiated during the early phase of LTP as well as contributing to activity-dependent structural changes (Fifkova *et al*., 1982).

The earliest demonstration that LTP was dependent on protein synthesis was in the dentate gyrus *in vivo*, in which the translation inhibitor anisomycin (ANI) was injected into the ventricles prior to tetanisation of the perforant path (Krug *et al*., 1984). This resulted in the induction of a much more decremental form of LTP than was induced in saline-treated control animals. More recent studies confirmed that protein synthesis is required for the late phase of LTP in different hippocampal regions, both *in vivo* and *in vitro* (Frey *et al*., 1988; Huang & Kandel, 1994; Nguyen & Kandel, 1996, 1997).

A particularly critical pathway required for new protein synthesis during LTP involves the activation of the transcription factor cAMP response element binding protein (CREB). CREB requires phosphorylation on Serine133 by various Ca^{2+}-dependent kinases to stimulate transcription (Impey & Goodman, 2001). A series of studies published in the 1990s demonstrated that disruption of CREB activity impaired

memory in both vertebrates and invertebrates and therefore gave CREB the title of 'the memory gene' (Bourtchuladze *et al*., 1994; Yin *et al*., 1994; Bartsch *et al*., 1995). The rudimentary view of CREBs role in LTP is that many different activity-dependent kinase pathways (most of which are CaMKII-dependent) converge upon CREB and that this activity elicits the expression of molecules that are necessary for the stabilisation of LTP and hence memory. This model is of course highly simplified; cAMP-dependent gene expression is not regulated solely by CREB, but also by other CREB family members that can replace or modulate its activity (Hummler *et al*., 1994; Blendy *et al*., 1996; Mantamadiotis *et al*., 2002) and other transcription factors are also crucial for cellular consolidation (Ramanan *et al*., 2005; Lemberger *et al*., 2008; Jancic *et al*., 2009).

2.4.5.2.1. Translation-dependent forms of LTP

Forms of LTP that are independent of new mRNA synthesis, yet dependent upon translation from pre-existing mRNA were first demonstrated in the dentate gyrus *in vivo* (Otani *et al*., 1989) and more recently identified in CA1 *in vitro* (Raymond *et al*., 2000; Gelinas & Nguyen, 2005). In these studies a form of LTP was blocked with the translation inhibitor ANI, but was unaffected by the mRNA transcription inhibitor Actinomycin D (Act D). This demonstrated the existence of a so-called 'intermediate' form of LTP that requires translation from a pool of pre-existing mRNA.

2.4.5.2.2. Transcription- and translation-dependent forms of LTP

Transcription- and translation-dependent LTP is historically referred to as L-LTP and has been extensively characterised in the hippocampus. Such forms of LTP are reliant upon a number of protein kinases, including PKA, CaMKII, Erk/MAPK, PTKs and PKC, which activate key transcription factors that may include CBP and so-called immediate-early genes such as c-Fos and Zif268/Egr-1 (Thomas & Huganir, 2004). These transcriptional complexes ultimately promote expression of key effector genes that are required for maintaining the synaptic enhancement (West *et al*., 2002).

2.4.5.2.3. Locus of translation

In the case of transcription- and translation-dependent LTP, mRNA can be translated at the soma and the newly synthesised proteins trafficked to the dendrites where they are utilised. Alternatively, the mRNA transcript itself can be trafficked to the dendrites where it is locally translated (Steward & Schuman, 2003).

Given that transcriptional activation at the soma is required for the late phase of LTP (Nguyen *et al*., 1994), initial hypotheses regarding the site of translation focused on the cell soma and results from early studies supported this notion (Frey *et al*., 1989). Later studies suggested that translation in the dendrites is critical and may even be sufficient for the maintenance of late phase LTP under certain conditions. For example, BDNF induces potentiation of CA3-CA1 transmission in hippocampal slices where the CA1 dendrites have been surgically removed from their cell bodies and this effect is dependent on translation (Kang & Schuman, 1996). More recent studies have demonstrated that isolated hippocampal dendritic fields can support protein synthesis-dependent forms of LTP (Cracco *et al*., 2005; Huang & Kandel, 2005; Vickers *et al*., 2005) and that focal dendritic application of translation inhibitors in slice preparations inhibits late phase LTP (Bradshaw *et al*., 2003). A number of mRNA transcripts have been identified in the dendrites, with a range of 40-100 candidates (depending on the methods used for mRNA detection; Eberwine *et al*., 2001; Steward & Schuman, 2001; Zhong *et al*., 2006). Discrepancies regarding the identity and numbers of local mRNA transcripts make it difficult to form inferences about the functions that local translation may regulate. Importantly, translational machinery has been identified near spines using electron microscopy (Steward & Reeves, 1988; Ostroff *et al*., 2002).

Interestingly, there is now mounting evidence to support a role for local translation in presynaptic terminals (Akins *et al*., 2009). Local presynaptic protein synthesis has been reported in both immature and mature rat hippocampal cultures (Sebeo *et al*., 2009), in forms of LTP and LTD in *Xenopus* nerve-muscle cultures (Zhang & Poo, 2002), in corticostriatal fibres (Yin *et al*., 2006) and in hippocampal mossy fibre-CA3 synapses (Huang & Hsu, 2004). Opponents of local presynaptic translation argue that translational components have not been detected in presynaptic compartments, however the failure to detect these structures may be due to their scarcity or their presence in subcellular domains that are not favourable for their ultrastructural detection (for example, electron dense regions; Koenig *et al*., 2000). It is now becoming increasingly apparent that local presynaptic and postsynaptic translation is likely to operate in conjunction with somatic translation to regulate changes in synaptic function.

2.4.5.2.5. Synaptic tagging hypothesis

An important question raised by the protein-synthesis dependence of certain forms of LTP is how input specificity is retained if plasticity related proteins and transcripts are synthesised at the cell soma? This dilemma led to the proposal of the synaptic tagging

hypothesis by Frey and Morris (1997, 1998b, a), in which newly synthesised plasticity proteins and mRNA are trafficked to the dendrites and then utilised only by potentiated synapses that have been previously 'tagged'. Such a process would restrict non-potentiated synapses from sequestering proteins and transcripts, and would allow for input specificity to be preserved. Frey and Morris (1998b) demonstrated that normally weak LTP inducing stimuli could also induce a more persistent form if a separate pathway had been strongly tetanised within a specific time window. Thus, the proteins produced in response to strong tetanisation can be utilised by previously tagged synapses on the weak pathway. They propose that the tag is only transiently active, with a half-life of approximately 30 min in the intact animal. Other work suggests that the plasticity related proteins are also characterised by a relatively short half-life, of approximately 1-2 hrs (Korz & Frey, 2004; Sajikumar *et al*., 2005a). Therefore, only when both processes (the synapse-specific tag and the plasticity related proteins) are available can the two interact and transform weak LTP into a more persistent form. The molecules mediating the tags remain unknown, however the tagging that occurs during LTP seems to be dependent upon CaMKII (Sajikumar *et al*., 2005b).

Importantly, results from *in vivo* studies are indirectly supportive of a role for synaptic tagging in learning (Redondo & Morris, 2011). For example, encouraging exploratory behaviour after prior LTP induction augments the persistence of LTP/LTD *in vivo* (Kemp & Manahan-Vaughan, 2004) and other behavioural experiences that upregulate the expression of plasticity related proteins also increase the persistence of previously induced LTP (Seidenbecher *et al*., 1997).

2.4.5.2.6. Epigenetic regulation of LTP and memory

Experience-dependent memory formation and storage requires long-lasting changes in memory-related neuronal circuits that extend well beyond the average 24 hour half-life of a protein or mRNA molecule (Mammen *et al*., 1997a). Recent investigations have examined the potential for epigenetic modifications in the long-term persistence of plasticity and memories, and in particular that DNA methylation and chromatin remodelling might underlie behavioural memory in the adult CNS (Borrelli *et al*., 2008; Day & Sweatt, 2010). Such alteration to the genome may be a means by which a lifelong change in phenotype is conferred.

Several lines of evidence indicate that DNA methylation is vital for normal memory function (Levenson *et al*., 2006; Lubin *et al*., 2008; Miller *et al*., 2010). Inhibition of the enzyme responsible for DNA methylation (DNMT) alters the status of

the plasticity related gene *Bdnf* (encoding the growth factor BDNF; Levenson *et al*., 2006) and DNMT expression is upregulated in adult rat hippocampus after a particular learning paradigm called contextual fear conditioning (Miller & Sweatt, 2007). The converse also holds true – blocking DNMT activity blocks contextual fear conditioning (Miller *et al*., 2008; Feng *et al*., 2010; Miller *et al*., 2010).

There is also evidence of a role for chromatin remodelling in synaptic plasticity. Histone posttranslational modifications are altered after LTP and LTD (Guan *et al*., 2002; Levenson *et al*., 2004) and mice deficient in key proteins required to initiate remodelling pathways exhibit memory deficits, whereas facilitation of the remodelling pathway can restore normal long-term memory formation in the mutants and even enhance it in normal animals (Alarcón *et al*., 2004).

The area of epigenetic regulation of long-term synaptic plasticity and memory is still in its infancy, however it is currently an attractive mechanism for the life long persistence of experience-dependent behavioural memories.

2.4.6. Role of LTP in memory and learning

A number of experimental strategies have been used to demonstrate a strong link between LTP and learning and memory (reviewed by Martin *et al*., 2000). Early studies of patients with hippocampal lesions provided the first evidence that the hippocampus was involved in the generation of new long-term memories (Scoville & Milner, 1957). Since this discovery, several behavioural experiments using animal models have supported the premise that hippocampal LTP is indeed the means by which memories are encoded.

For example, NMDA antagonists that prevent the induction of some forms of LTP also impair the ability of rats to perform the Morris water maze, a spatial memory task that requires the hippocampus for successful completion (Morris *et al*., 1986; Morris, 1989; Davis *et al*., 1992). Other experiments are based upon the premise that if synaptic plasticity is necessary for the formation of new memories, then artificially inducing LTP in the hippocampus at as many synapses as possible should disrupt subsequent acquisition of new memories. In a study by McNaughton et al. (1986) animals were chronically implanted bilaterally with electrodes in the perforant path and dentate gyrus. The perforant pathway was repeatedly tetanised over a 34-day period, inducing persistent LTP in the dentate gyrus. The rats were then trained to decipher a particular maze and demonstrated impaired ability to learn the spatial task in comparison to controls. Other studies have reported impaired spatial learning after

interfering with synthesis of NO (Chapman *et al*., 1992; Böhme *et al*., 1993; Hölscher *et al*., 1996). Genetic studies have also implicated a role for LTP in learning and memory. One such study introduced a point mutation into the gene encoding CaMKII in mice to partially block its activity (Giese *et al*., 1998). Under these conditions, LTP could not be elicited across a range of stimulation frequencies and the animals exhibited profound deficits in spatial learning in a water maze. If LTP is indeed a learning and memory mechanism, then activities that induce learning and memory formation should also induce LTP. This has been observed in the extrinsic connections of the hippocampal formation. Mice trained in two different tasks in a spatial maze exhibited task-dependent potentiation within certain circuits (Jaffard *et al*., 1996). Other studies have reported enhanced EPSP amplitude in slices taken from animals that have been exposed to a learning situation in comparison with animals that have been left unattended (Tang & Zou, 2002). Of particular significance is a set of experiments published by Whitlock *et al*. (2006) in which a form of inhibitory avoidance training was shown to induce spatially restricted LTP in CA1 *in vivo*. Such work has proven crucial in establishing a direct causal relationship between LTP and learning and memory.

2.5. LTP1, 2 and 3

2.5.1. Overview of different forms of LTP

It has long been clear that several different forms of LTP can be classified, depending on the preparation type, stimulation protocol used for induction, and developmental stage of the animal. For example, some forms of LTP depend upon NMDARs (Harris *et al*., 1984; Bliss & Collingridge, 1993) whereas others are completely NMDAR-independent (reviewed by Johnston *et al*., 1992; Isaac *et al*., 1995) and both forms can be induced at the synapses between Schaffer collaterals and pyramidal neurons in area CA1 (Johnston *et al*., 1992). LTP is also frequently classified on the basis of its persistence as either E-LTP (Malinow *et al*., 1988; Malenka *et al*., 1989a; Lu *et al*., 1999; Abraham & Williams, 2003) or L-LTP (Frey *et al*., 1988; Nguyen *et al*., 1994; Lu *et al*., 1999). These two forms differ in their magnitude, persistence and the underlying biochemical pathways.

It is now also recognised that three mechanistically distinct forms of LTP can be induced in situations where the cell type, animal age and other experimental conditions are kept constant (reviewed by Raymond, 2007). Analysis of *in vivo* dentate gyrus studies by Abraham and Otani (1991) revealed the existence of three distinct groups of LTP decay functions, with time constants of 2 hours, 4 days and 20 days (Figure 2.10B). Based upon the nomenclature of Racine et al. (1983), they called these three groups LTP1, 2 and 3 in order of increasing persistence. Under this classification system, E-LTP is similar to LTP1 and is short lasting, LTP2 can be thought of as an intermediary phase of L-LTP and LTP3 represents a separate phase of L-LTP that is durable in the long-term and perhaps even permanent *in vivo* (Abraham *et al*., 2002). Whilst useful to relate the three forms to the classical E-LTP/L-LTP nomenclature, it is important to note that LTP1, 2 and 3 differ fundamentally in that they are proposed to represent discrete phenomena, rather than progressive phases (Raymond, 2007). All three forms of LTP are NMDAR-dependent and can be induced at CA3-CA1 synapses *in vitro* by increasing numbers of a theta-patterned stimulation protocol (Figure 2.10A). Each form has been characterised on the basis of its induction and maintenance mechanisms (Raymond *et al*., 2000; Raymond & Redman, 2002, 2006; Raymond, 2007; Reymann & Frey, 2007; Raymond, 2008), however the expression mechanisms of the three forms remain to be determined.

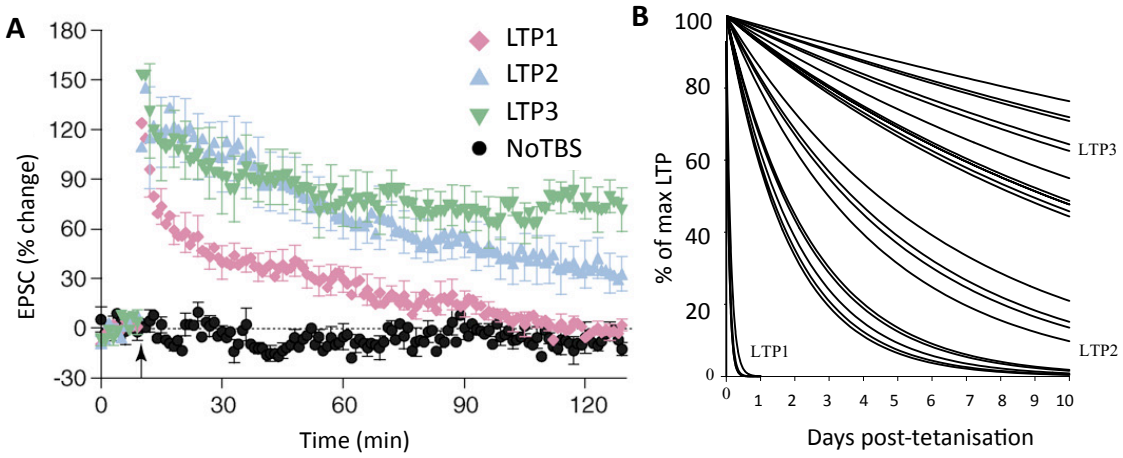

Figure 2.10. LTP1, 2 and 3 can be induced *in vivo* and *in vitro*. A) LTP1, 2 and 3 measured using whole-cell patch-clamp recording technique *in vitro*. TBS patterned stimulation was delivered at 10 min (indicated by arrow), with increasing numbers of repetitions inducing more persistent forms of LTP. Modified from Raymond (2007). **B)** LTP decay curves obtained from analysis of *in vivo* dentate gyrus studies demonstrate the existence of three discrete families of curves. These families have been called LTP1, 2 and 3 in order of their persistence. All curves are normalised to 100% LTP immediately following tetanisation. (Taken from Abraham, 2003 [modified from Abraham & Otani, 1991]).

2.5.2. Induction mechanisms of LTP1, 2 and 3

2.5.2.1. Unique source and locus of Ca^{2+}signal

Using a combination of electrophysiological and two-photon Ca^{2+} imaging techniques, Raymond and Redman (2002, 2006) showed for the first time that different forms of LTP require different sources of Ca^{2+} for their induction and that these unique sources are spatially segregated (Table 2-1).

Using ruthenium red to block RyRs they demonstrated that RyRs are required for the selective induction of LTP1 (ruthenium red had no effect on LTP2 and 3 decay). Moreover, this inhibition of RyRs selectively inhibited a component of TBS-evoked spine Ca^{2+} signals, demonstrating that activation of RyRs in the spine head is required for LTP1 induction. It is thought that activation of postsynaptic NMDARs during 1TBS triggers CICR via RyRs in the spine, thereby activating signalling molecules such as CaMKII, a key enzyme in the posttranslational modifications that maintain LTP1 (Bliss & Collingridge, 1993). This hypothesis is strengthened by the finding that NMDARs and RyRs may be colocalised together in a Ca^{2+} microdomain via interactions with key scaffolding proteins (Feng *et al*., 2002).

A similar investigation into the Ca^{2+} source and location required for LTP2 induction revealed a role for IP_3Rs located in the dendrites (Raymond & Redman, 2006). Inhibition of IP_3Rs with xestospongin-C selectively blocked LTP2 induction, without affecting LTP1 or 3. This inhibition was found to interfere only with dendritic Ca^{2+} signals, demonstrating that activation of IP_3Rs in the dendrite is required for induction of LTP2. Similar to the scenario for LTP1, it seems that activation of NMDARs during the TBS protocol mediates Ca^{2+} release from the ER. Delivery of 4TBS may initiate an IP_3- dependent signalling pathway via the activation of Group I mGluRs, which are essential for the induction of LTP2 (Raymond *et al*., 2000) and stimulate IP_3 formation (Nakanishi, 1992). It remains unclear whether Ca^{2+} entering via the NMDAR acts directly on IP_3Rs, or alternatively on another step of the transduction cascade. Since synaptic NMDARs are a considerable distance from the parent dendrite, a role for extrasynaptic NMDARs has been postulated and it is thought that activation of such receptors by glutamate spillover during repetitive TBS may be important (Lohmann & Raymond, unpublished).

LTP3 was found to be dependent upon activation of somatic L-type VGCCs for its induction (Raymond & Redman, 2006). Inhibition of L-type VGCCs with nifedipine had no effect on LTP1 or 2 decay, but significantly reduced the persistence of LTP3. This finding is reinforced by similar results showing that L-type VGCCs are important for the induction of persistent forms of LTP induced by intense conditioning stimulation (Grover & Teyler, 1990; Impey *et al*., 1996; Morgan & Teyler, 2001). This inhibition was found to inhibit only somatic Ca^{2+} signals, demonstrating that activation of L-type VGCCs at the soma are required for LTP3 induction. This form of LTP was compound in nature, incorporating a significant NMDAR-independent component.

2.5.2.3. Requirement for postsynaptic action potentials

Given that LTP1, 2 and 3 differ in their requirements for Ca^{2+}, it was of interest to determine whether they also differed in their requirement for postsynaptic action potentials. It was found that inhibition of action potentials during TBS had no effect on the persistence of LTP1, but reduced the persistence of LTP2 and 3 (Raymond, 2008). This indicates that LTP1 is either non-Hebbian, or utilises alternative associative cues. Another important finding was that LTP1 could be 'transformed' into LTP3 by following the 1TBS stimulation with delivery of 7 trains of TBS-patterned action potentials to the soma. This suggests that postsynaptic action potentials alone are sufficient to activate LTP3 maintenance, a finding that has substantial implications for

late-associativity whereby a weaker form of LTP can be strengthened by more persistent forms of LTP at other inputs (Frey & Morris, 1998b, a; Raymond, 2008).

2.5.3. Maintenance mechanisms of LTP1, 2 and 3

Several results have demonstrated that different biochemical processes maintain LTP1, 2 and 3 (Table 2-1), although a comprehensive study of these mechanisms has yet to be published. Like E-LTP, LTP1 may be maintained by posttranslational modifications such as phosphorylation. These modifications are likely mediated by CaMKII and PKC (Bliss & Collingridge, 1993) and the current hypothesis is that CaMKII (although important for multiple forms and phases of NMDAR-dependent LTP) is the primary effector for LTP1 (Raymond, 2007). CaMKII is a major component of the postsynaptic density and is preferentially activated by pulses of Ca^{2+} (Kennedy, 2000). Both of these properties make it an ideal target for the RyR-dependent, spine-mediated Ca^{2+} required for the induction of LTP1.

Table 2-1. Characteristics of LTP1, 2 and 3. Information taken from Raymond and Redman (2002) and Raymond (2007).

	LTP1	**LTP2**	**LTP3`**
Duration	Short	Intermediate	Long
Source of Ca^{2+}	NMDAR RyR (on ER)	NMDAR IP_3R (on ER)	NMDAR L-VGCC
Spatial Origin of Ca^{2+}	Spine	Dendrite	Soma
Maintenance Mechanism	Requires phosphorylation of existing proteins Likely to involve CaMKII	Dependent on protein synthesis Likely to involve group I mGluRs	Dependent on gene transcription and protein synthesis Likely to involve CRE-mediated transcription, cAMP-PKA and Erk/MAPK signalling pathways

Several studies highlight a distinction between transcription-dependent and transcription-independent L-LTP, supporting the existence of the intermediary phase LTP2. An LTP2-like form of potentiation that was transcription-independent and translation-dependent was first observed in the dentate gyrus *in vivo* (Otani *et al.*, 1989) and a similar form of LTP was observed in area CA1 *in vitro* that was dependent on activation of mGluRs (Raymond *et al.*, 2000). In this experiment, LTP1 could be

transformed into a translation-dependent form of LTP by prior activation of mGluRs. LTP2 is presumably reliant upon local translation of pre-existing mRNA in the dendritic shaft (see *Section 2.4.5. – Long-Term Potentiation – LTP maintenance*). Indeed, pairing an LTP inducing protocol with beta-adrenoceptor activation induced LTP2 in area CA1 and this form of LTP was preserved in physically isolated dendrites (Gelinas & Nguyen, 2005). CaMKII also seems to be critical for LTP2 induction. It is known that CaMKII mRNA is trafficked into the dendrites and that its local translation is important for the maintenance of LTP induced by multiple tetanisations, but not for LTP1 (Ouyang *et al*., 1999; Miller *et al*., 2002; Håvik *et al*., 2003).

LTP3 can be thought of as the classical transcription and translation-dependent form of L-LTP. This form of LTP requires CREB-dependent transcription for its activation (West *et al*., 2002) and recruitment of the co-activator CBP. LTP3 is a compound form of LTP, comprising NMDAR-dependent and L-type VGCC-dependent components (Morgan & Teyler, 2001; Raymond & Redman, 2002). Although both NMDARs and L-type VGCCs can lead to CREB phosphorylation, only L-type VGCCs produce sufficient Ca^{2+} signal for the recruitment of CBP (Hardingham *et al*., 1999). CRE-mediated gene expression therefore requires activation of L-type VGCCs (Impey *et al*., 1996), supporting the notion that LTP3 is indeed a transcription-dependent form of L-LTP. It is thought that the NMDAR-activated Ca^{2+} influx that plays a role in LTP3 maintenance may help prolong the phosphorylation of CREB, increasing the time for CREB-CBP interaction and facilitating transcription (Raymond & Redman, 2006). NMDARs are also potent activators of serum-responsive element (SRE)-dependent transcription (Bading *et al*., 1993), which could act in parallel with CREB-mediated transcription to promote the late phase of LTP3 maintenance.

2.6. Aim and Hypothesis

Whilst LTP1, 2 and 3 are reasonably well characterized in terms of their induction and maintenance, the exact expression mechanisms and locus of expression of the three forms of LTP have yet to be elucidated. The aim of these experiments is to investigate the mechanisms involved in the expression of each form of LTP at CA3-CA1 synapses.

To determine whether presynaptic mechanisms are involved in the expression of LTP1, 2 and 3 the rate of exocytosis and the level of PPF will be measured in area CA1 of the rat hippocampus. Existing evidence suggests that the more durable forms of LTP induced with strong tetanisation are associated with a presynaptic enhancement of vesicle exocytosis (Zakharenko *et al*., 2001; Zakharenko *et al*., 2003; Huang *et al*., 2005b; Stanton *et al*., 2005). It is therefore hypothesized that LTP3 (and possibly even LTP2) will involve some form of presynaptic expression mechanism, whereas LTP1 will be predominantly postsynaptic.

2.7. Organisation of Thesis

The experiments contained within this thesis have been designed to investigate the involvement of presynaptic mechanisms in the expression of LTP1, 2 and 3 in area CA1 in rat hippocampal slices and their dependence upon NO signalling and protein synthesis. **Chapter 3** outlines the experimental materials and methodology used to acquire the results. **Chapter 4.1** presents data on the differing degree of presynaptic expression in LTP1, 2 and 3. **Chapter 4.2** introduces the effects of disruption of NO signalling on the enhanced presynaptic release. **Chapter 4.3** presents results showing that more persistent forms of LTP are dependent on protein synthesis and that the enhanced exocytosis associated with these more persistent LTP forms is also protein synthesis-dependent**. Chapter 4.4** presents data demonstrating that presynaptic enhancement is dependent on postsynaptic mRNA translation. Finally, a discussion of the results is presented in **Chapter 5**.

3. Methods

3.1. Animals

Young adult male Wistar rats (6-9 weeks) were used for all experiments described here. The animals were housed in a climate controlled holding room and were exposed to 12 hour light-dark cycles. They were held 4 to a cage and had access to food and water *ad libitum*. Animals were treated in accordance with the Australian National University Animal Experimentation Ethics Committee Guidelines.

3.2. Hippocampal Slice Preparation

3.2.1. Field recording experiments

Animals were anaesthetised with isoflurane and decapitated. The brain was rapidly removed from the skull and chilled in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (124), KCl (3.2), NaH_2PO_4 (1.25), $NaHCO_3$ (26), $CaCl_2$ (0.5), $MgCl_2$ (7) and D-glucose (10) equilibrated with 95% O_2-5% CO_2. Following hemisection, the frontal cortex and cerebellum were removed to roughly isolate the hippocampi. Transverse slices (400 μm in thickness) were prepared using a vibrotome (Campden Instruments, MA752 Motorised Advanced Vibroslice) and area CA3 was removed to reduce potential hyperexcitability. The slices were then transferred to a 34°C holding chamber in which they were submerged in a holding solution of ACSF (as described above, except with the Ca^{2+} and Mg^{2+} concentrations adjusted to 2.5 mM and 1.3 mM respectively) that was continuously bubbled with 95% O_2-5% CO_2. Slices were held at 34°C for 90 min, or until required for transfer to the recording chamber.

3.2.2. Whole-cell patch-clamp recording experiments

The procedure for slice preparation was the same as described above, with the following adjustments:

1) The dissecting solution contained 110 mM choline chloride in place of NaCl to reduce potential excitability. In addition, 2 mM ascorbate and 3 mM pyruvate were included to preserve slice surface integrity.
2) Slices were cut 300 μm thick to assist with imaging.
3) Slices were transferred to a holding chamber containing the same holding solution as described above for the field recording experiments, except with the addition of 2 mM ascorbate and 3 mM pyruvate.

4) Slices were held at 34°C for 40 min and then at room temperature for a further 30 min, or until required in order to improve slice surface health

3.3. Electrophysiology

3.3.1. Experimental procedure

Slices were perfused in a continuous flow (~2 ml min^{-1}) of ACSF recording solution (as per holding solution described above for field recording experiments) bubbled with 95% O_2-5% CO_2 and heated to 32°C.

3.3.1.1. Field recordings

For all experiments described here, field excitatory postsynaptic potentials (fEPSPs) were recorded from stratum radiatum in area CA1. Recordings were made on a standard patch-clamp setup with a Zeiss LSM 510 laser-scanning microscope with a water immersion objective (Achroplan 40x/0.75 W). The microscope was mounted on a vibration-isolated table together with two micromanipulators (MP-285; Sutter Instrument Company, Novato, CA, USA). Responses were recorded using borosilicate glass microelectrodes (GC150F; Harvard Apparatus) pulled on a computer controlled micropipette puller (P-97, Sutter) to a tip resistance of 2-9 MΩ when filled with ACSF. Filled electrodes were mounted in a pipette holder connected to the amplifier headstage, with a chlorided silver wire to establish electrical contact. The electrode was placed in stratum radiatum approximately halfway between stratum lacunosum moleculare and stratum pyramidale (Figure 3.1). Signals from the recording electrode were amplified using a Multiclamp 700B amplifier. A bipolar Teflon-insulated tungsten stimulating electrode was positioned approximately 300 μm from the recording site in the Schaffer collateral input pathway (Figure 3.1). Electrical stimulation of this pathway evoked synaptic responses in the apical dendrites of CA1 pyramidal cells.

Baseline synaptic responses were evoked by delivery of stimuli at a frequency of 0.033 Hz (square pulse, 0.1 ms pulse-width). The recording electrode was slowly lowered into the slice until the amplitude of the fEPSP reached a maximum. The stimulation current was adjusted between 60 and 250 μA to evoke fEPSPs of approximately two-thirds of maximum amplitude. Only slices with maximum fEPSP amplitude greater than 1.5 mV were used.

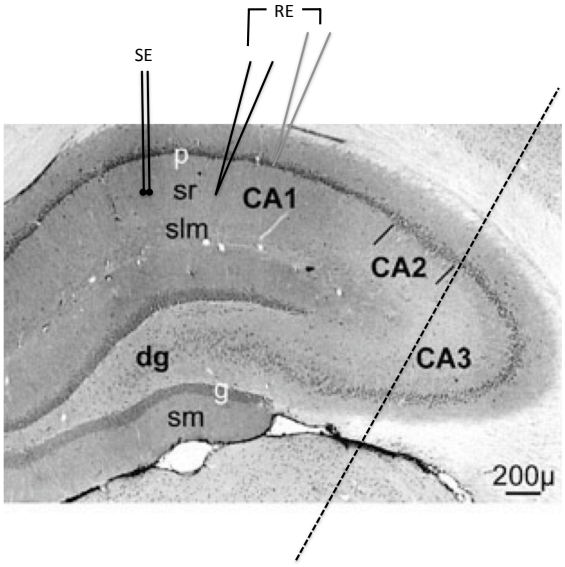

Figure 3.1. Electrode arrangement in the hippocampal slice. For field recordings both the stimulating electrode (SE) and the recording electrode (RE, black) were placed in stratum radiatum. For whole cell experiments the positioning of the SE remained the same and the RE (grey) was situated in the pyramidal cell layer (p). Area CA3 was removed from all slices during the dissection procedure (dashed line). CA1-3, hippocampal fields CA1-3; dg, dentate gyrus; g, granular cell layer; sm, stratum moleculare; slm, stratum lacunosum moleculare; sr, stratum radiatum. Modified from Kauselmann *et al*. (1999).

3.3.1.2. Whole-cell patch-clamp recordings

Whole-cell voltage-clamp recordings from CA1 pyramidal cells were made using glass electrodes pulled to a tip resistance of 4-6 MΩ when filled with an internal solution containing (mM): $KMeSO_4$ (127), KCl (8), Hepes (10), sodium phosphocreatine (10), Mg-ATP (4), Na-GTP (0.4), Alexa Fluor 488 (0.05) and in some cases Gelonin (0.0035).

All recordings were performed on the same patch-clamp setup as described above for field recordings. A pyramidal cell of interest was visually identified using infrared differential interference contrast (IR-DIC) optics with a CCD camera (Hamamatsu) connected to a video monitor. After identifying a cell, the bipolar stimulating electrode was positioned on the surface of the slice in the Schaffer collateral input pathway at a site that would not impede imaging of the selected cells dendrites (Figure 3.1). As for the field recordings, the filled electrodes were mounted in a pipette holder connected to the amplifier headstage and the chlorided silver wire inserted into the internal solution.

Synaptic responses were evoked by stimulation of the Schaffer collaterals (0.1 ms pulse width) via the stimulating electrode. EPSCs were recorded in whole-cell voltage-clamp mode and the stimulus amplitude was adjusted to produce responses of approximately 200 pA. It was ensured that this stimulus intensity was not sufficient to evoke action potential firing with the somatic membrane potential voltage-clamped at -

66 mV. As for field recordings, signals from the recording electrode were amplified using a Multiclamp 700B.

Baseline recordings (10 min) were initiated no later than 5 min after the establishment of whole-cell configuration, to avoid the 'wash-out' effect that is commonly observed with whole-cell LTP experiments (Malinow & Tsien, 1990). Baseline synaptic responses were evoked by delivery of stimuli at a frequency of 0.066 Hz (0.1 ms pulse-width). Every 2.5 mins a second pulse was administered 20 ms after the first to enable measurements of paired-pulse facilitation (PPF) and the paired-pulse ratio (PPR). Series resistance (R_s) was monitored on-line and recordings were abandoned if this varied by more than 20% from the baseline value, or alternatively rose above 25 MΩ.

3.3.2. LTP induction protocols

LTP1, 2 and 3 were induced by TBS, consisting of trains of 10 x 100-Hz bursts (5 pulses/burst) with a 200 ms inter-burst interval (Figure 3.2). One train of 10 bursts

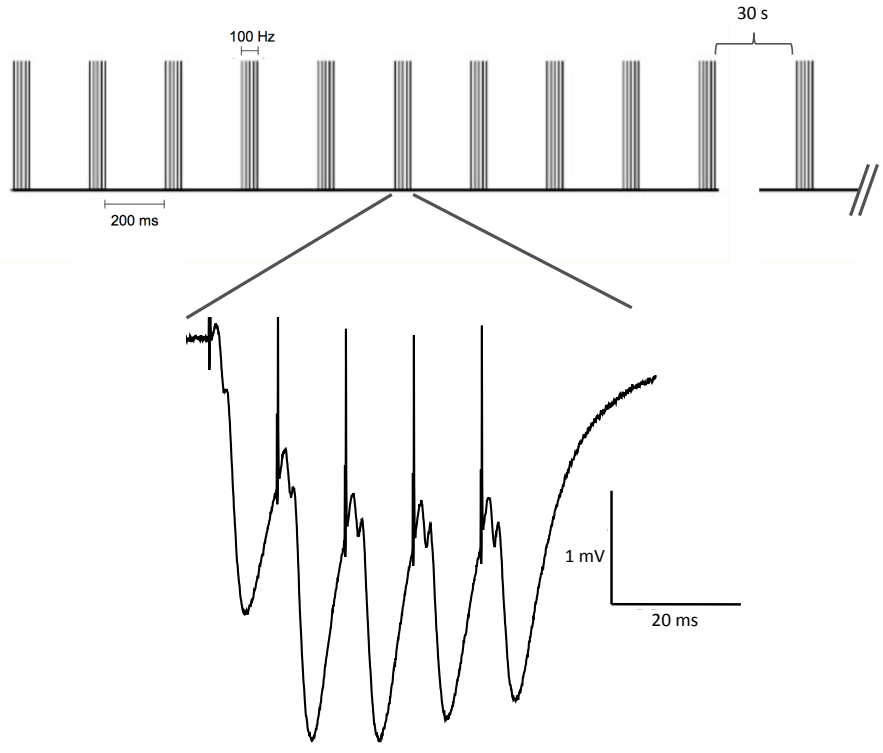

Figure 3.2. Pictorial representation of the TBS protocol used to induce LTP. When more than one train is used (i.e. 4 TBS and 8 TBS) there is a 30 sec inter-train interval. Modified from Raymond (2007). An example record of 5 fEPSPs recorded during the 100 Hz burst is shown below the protocol.

is denoted as 1TBS. When more than one train was used (i.e. 4TBS and 8TBS) the inter-train interval was 30 sec. All TBS trains were delivered at the test-pulse intensity.

3.4. Data Acquisition and Analysis

For both field and whole-cell LTP experiments, 10-40 min of baseline data was acquired prior to LTP induction. Recorded signals were low-pass filtered at 10 kHz using the Multiclamp's built in 4-pole Bessel filter and were digitised at 50 kHz and stored on computer for later off-line analysis. Data was acquired using AxoGraph (v. 4.9) and analysed using AxoGraph X (v. 1.3.1) software on a Macintosh computer.

3.4.1. Field recording analysis

Initial maximum slopes of the fEPSPs were measured off-line using AxoGraph (Figure 3.3) and the baseline average was calculated as the mean of the baseline points immediately prior to LTP induction. Field EPSP slopes were expressed as a percentage change from the baseline average by the formula (x – baseline average)/baseline average x 100, where x is a recorded fEPSP slope. LTP magnitude was measured as the mean percent change in fEPSP slope over the last 5 min of the recording period. In most cases the persistence of LTP was determined by measuring the average time constant (τ) of decay by fitting a double negative exponential to post-TBS data. The time constant of the second, slower exponential was used as an indicator of persistence and was averaged for each TBS group. Only slices in which fEPSP slope varied less than 15% over the entire baseline period were included in analysis.

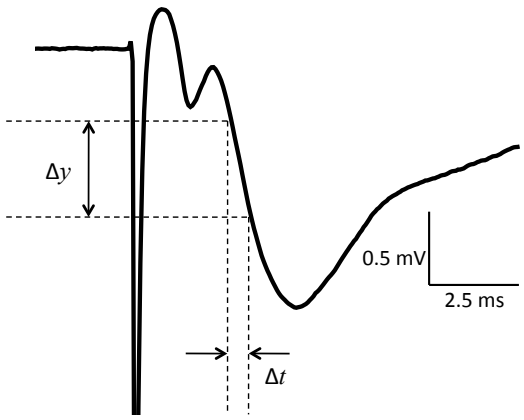

Figure 3.3. Measurement of fEPSP slope. Example of a fEPSP recorded from stratum radiatum in area CA1. Slope was measured from a portion of the fEPSP that appeared linear by eye. The slope was measured in mV/ms ($\Delta y/\Delta t$), where Δy is the change in amplitude and Δt is the period of time in which Δy occurred.

3.4.2. Whole-cell patch-clamp recording analysis

Maximum amplitudes of the EPSCs were measured off-line using AxoGraph and the baseline value was calculated as the mean of the first 40 points (i.e. 10 min) immediately prior to LTP induction. Amplitudes were expressed as the percentage change from the average baseline EPSC amplitude. All whole-cell recording analysis was the same as is described for field recordings, except that in addition to the requirement for less than 15% variation during the baseline period, only cells that exhibited a stable series resistance were included in analysis.

3.5. Imaging Experiments

3.5.1. FM 1-43 kinetics

FM dye destaining was used in several experiments here to assess presynaptic function. These experiments were performed simultaneously with the LTP electrophysiological experiments described above, in order to determine the involvement of presynaptic expression in different forms of LTP.

FM dyes are styryl fluorescent dyes that fluoresce particularly strongly when embedded in a lipid membrane and for this reason are frequently used to assess vesicle cycling. Their structure makes them ideal for this usage (Figure 3.4). They have a lipophilic tail region which partitions into lipids, a middle region containing aromatic rings that contain the fluorophore and a positively charged head group that prevents the dye from flipping across the membrane (Gaffield & Betz, 2006). Therefore once a dye is embedded within a vesicle membrane, its only means of release is via exocytosis. The most widely used FM dye is FM 1-43, as it provides optimum dye unloading and fluorescent signal (Gaffield & Betz, 2006).

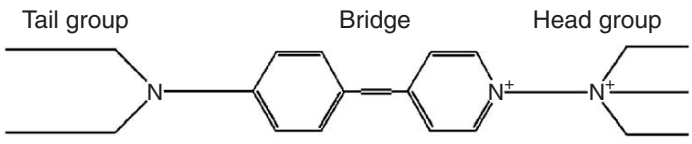

Figure 3.4. FM dye structure. The lipophilic tail region inserts in vesicle membranes, the bridge region contains the fluorophore and the charged head group keeps the molecule positioned in the membrane. Taken from Gaffield & Betz (2006).

When FM dyes are applied during periods of either chemical or electrical stimulation, recycling vesicles are labelled as they endocytose (Figure 3.5). Any externally-bound dye can then be washed away using a dye-free medium, allowing the labelled vesicles to be easily visualised. Further periods of stimulation cause the labelled vesicles to once again be exposed to the bathing medium, resulting in loss of dye. Imaging during this 'destaining' period allows the rate of dye loss to be measured, which directly corresponds to rate of exocytosis.

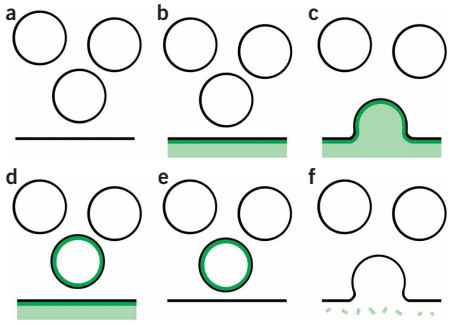

Figure 3.5. Typical FM dye experiment. (a) Synaptic vesicles within a terminal. (b) FM dye is added to the bath. (c) Upon stimulation, the lumen of fusing vesicles is exposed to the dye. (d) The vesicle is endocytosed with dye inside. (e) The FM dye is washed out and labelled vesicles are imaged. (f) The preparation is stimulated again in dye-free medium whilst labelled vesicles are imaged. The rate of vesicle exocytosis is determined by measuring the rate of vesicular dye loss. Taken from Gaffield & Betz (2006).

3.5.2. FM 1-43 field recording experiments

3.5.2.1. Loading and unloading

FM 1-43 (5 μM) was added to the bathing solution via a recirculation system and electrical loading stimulation (10 Hz, 2 min) was delivered to Schaffer collaterals to incorporate dye into vesicle membranes (Figure 3.6). The slice was then washed with ACSF containing ADVASEP-7 (0.05 mM) for 20 min. ADVASEP-7 is a compound that has a higher affinity for FM 1-43 than plasma membranes and helps reduce all non-specific FM binding (Kay *et al*., 1999). Electrical destaining stimulation (1 Hz for 3 – 8 min) was used to unload the dye. All loading and unloading procedures were performed in the presence of the NMDAR antagonist D-(-)-2-amino-5-phosphonopentanoic acid (D-APV, 100 μM), which was washed in 15 min prior to stimulation to reduce synaptic plasticity. Where possible three load/unload protocols were conducted within the same slice – before LTP induction, 80 min post-induction and 160 min post-induction and the

same terminals were imaged at each time point. This allowed for temporal changes in presynaptic function to be assessed.

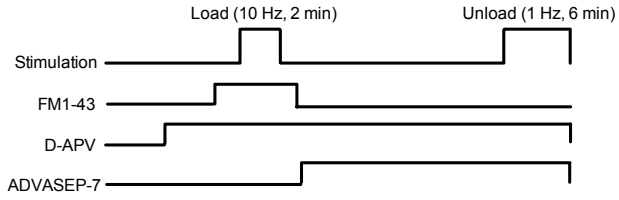

Figure 3.6. Protocol used to load synaptic terminals with FM 1-43 and measure the kinetics of dye release. Where possible this protocol was repeated 3 times within a single slice, once before LTP induction and then 80 min and 160 min post-TBS.

3.5.2.2. Two-photon imaging

3.5.2.2.1. Overview of technique

Two-photon laser scanning microscopy is a fluorescence imaging technique that is frequently used for the imaging of neural tissue, as it overcomes the strong scattering that occurs with short wavelength visible light (Denk *et al*., 1990; Denk *et al*., 1994; Svoboda *et al*., 1997; Mainen *et al*., 1999). The technique relies upon two-photon excitation, in which two long-wavelength excitation photons produced by a mode-locked pulsed laser combine to excite a fluorophore (Denk *et al*., 1990). A two-photon microscope requires a pulsed laser source that has a tuneable wavelength over a large range (700 – 1000 nm; Helmchen & Denk, 2005). The excitation pathway for such a system is as follows (Figure 3.7; Helmchen & Denk, 2005). Starting from the laser, the beam intensity is adjusted using a polariser and is then expanded using either curved mirrors or lenses. The beam is then scanned by a *xy*-deflection module (typically a pair of galvanometric scanners) and is further expanded using the scan and tube lens in order to fill the back aperture of the microscope objective, which focuses the light onto the sample. The two-photon-excited fluorescence can then be collected using either reflected and/or transmitted modes by photomultiplier tubes (Denk *et al*., 1994). There are two main advantages of two-photon laser scanning microscopy. First, infrared excitation provides greater depth penetration as the longer wavelength excitation light is scattered less throughout the tissue than single photon techniques (Mainen *et al*., 1999). Tissue penetration depths up to 500 μm have been demonstrated, even in the intact brain (Svoboda *et al*., 1997). Second, excitation is localised to a small focal region at which the two photons combine (less than 1 μm^3), providing three-dimensional optical

sectioning and resolution equivalent to that of a single photon system, but without any loss of signal due to a detector pinhole (Denk *et al*., 1994). This results in much less phototoxicity and photobleaching (Denk *et al*., 1990; Stelzer *et al*., 1994). In the experiments outlined here, two-photon microscopy was used to image FM 1-43 and Alexa Fluor 488 probes.

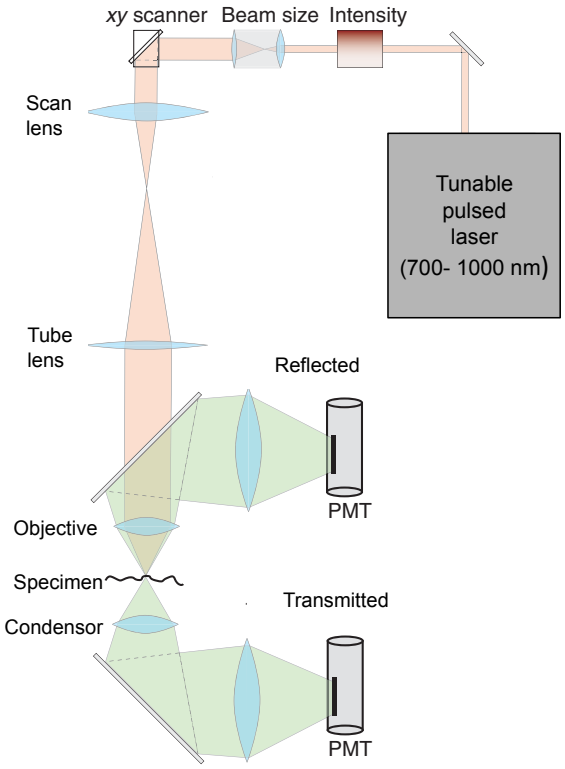

Figure 3.7. Excitation pathway for two-photon microscopy. A laser source provides short pulses and the intensity and beam size are adjusted before reaching the microscope. Two-photon excited fluorescence can be collected in reflected and transmitted modes using photomultiplier tubes (PMT). See text for specific details. Modified from Helmchen and Denk, 2005.

3.5.2.2.2. Methodology

Fluorescence of FM 1-43 labelled release sites was evoked by two-photon excitation and visualised using a Zeiss LSM 510 two-photon laser-scanning microscope with a water immersion objective (Achroplan 40x/0.75 W). Two-photon excitation was achieved with a Ti:Sapphire laser tuned to 840 nm wavelength. Laser intensity was controlled to use the lowest intensity necessary for adequate signal-to-noise ratio in order to reduce photodamage and photobleaching.

3.5.2.3. Data acquisition and analysis

Zeiss LSM 510 software was used to acquire 512 x 512 pixel images, 0.15 μm/pixel in the *x*-*y* axes with a scan time of 1.57 sec. Images were captured in 0.5-2 μm steps in the *z*-direction and a 3D *z*- stack of the fluorescent terminals was constructed offline (Figure 3.8). The number of images in a *z*-stack varied from 4 – 13, in order to

maximise the number of puncta that could be captured in the *z* direction, without risking photobleaching or photodamage. All fields imaged were typically 10-20 μm from the surface of the slice and were 40-80 μm away from the stimulating electrode. A *z*-stack of images was taken every 30 sec so that a time-series of destaining could be constructed over the entire unload period (Figure 3.9). A total of 15 *z*-stacks were captured, with the first 3 *z*-stacks serving as baseline points prior to the commencement of unloading stimulation. In offline analyses putative terminals were examined using circular regions of interest defined around the centre of brightly stained punctate fluorescent spots. A range of 2-69 terminals and 3 background fields were measured in each slice (Figure 3.10).

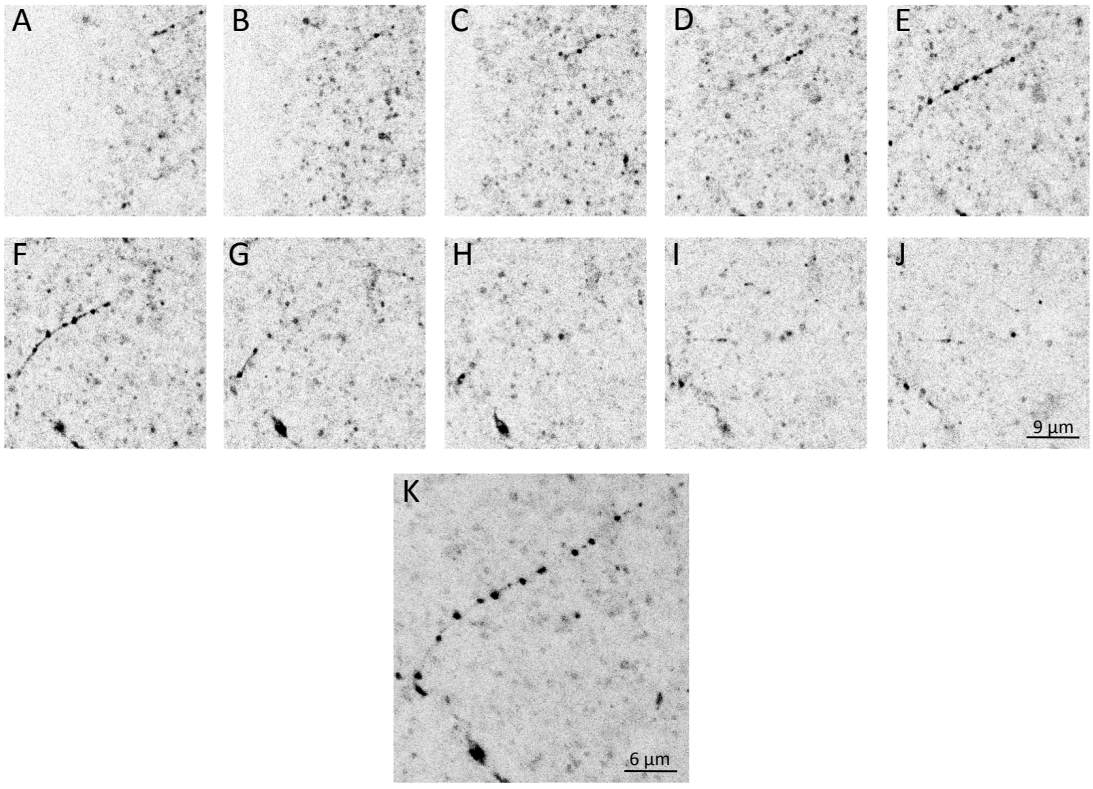

Figure 3.8. Example demonstrating the generation of a single 3D projected image from a *z*-series stack of images. A-J: 10 source images of FM 1-43 labelled terminals in area CA1, taken at 0.8 μm increments in the *z* direction. K: Projected image generated by LSM 510 software in which the 10 source images shown in A-J have been 'stacked' together. Image colours are inverted.

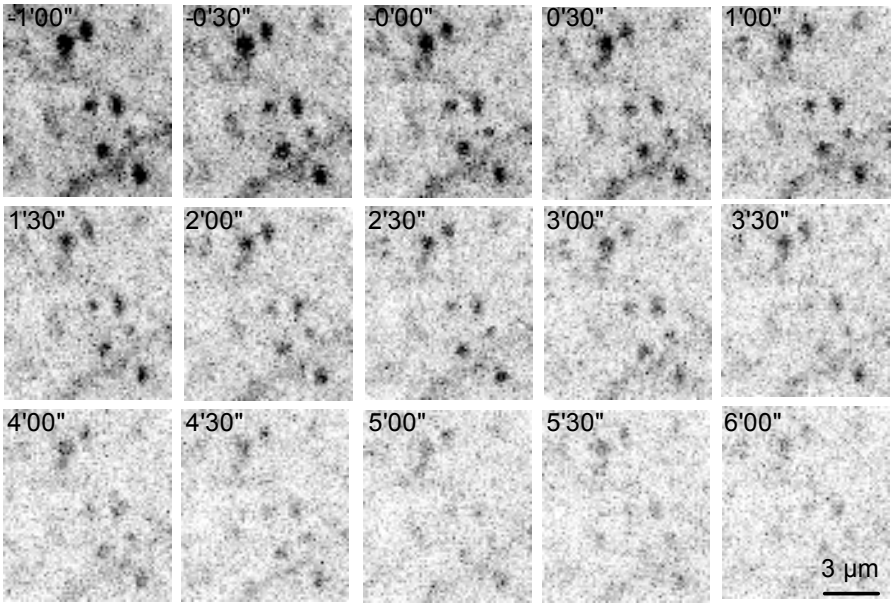

Figure 3.9. Example time-series of destaining during the unload period. Two-photon fluorescent images of terminals loaded with FM 1-43 in stratum radiatum of area CA1. Images are of the same field at different times during unloading. Numbers indicate time in minutes and seconds, with zero representing the onset of unloading stimulation (1 Hz, 6 min). Image colours are inverted.

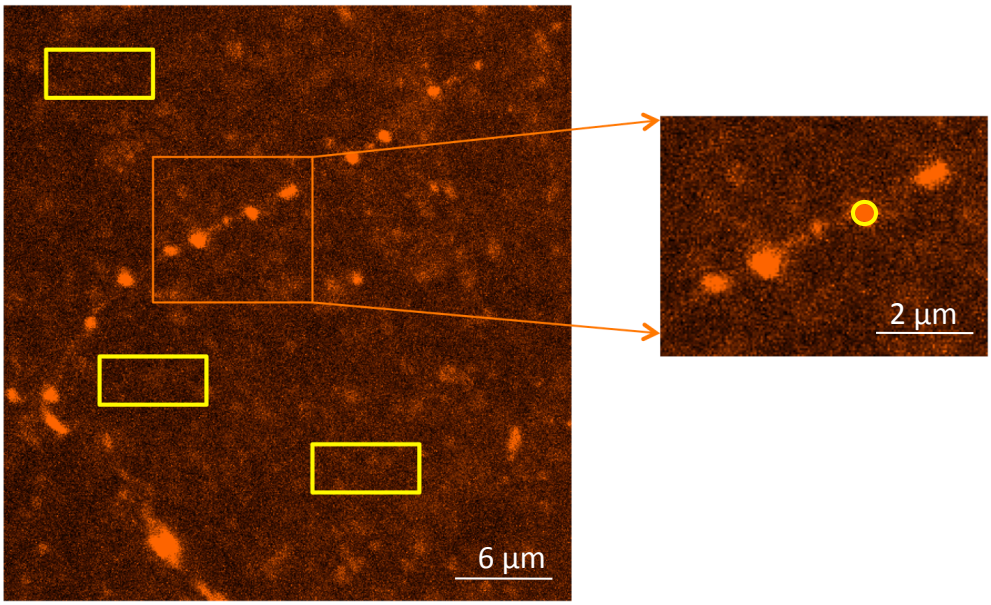

Figure 3.10. Regions of interest and background fields used in FM analysis. Two-photon image of FM 1-43-labelled terminals in area CA1. Three background fields were measured in each slice (yellow rectangles). Background fields were chosen that contained few puncta and were qualitatively representative of the non-specific staining. Magnified image on the right shows example placement of a region of interest for a single puncta.

Only puncta that satisfied 6 predetermined criteria were included in analysis. These were:

(1) Diameter of 0.5 – 2 μm.
(2) Approximately circular shape.
(3) Minimal *x-y* movement during imaging.
(4) Stable baseline fluorescence.
(5) Activity dependent destaining that was well fit by a first order exponential decay function.
(6) At least 70% loss of dye by the end of the unloading stimulation.

The combined use of ADVASEP-7 with two-photon imaging techniques substantially reduced background non-specific staining, so that puncta were essentially surrounded by dark background. The fluorescence intensity values obtained for each punctum were therefore not corrected for background staining in accordance with similar analytical procedures published by others (Stanton *et al*., 2003; Stanton *et al*., 2005). A dye-bleaching time course was constructed from the average of the 3 background fields and photobleaching was corrected for by dividing by the bleach value at the corresponding time point throughout the unload stimulation (Figure 3.11; Stanton *et al*., 2005). This corrected for any changes in fluorescence that were not due to activity-dependent destaining. A fluorescence time-course was generated by normalising the photobleaching-corrected fluorescence of a punctum at each time point to the average of the pre-stimulus values. The halftime of decay of fluorescence intensity during unloading ($t_{1/2}$) was calculated for each punctum from single exponential decay curves fitted to the period of stimulus-induced destaining.

3.5.3. FM 1-43 whole-cell recording experiments

In some experiments, FM 1-43 loading and unloading were performed after having established a whole-cell recording from a CA1 pyramidal cell filled with Alexa Fluor 488 (50 μM). In these experiments LTP was induced and then at 160 min post-TBS an FM load/unload was performed in order to image terminals making putative contact with distal dendrites (100-200 μm from the soma) of the filled cell. All image acquisition and analysis procedures are as described above, except with the following adjustments:

1) Two-photon excitation of both fluophores was achieved by tuning the laser wavelength to 810 nm.

2) Images were captured in smaller increments in the *z*-direction, with an average distance of 0.3 μm.

3.6. Statistics

Statistical analysis of all data was performed using Excel (Microsoft) and Prism (Graph Pad). In all analyses Student's two-tailed *t*-tests were used for statistical comparison unless otherwise stated and a P value of 0.05 or less was considered to represent a significant difference. The sample size, n, refers either to the number of slices, the number of cells, or the number of FM-labelled puncta sampled. All results are presented as mean ± standard error of the mean, unless otherwise stated.

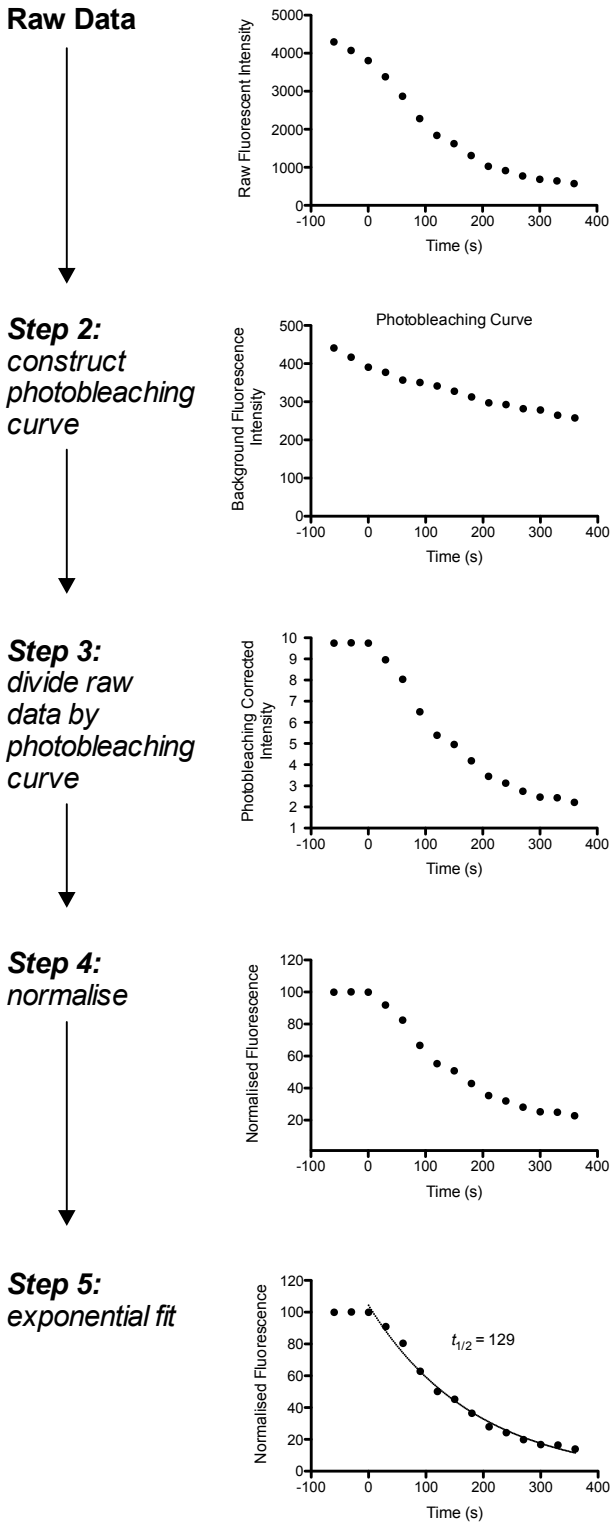

Figure 3.11. Procedure used to generate a normalised destaining curve from raw destaining data. A photobleaching curve is constructed from the average of 3 background regions (Step 2) and the raw fluorescent intensity values are divided by the bleach value at the corresponding time point (Step 3). The data are then normalised to a percentage of the original pre-stimulus fluorescence (Step 4) and a single exponential is fitted to the data from which the half-life of destaining ($t_{1/2}$) is measured (Step 5).

4. Results

4.1. More persistent forms of LTP involve enhanced vesicle turnover

In these experiments forms of LTP induced by 1, 4 and 8TBS in hippocampal area CA1 were characterised on the basis of their persistence and magnitude. The use of FM 1-43 destaining as a valid measure of release rate was verified and the association of each form of LTP with enhanced presynaptic release was determined by measuring FM 1-43 destaining pre- and post-induction.

4.1.1. Increasing numbers of TBS trains induces LTP of increasing persistence

In order to compare results with those previously published on LTP1, 2 and 3 in area CA1 (Raymond & Redman, 2002, 2006; Raymond, 2008) it was important to ensure that all three forms of LTP could be reliably induced by 1, 4 and 8TBS respectively. In control slices 1TBS resulted in weak LTP that decayed back to baseline approximately 180 min post-TBS (Figure 4.1A), with a magnitude in the last 5 mins of recording of 5 ± 1% (n=5; Figure 4.3A). Four trains of TBS resulted in LTP that had a similar initial magnitude to that induced by 1TBS (Figure 4.1B), but a significantly larger magnitude in the last 5 mins of recording, measuring 27 ± 7% (n=11; Figure 4.3A). Eight trains of TBS induced LTP that had a similar initial magnitude to that produced induced by 4TBS (Figure 4.1C), but a significantly larger final magnitude, measuring 83 ± 13% (n=7) in the last 5 mins of recording (Figure 4.3A). Summary data of the mean percent change in fEPSP slope with 1, 4 and 8TBS is shown in Figure 4.2. Importantly, slices that were not exposed to TBS demonstrated stable recordings over the entire recording period (Figure 4.2).

In relation to its proposed role as a Hebbian learning and memory mechanism, LTP is often best classified on the basis of persistence rather than magnitude. The persistence of LTP induced by 1, 4 and 8TBS was determined by fitting the post-TBS fEPSP data for each individual slice with the sum of two negative exponentials described by the following equation: $y = a^{(-bx)} + c^{(-dx)}$ (Cohen & Abraham, 1996; Raymond *et al*., 2000; Raymond & Redman, 2002, 2006). The time constant (τ) of the second, slower component was used as a measure of persistence and the mean τ was compared under different experimental conditions. In some cases the post-TBS could not be fit with a two-phase exponential decay function, therefore these slices were excluded from persistence analyses.

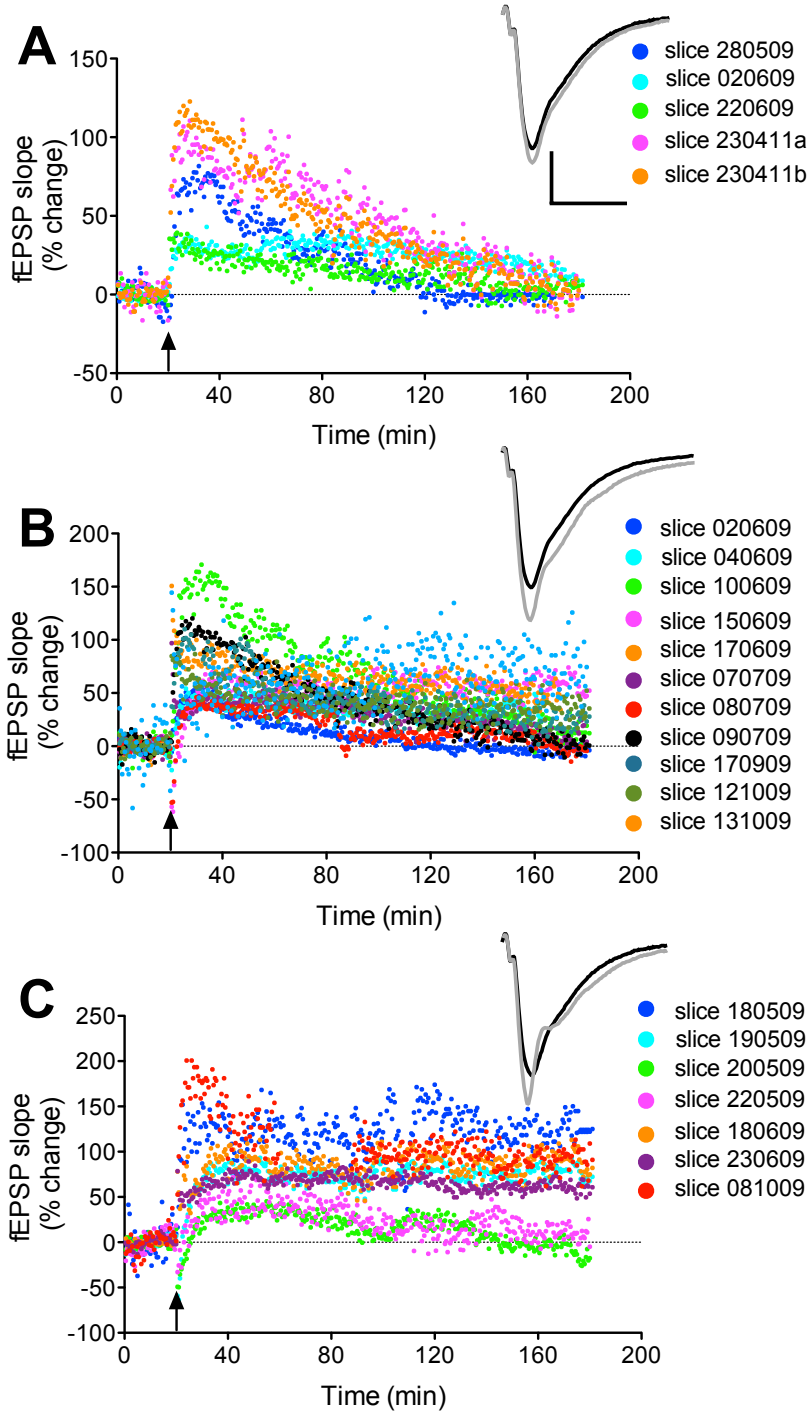

Figure 4.1. Percent change of field excitatory postsynaptic potential (fEPSP) slope from slices receiving 1TBS (A), 4TBS (B), or 8TBS (C).

LTP was induced (arrow) after a 20 min baseline period and recordings continued for 160 min post-TBS. *Inset*, representative fEPSPs averaged from 5 consecutive stimuli are shown for the period immediately prior to LTP induction (black) and upon recording termination (grey). Scale is 1 mV, 10 ms.

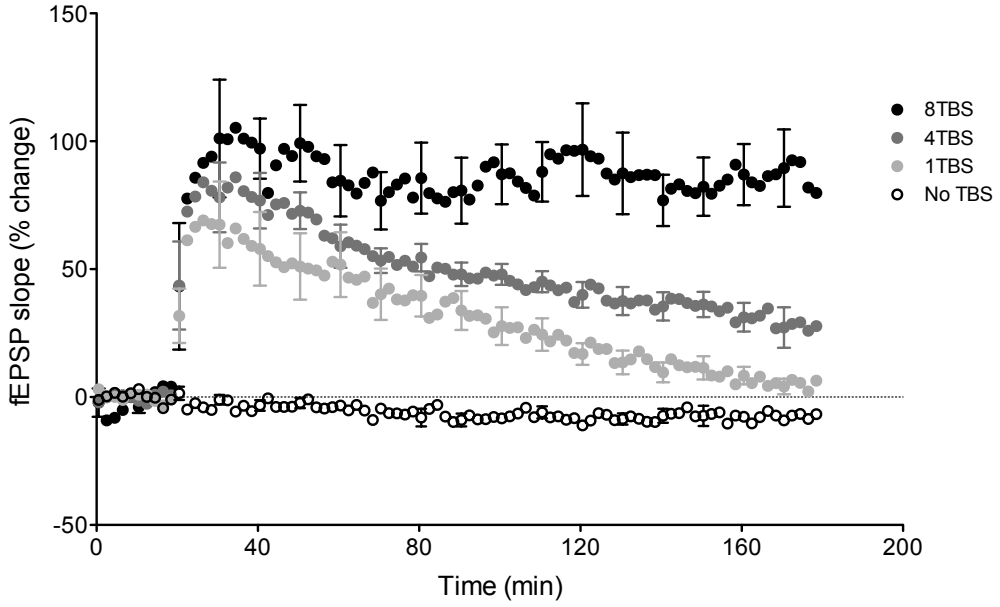

Figure 4.2. Mean percent change of field excitatory postsynaptic potential (fEPSP) slope from slices receiving 1TBS, 4TBS, 8TBS or no TBS.

LTP was induced (arrow) after a 20 min baseline period and recordings continued for 160 min post-TBS. 1TBS(n=3), 4TBS(n=11), 8TBS(n=7), no TBS(n=4).

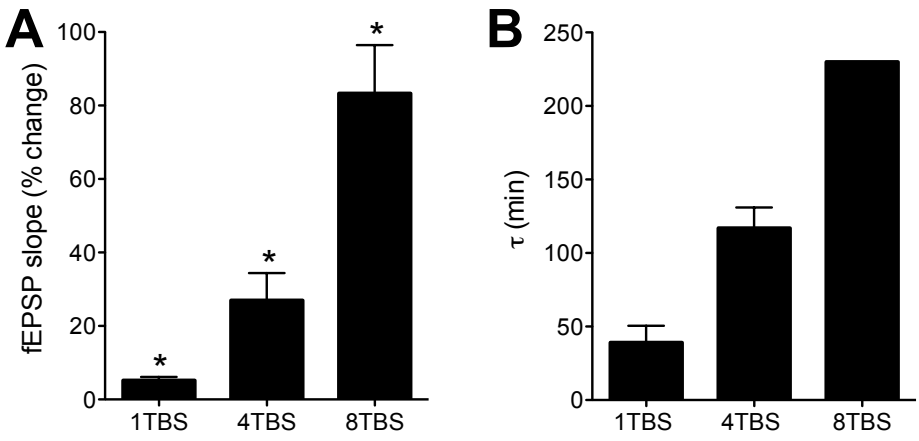

Figure 4.3. Measures of LTP Robustness.

A, Magnitudes of LTP1, 2 and 3. The mean magnitude of LTP1, 2 and 3 was calculated in the last 5 min of recording. All magnitudes were significantly different from one another (one-way ANOVA, *p<0.05). **B**, Decay of LTP1, 2 and 3. The mean time constant of decay, τ was calculated for LTP induced with 1 and 4TBS. A τ value could not be calculated for LTP induced by 8TBS due to the robust nature of the LTP, therefore a value has been reproduced from Raymond and Redman (2002).

In control slices 1TBS was associated with LTP with a τ of 39 ± 12 min (n=4; Figure 4.3B). Four trains of TBS induced a more persistent form of LTP, with τ measuring 117 ± 14 min (n=9; Figure 4.3B). Eight trains of TBS induced LTP that was qualitatively more persistent than that induced by 4TBS. A τ value for 8TBS LTP could not be determined in these experiments because all control LTP was essentially non-decremental over 2 hrs (Figure 4.1C) and could not be fit by exponential decay curves. For illustrative purposes the τ value obtained from a previous set of experiments (Raymond & Redman, 2002) has been displayed (Figure 4.3B). This 8TBS τ value is therefore an underestimate and is not used for statistical analysis. Based on these measurements and previously published data using 1, 4 and 8TBS, these forms of LTP are henceforth classified as LTP1, LTP2 and LTP3 respectively.

4.1.2. FM 1-43 destaining as a valid measure of release rate and justification of methods

FM 1-43 was loaded into terminals of CA3 neurons using a train of electrical stimulation applied to the Schaffer collateral pathway, following bath application of the dye (Figure 3.6). This method resulted in bright, punctate staining that could be visualised using two-photon microscopy. The fluorescence of these putative terminals decreased rapidly following subsequent electrical stimulation, corresponding to diffusion of dye from synaptic vesicles (Figure 3.9).

During a train of electrical unloading stimulation, the fluorescence of terminals decayed with an approximately exponential time course, reflecting the first-order kinetics of exocytosis (Ryan *et al*., 1993; Stevens & Tsujimoto, 1995). To determine the optimal stimulation frequency to elicit unloading, the effect of different frequencies on the rate of unloading was measured (Figure 4.4). The time required for fluorescence intensity to decay to half of its original value ($t_{1/2}$) decreased as the frequency of electrical stimulation increased between 1 and 3 Hz. At stimulation frequencies above 3 Hz, the rate of unloading approached a maximal value, perhaps due to the rate limiting kinetics of FM 1-43 release from membranes (Klingauf *et al*., 1998). A stimulation frequency of 1 Hz was used in all subsequent experiments as this resulted in an intermediate rate of unloading that allowed for the detection of changes in release kinetics.

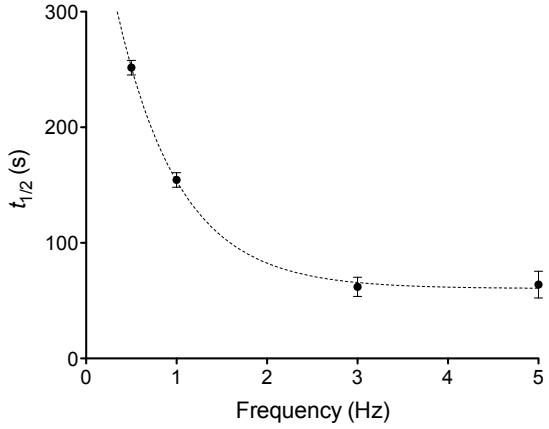

Figure 4.4. Effect of stimulation frequency on the half time ($t_{1/2}$) of FM 1-43 unloading.

Increasing frequency of stimulation increased the rate of unloading up to 3 Hz, after which the rate approached a maximal value. In the following experiments, a stimulation frequency of 1 Hz was used, as this allowed for changes in destaining kinetics to be detected. The data were will fit by a first order exponential decay function (R squared = 0.99)

Although the kinetics of destaining described here have previously been shown to strongly correlate with transmitter release at CA3-CA1 synapses (Zakharenko *et al*., 2001), it was important in these experiments to confirm this under our conditions by artificially manipulating P_r and examining the effects on FM 1-43 destaining.

Elevation of the Ca^{2+}/Mg^{2+} ratio in the external solution, which increases the probability of vesicle fusion and transmitter release, resulted in an increased rate of FM 1-43 destaining (Figure 4.5A, B; 2.5 mM Ca^{2+} $t_{1/2}$ = 154 ± 3 s, n=3 slices, 41 terminals; 5.0 mM Ca^{2+} $t_{1/2}$ = 48 ± 5 s, n=3 slices, 63 terminals; p<0.05), consistent with previous studies (Zakharenko *et al*., 2001). Changes in recording temperature were also used to examine the use of FM 1-43 as a predictor of presynaptic function, as reductions in recording temperature are known to reduce vesicle turnover (Gaffield & Betz, 2007; Ruiz *et al*., 2011). At room temperature (21.5°C) the rate of FM 1-43 destaining was diminished compared to normal recording temperature of 32°C (Figure 4.5C, D; 21.5°C $t_{1/2}$ = 194 ± 3 s, n=4 slices, 64 terminals; 32°C $t_{1/2}$ = 158 ± 3 s, n=3 slices, 47 terminals; p<0.05). Both experiments suggest that the measurements of FM 1-43 destaining described here accurately predict exocytotic rate.

It was also critical to verify the use of the NMDAR antagonist D-APV as an adequate inhibitor of synaptic plasticity during loading and unloading stimulation. Control experiments in the absence of TBS demonstrated that even following inclusion of D-APV (100 μM), a small degree of potentiation occurred as a result of the loading and unloading protocol (Figure 4.6A). However this was transient and fEPSP size returned to baseline amplitude after approximately 5-10 min, well before the next load/unload protocol occurred.

Also, despite this small short-lived plasticity, the LTP decay characteristics with multiple bouts of loading/unloading were not significantly different from controls (Figure 4.6B, C). In control slices, 1TBS resulted in LTP with a τ of 39 ± 12 min (n=4) and in slices with multiple load/unloads a τ of 30 ± 3 min (n=7; Figure 4.6C). Control 4TBS induced LTP with a τ measuring 101 ± 32 min (n=9, Figure 4.3B, 4.6C) and 4TBS in slices with multiple load/unload induced LTP with a mean τ of 94 ± 22 (n=8; Figure 4.6C). As for the 8TBS group of slices without multiple load/unloads, a τ value could not be measured for slices exposed to load/unload protocols, as the LTP was essentially non-decremental and could not be fit by a second order decay function.

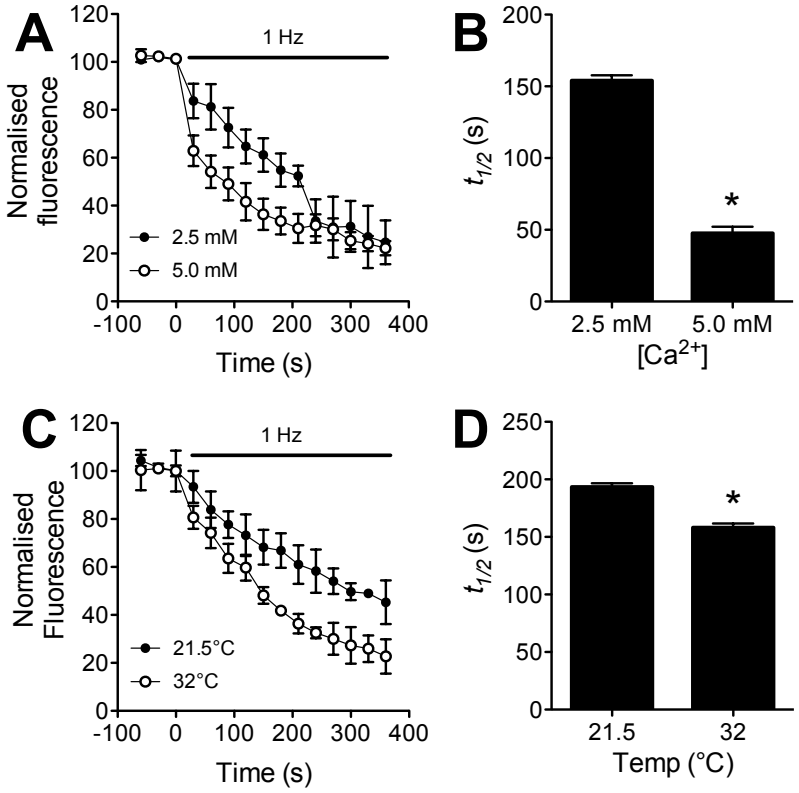

Figure 4.5. The rate of FM 1-43 unloading was predictably altered with changes in release probability.

A, Increasing release probability by elevating extracellular Ca^{2+} enhanced FM 1-43 destaining. Mean time course of FM 1-43 destaining in normal ACSF (2.5 mM) and elevated (5.0 mM) extracellular Ca^{2+}. **B**, Summary bar graph showing average half time of decay ($t_{1/2}$) of FM 1-43 fluorescence for unload in normal (2.5 mM) ACSF and elevated (5.0 mM) extracellular Ca^{2+}. **C**, Decreasing release probability by lowering recording temperature slowed FM 1-43 destaining. Mean time course of FM 1-43 destaining at normal (32°C) and reduced (21.5°C) recording temperature. **D**, Summary bar graph showing average half time of decay ($t_{1/2}$) of FM 1-43 fluorescence for unload at normal and reduced recording temperature (*p<0.05).

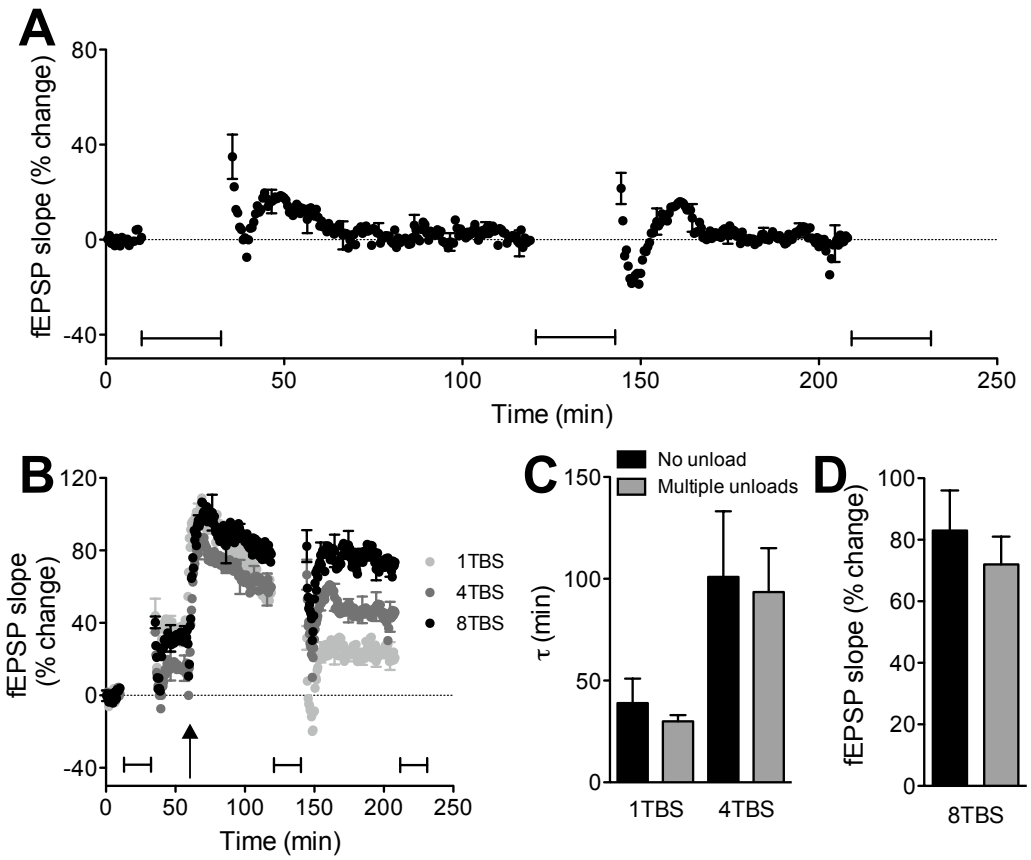

Figure 4.6. Effect of multiple loads/unloads on LTP decay.

A, Mean percent change in field excitatory postsynaptic potential (fEPSP) slope from slices receiving no TBS with a load/unload (indicated by capped lines) beginning at 10, 120 and 210 min to mimic those received by TBS exposed slices. D-APV (100 μM) was present during the load/unload protocols. **B**, Mean percent change in fEPSP slope in slices receiving 1, 4 or 8TBS with a load/unload beginning 60 min and 120 min post-TBS, as well as during the baseline. LTP was induced after a 60 min baseline period (arrow). **C**, The mean time constant of decay, τ is shown for LTP induced with 1 and 4TBS in slices exposed to multiple loads/unloads (grey bars) and without such protocols (black bars). Decay of LTP remained the same whether or not load/unload procedures occurred during the recording. **D**, There was no difference in magnitude of LTP for 8TBS slices exposed to multiple load/unload protocols (grey bars) and without such protocols (black bars).

A magnitude comparison for 8TBS slices demonstrated that load/unload protocols did not significantly affect the magnitude of 8TBS-LTP in the last 5 mins of recording (Figure 4.6D) with a mean fEPSP magnitude of 83 ± 13% (n=7) in control slices and 73 ± 9% (n=15) in slices exposed to multiple load/unloads.

Importantly, unloading experiments in the absence of TBS showed no change in destaining rate between 'baseline' ($t_{1/2}$ = 120 ± 27 s, n=4 slices, 67 terminals), 80 min ($t_{1/2}$ = 140 ± 16 s, n=4 slices, 58 terminals) and 160 min unloads ($t_{1/2}$ = 152 ± 27 s, n=3 slices, 46 terminals; Figure 4.7). There are two important implications of this finding. First, the load/unload protocol does not itself affect presynaptic release rate as measured via subsequent unloads and second, there is no time-dependent change in release rate in the absence of TBS. Therefore any changes observed in presynaptic release must be due to changes in expression associated with LTP1, 2 and 3 and not due to the short-term plasticity associated with the load/unload protocol or due to other factors related to the experimental design.

4.1.3. More persistent forms of LTP are associated with enhanced exocytosis

To determine whether presynaptic changes are associated with LTP1, 2 and 3 we simultaneously measured FM 1-43 release from potentiated CA3 terminals in the same slices as the LTP experiments shown in Figure 4.6B.

Under baseline conditions, FM 1-43 destaining kinetics were consistent across each experimental group (Figure 4.8; Baseline $t_{1/2}$ 1TBS = 156 ± 27 s, n=5 slices, 76 terminals; 4TBS = 153 ± 27 s, n=8 slices, 115 terminals; 8TBS = 145 ± 17 s, n=15 slices, 228 terminals). LTP1 was not associated with a change in FM 1-43 destaining at either 80 min ($t_{1/2}$ = 147 ± 19 s, n=7 slices, 108 terminals) or 160 min post-induction ($t_{1/2}$ = 160 ± 31 s, n=5 slices, 76 terminals; Figure 4.8A, B). LTP2 was associated with enhanced exocytosis at 160 min after induction ($t_{1/2}$ = 45 ± 9 s, n=6 slices, 90 terminals; p<0.05), but not at 80 min ($t_{1/2}$ = 114 ± 22 s, n=8 slices, 118 terminals; Figure 4.8C, D). Finally, LTP3 was associated with enhanced exocytosis at both 80 min ($t_{1/2}$ = 39 ± 8 s, n=14 slices, 212 terminals) and 160 min post-induction ($t_{1/2}$ = 47 ± 6 s, n=10 slices, 148 terminals; p<0.05; Figure 4.8E, F). These findings demonstrate that short-lasting LTP1 is predominantly expressed postsynaptically and that more persistent LTP2 and 3 recruit a presynaptic component of expression.

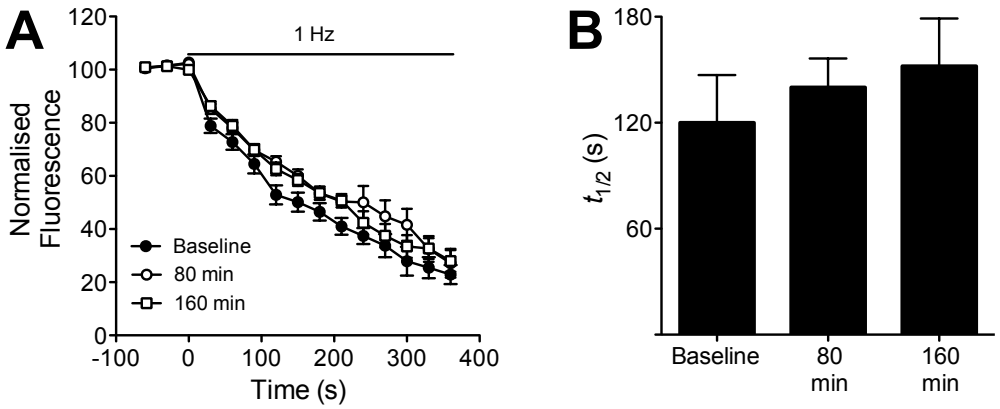

Figure 4.7. Basal release rate does not change in response to multiple load/unloads.

A, Mean time course of FM 1-43 destaining during the 'baseline' period and at 80 min and 160 min post-baseline in the absence of TBS. **B**, Summary histogram showing average half time of decay ($t_{1/2}$) of FM 1-43 fluorescence for the baseline, 80 min and 160 min unload.

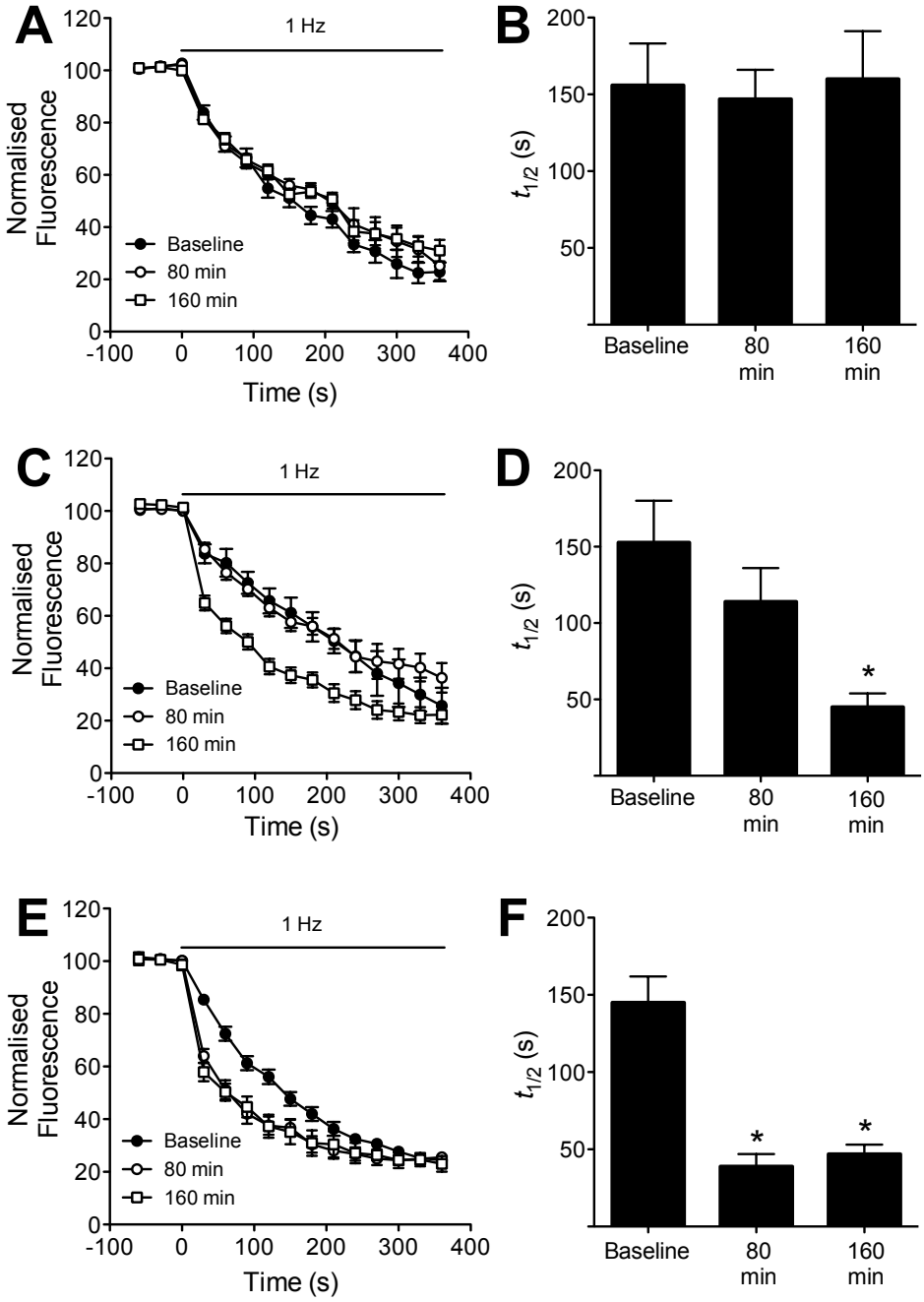

Figure 4.8. More persistent forms of LTP are associated with an enhancement of presynaptic function.

A, LTP1 is not associated with an increased rate of FM 1-43 release from CA3 terminals. Mean (±SEM) time course of FM 1-43 destaining prior to LTP1 induction (baseline), 80 min and 160 min post-induction. **B**, Summary histogram showing average half time of decay ($t_{1/2}$) of FM 1-43 fluorescence for the baseline, 80 min and 160 min unload for LTP1 slices. **C**, FM 1-43 destaining time course shows LTP2 is associated with an enhancement of release 160 min post-induction. **D**, Summary histogram of average $t_{1/2}$ in LTP2 experiments. **E**, FM 1-43 destaining time course shows LTP3 is associated with enhanced release at 80 min and 160 min post-TBS. **F**, Summary histogram of average $t_{1/2}$ in LTP3 experiments (*p<0.05).

4.1.4. Induction of LTP3 involves the uptake of FM 1-43 into non-releasing compartments

Interestingly, following the induction of LTP with 8TBS, there was a notable increase in the number of terminals that did not destain. There normally exists a population of terminals that take up dye, yet do not exhibit activity-dependent destaining (defined by our criteria as losing less than 70% of the dye upon termination of the unload stimulation). This is presumably because the 1 Hz unload stimulation is insufficient to induce appreciable dye loss. The proportion of these terminals remains unchanged in slices stimulated with 1 and 4TBS, however is significantly increased following 8TBS at both the 80 min (40 ± 6%, n=14 slices, 211 terminals) and 160 min (57 ± 8%, n=10 slices, 163 terminals) time points (Figure 4.9A; two-way ANOVA, Bonferroni Posttests, $p<0.05$). There are several potential explanations for why such a phenomenon might occur. These include an increased uptake of dye into other non-releasing vesicle pools or into non-vesicular compartments such as endosomes. Alternatively such an increase could result from a change in the mode of transmitter release from a full fusion event (that allows FM 1-43 diffusion) to a more rapid kiss-and-run type release that would cause FM trapping. To distinguish between these possibilities, it was important to quantify the average maximal fluorescence of terminals immediately following loading to determine whether other synaptic compartments were taking up dye during the loading procedure. The maximum fluorescence was found to be significantly larger in 8TBS non-destaining terminals (Figure 4.9B) at both 80 min (4.5 ± 1.3 a.u., n=14 slices, 211 terminals) and 160 min (4.7 ± 1.4 a.u., n=10 slices, 163 terminals) post-TBS, supporting a role for trapping of dye in non-releasing vesicle pools or endosomal compartments and thereby making it difficult to detect destaining. Also important to note is that the absolute fluorescence remained unchanged in the other destaining groups (Figure 4.9B), suggesting that there was no temporal change in the amount of dye that could be internalised within a terminal.

The population of non-destaining terminals were further investigated to establish if other stimuli could induce release of the dye. Extending the 1 Hz unloading protocol to 12 minutes and use of both higher frequency 5 and 10 Hz unload stimulation all failed to promote release of the dye (Figure 4.10A). Bath application of KCl solution (which is known to deplete the total pool of vesicles within a terminal) did produce significant loss of dye from nondestaining terminals (Figure 4.10B), with average fluorescent intensity being reduced to 1.7 ± 0.2 a.u. (n=2 slices, 28 terminals; $p<0.05$).

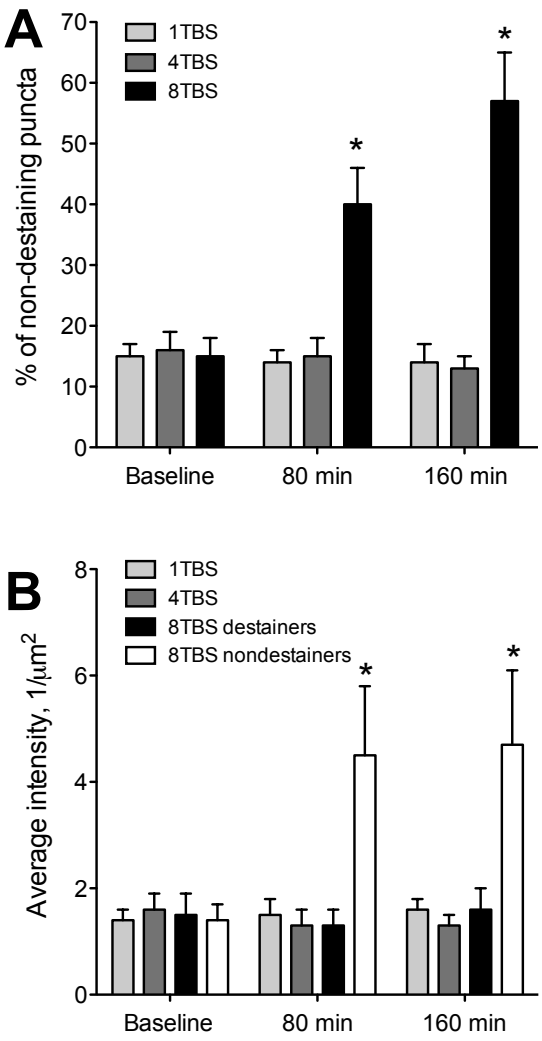

Figure 4.9. The proportion of nondestaining puncta is increased following 8TBS.

A, Mean percentage of nondestaining terminals are shown for the baseline, 80 min and 160 min unload in slices stimulated with 1, 4 and 8TBS. There were significantly more terminals that did not destain following 8TBS (two-way ANOVA, Bonferroni Posttests, *p<0.05). **B**, Average fluorescent intensity of terminals (post-loading) following 1, 4 and 8TBS. Average intensity was calculated by dividing total fluorescence intensity of individual terminals by their area. Average resting intensity was significantly larger in the population of non-destaining terminals exposed to 8TBS (two-way ANOVA, Bonferroni Posttests, *p<0.05).

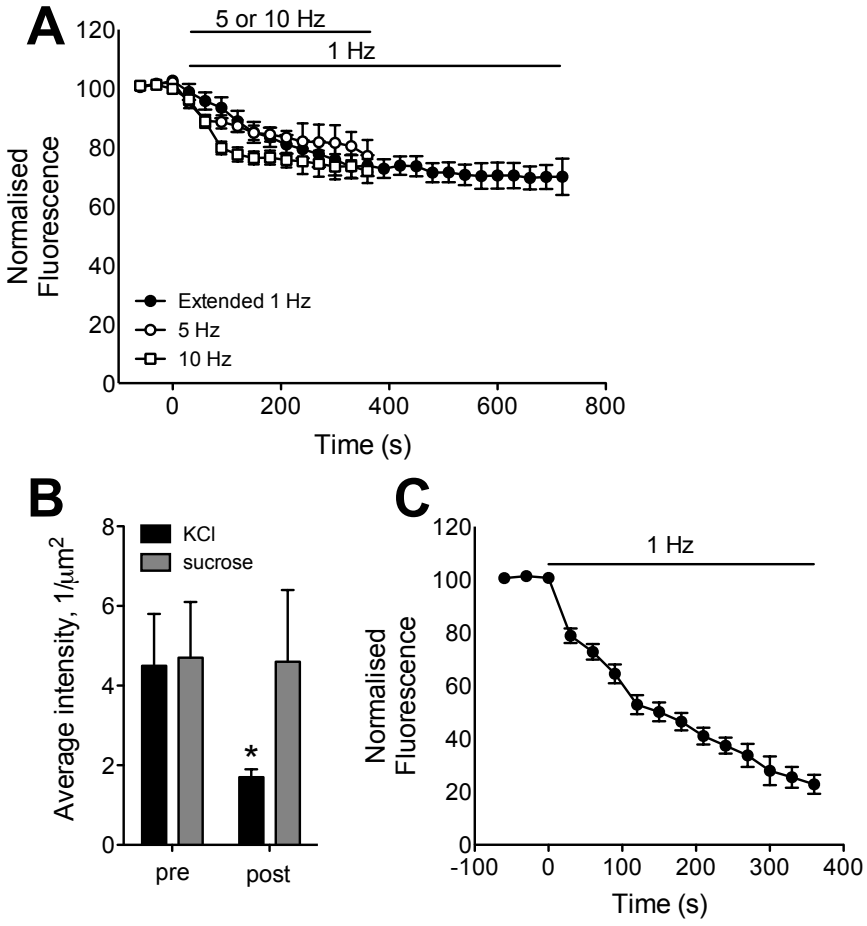

Figure 4.10. Loading stimulation following 8TBS causes uptake of dye into non-releasing compartments.

A, Mean time course of FM 1-43 destaining in non-destaining terminals in 8TBS slices following subsequent 1 Hz stimulation for 12 min, 5 Hz stimulation for 6 min and 10 Hz stimulation for 6 min. All of these protocols failed to induce appreciable destaining. **B**, Average fluorescent intensity of non-destaining terminals in 8TBS slices following KCl and sucrose application. Depletion of the RRP with sucrose did not induce release of the sequestered dye, whereas depletion of the total pool with KCl caused significant dye loss (*p<0.05). **C**, Subsequent 1 Hz stimulation following sucrose depletion of the RRP resulted in activity-dependent destaining, presumably due to recruitment of dye-containing reserve pool vesicles to the active zone.

This indicates that the dye was being sequestered in nonreleasing vesicles, rather than endosomes or other non-vesicular compartments. To further elucidate which vesicle population the dye was trapped in, the RRP was depleted with bath application of 500 mM sucrose solution (Rosenmund & Stevens, 1996; Pyle *et al*., 2000; Mozhayeva *et al*., 2002). This alone did not result in further dye loss (Figure 4.10B), however subsequent 1 Hz stimulation following sucrose application did induce activity-dependent destaining, presumably as depletion of the RRP allowed for recruitment of dye-containing reserve pool vesicles to the active zone (Figure 4.10C). Therefore it is highly likely that loading stimulation following induction of LTP with 8TBS results in uptake of dye into the RRP as well as the reserve pool of vesicles.

4.1.5. There exists a single, stable population of terminals before and after LTP

It was important to verify that sampled puncta were primarily representative of CA3 terminals and did not include large numbers of GABAergic inhibitory interneurons or astrocytes, both of which endocytose FM 1-43 (Hablitz *et al*., 2009; Li *et al*., 2009) and are frequently overlooked in hippocampal FM experiments. If this were the case one might expect different populations of destaining kinetics to be apparent in our data, as both inhibitory interneurons and astrocytes exhibit heterogeneity in their release properties (McBain & Fisahn, 2001; Moulder *et al*., 2007; Sakaba, 2008). However, we found only one population of destaining kinetics, with an average basal $t_{1/2}$ of 131 ± 4 s (n=28 slices, 420 terminals; Figure 4.11A). It was therefore concluded unlikely that release from astrocytes or inhibitory interneurons were appreciably affecting measurements of destaining from CA3 terminals. This is supported by evidence indicating that only 2.5% of terminals in the CA3 region are GABAergic (Hiscock *et al*., 2000).

There was also no change in the mean number of release sites measured before and after LTP induction (Figure 4.11B; Basal: 15 ± 1, n=28 slices, 420 terminals; LTP1: 16 ± 1, n=5 slices, 76 terminals; LTP2: 14 ± 2, n=8 slices, 118 terminals; LTP3: 15 ± 3, n=10 slices, 148 terminals). This stability in the number of terminals loaded with FM 1-43 suggests that, under these conditions, induction of LTP1, 2 and 3 does not 'unsilence' presynaptically inactive synapses (Voronin *et al*., 2004).

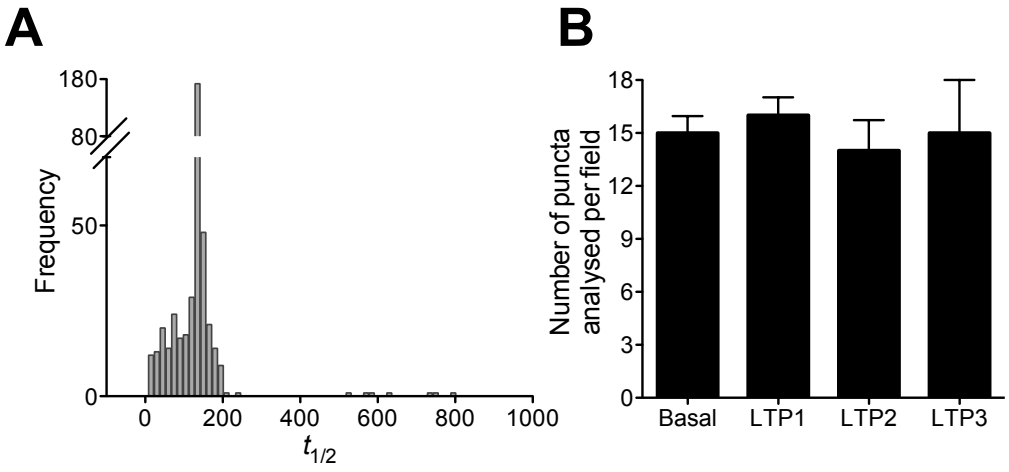

Figure 4.11. There exists a single, stable population of terminals before and after LTP induction.

A, Frequency histogram of half time of intensity decay ($t_{1/2}$) of individual terminals suggests that measurements are taken from a single population of synaptic vesicles. Data not well fit by a Gaussian distribution ($p<0.05$, D'Agostino and Pearson omnibus normality test). **B**, No difference in the number of terminals analysed per field before LTP induction (basal) and 160 min post induction of LTP1, LTP2 and LTP3 indicates there is no 'unsilencing' of presynaptically inactive synapses post-LTP (p=0.88, one-way ANOVA).

4.1.6. Enhanced release is an 'all-or-none' phenomenon

The enhanced release that occurs with LTP2 and 3 appears to be an 'all-or-none' phenomenon, in that release is always elevated to a certain level and does not occur in a graded fashion. This can be seen by comparing the half-life of destaining at the 160 min unload with the percentage of LTP in the last 5 min of recording for individual terminals for all three forms of LTP (Figure 4.12). The relationship resembles a step-function, indicating that there is a critical level of LTP robustness that must be reached in order for enhanced release to occur. Only slices in which magnitude in the final 5 min of recording was larger than approximately 30% exhibited enhanced release. This relationship also seems to correlate with measures of persistence. Although τ values could not be measured for LTP3 slices, it seems that there is also a critical τ threshold (approximately 123 min) and at decay rates faster than this enhanced release does not occur. It is also important to note that there is a division within the LTP2 slices, with some showing enhanced release while others do not. This appears to be dependent upon the final LTP magnitude.

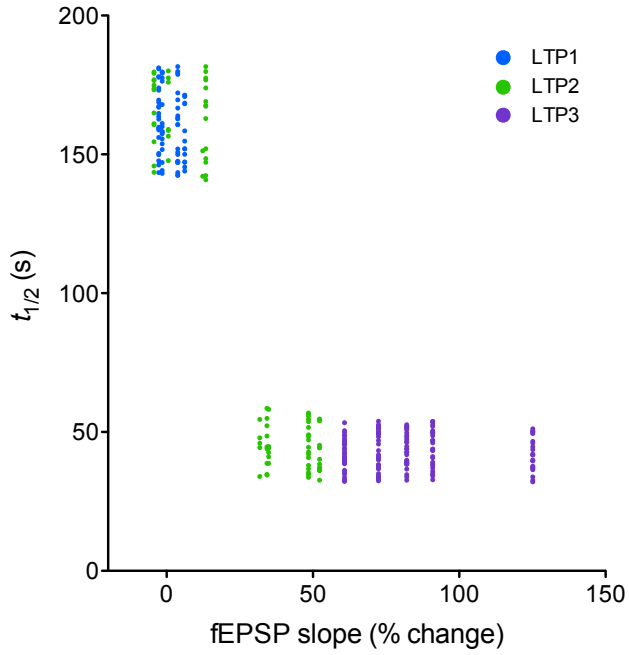

Figure 4.12. Dependence of half-life of destaining on final LTP magnitude.

Correlation between $t_{1/2}$ and LTP magnitude in the final 5 min of recording for individual LTP1 (n=76), LTP2 (n=90) and LTP3 (n=148) terminals. The data resemble a step-function, indicating that only LTP of a critical magnitude will be associated with enhanced release.

4.2. LTP2 and LTP3 are nitric oxide dependent

In order for persistent forms of LTP to recruit a presynaptic component to their expression it is logical that this may require a retrograde method of signalling from the postsynaptic dendrite to the presynaptic terminal. A particularly attractive candidate to mediate this signalling is NO and several studies have shown that inhibition of NO signalling in CA1 prevents the induction of LTP (Böhme *et al*., 1991; O'Dell *et al*., 1991a; Schuman & Madison, 1991; Haley *et al*., 1992). However, it is not clear whether NO signalling is important for all forms of LTP now identified at these synapses. Furthermore, evidence for the prevailing view that during LTP induction NO is released from the postsynaptic cell and acts on the presynaptic terminal to enhance transmitter release (reviewed by Regehr *et al*., 2009) is largely based on indirect measurements of presynaptic function. In the next set of experiments we therefore determined whether inhibition of NO signalling differentially affects the persistence of LTP1, 2 and 3 and if so, whether this correlates with direct measurements of exocytosis. Given that significant increases in exocytosis are observed at the 160 min time point for both LTP2 and 3, we confine all subsequent pharmacological analyses to this time point.

NO signalling was disrupted by inhibiting either nitric oxide synthase (NOS) with *N*-ω-nitro-L-arginine methyl ester (L-NAME, 100 μM) or inhibiting extracellular NO signalling with the membrane impermeable NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 40 μM). Both drugs were bath applied for 40 min, beginning 20 min prior to LTP induction. Importantly neither L-NAME nor cPTIO significantly affected fEPSP slope (Figure 4.13A) or basal release rate in the absence of TBS (Figure 4.13B; basal $t_{1/2}$ = 144 ± 22 s, n=3 slices, 47 terminals; 200 min = 152 ± 17 s, n=3 slices, 53 terminals).

Inhibition of NOS had no significant effect on either the magnitude or persistence of LTP1 (Figure 4.14A, C; Control τ = 39 ± 12 min, n=4; L-NAME = 59 ± 16 min, n=3). Likewise, LTP1 was not affected by cPTIO (Figure 4.14B, C; cPTIO τ = 63 ± 9 min, n=4). Unsurprisingly, exocytotic rates measured at 160 min after 1TBS were also unaffected by inhibition of NO signalling with either L-NAME (Figure 4.14D, E; Control $t_{1/2}$ = 160 ± 31 s, n=5 slices, 76 terminals; L-NAME = 138 ± 6 s, n=4 slices, 83 terminals) or cPTIO (143 ± 4 s, n=4 slices, 162 terminals).

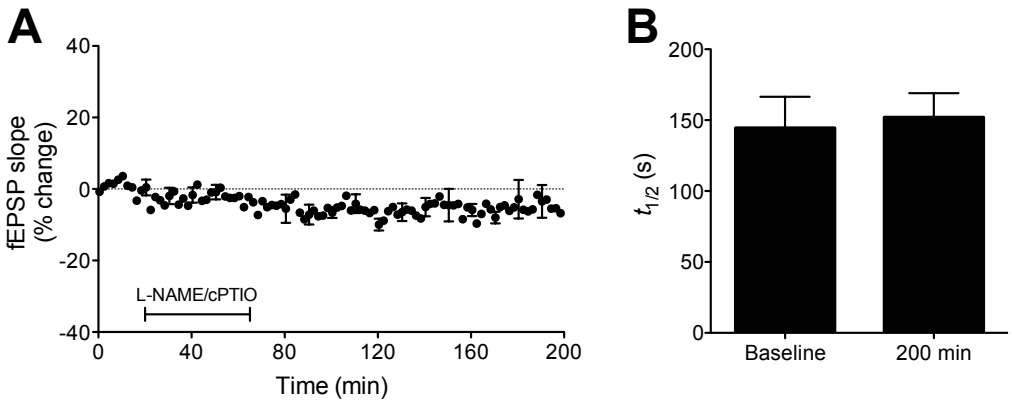

Figure 4.13. L-NAME and cPTIO have no effect on basal fEPSPs or exocytotic rate.

A, Mean percent change in fEPSP slope from slices exposed to a combination of L-NAME and cPTIO over a 40 min period with no TBS. **B**, Summary histogram showing average half time of decay ($t_{1/2}$) of FM 1-43 fluorescence for the Baseline and 200 min unload.

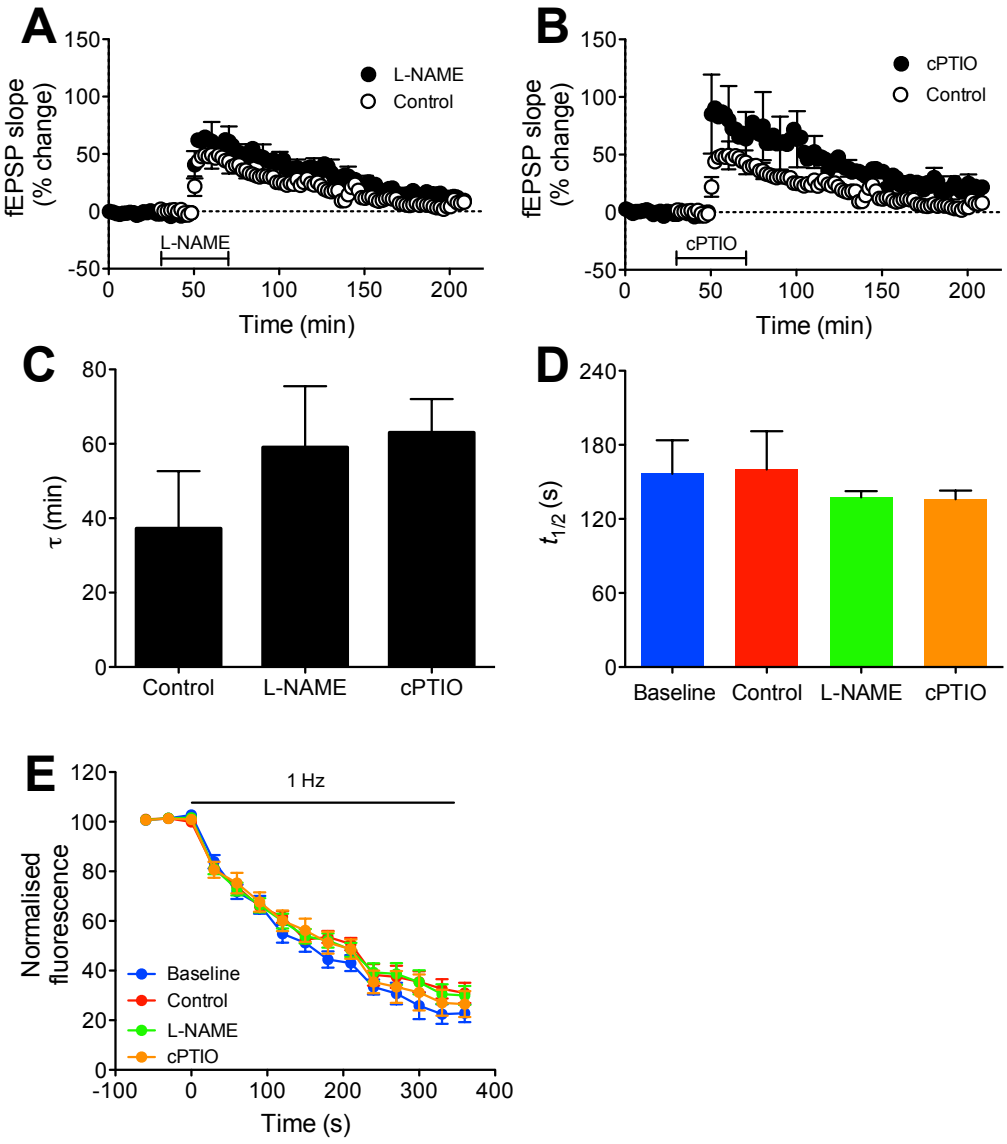

Figure 4.14. LTP1 is independent of NO signalling.

A, Mean percent change in fEPSP slope in controls and slices incubated with L-NAME (100 μM) during the 1TBS induction protocol. **B**, Mean percent change in fEPSP slope in controls and slices incubated with cPTIO (40 μM) during the induction period. **C**, Application of L-NAME and cPTIO did not affect LTP1 decay. **D**, Histogram of mean FM 1-43 destaining kinetics ($t_{1/2}$) in LTP1 experiments showing that application of L-NAME and cPTIO did not affect exocytotic rate 160 min post-TBS. **E**, Mean (±SEM) time course of FM 1-43 destaining prior to LTP induction (Baseline), 160 min post-induction (Control) and 160 min post-induction with L-NAME/cPTIO present during the induction period.

In contrast, inhibition of NO signalling significantly reduced the persistence of LTP2 (Figure 4.15A, B, C; Control τ = 117 ± 14 min, n=9; L-NAME = 72 ± 8 min, n=4; cPTIO = 80 ± 10 min, n=4; $p<0.05$). Both inhibitors also abolished the enhanced exocytosis associated with LTP2 measured 160 min post-TBS (Figure 4.15D, E; Control $t_{1/2}$ = 45 ± 9 s, n=6 slices, 90 terminals; L-NAME = 140 ± 9 s, n=4 slices, 53 terminals; cPTIO = 147 ± 6 s, n=4 slices, 57 terminals; $p<0.05$).

Inhibition of NO signalling with L-NAME and cPTIO dramatically reduced LTP3 persistence (Figure 4.16A, B, D; Control τ = 230 min [from Raymond & Redman, 2002]; L-NAME = 69 ± 8 min, n=4; cPTIO = 77 ± 10 min, n=3). Since statistical analysis of τ values for LTP3 was not possible in this case we compared the magnitude of LTP over the last 5 min of recording. Both inhibitors significantly reduced LTP magnitude at 160 min post-8TBS (Figure 4.16C; Control = 83 ± 13%, n=7; L-NAME = 1 ± 6%, n=4; cPTIO = 34 ± 18%, n=3; $p<0.05$). Inhibition of NO signalling also prevented the enhanced exocytosis associated with LTP3 at 160 min post-TBS (Figure 4.16E, F; Control $t_{1/2}$ = 47 ± 6 s, n=10 slices, 148 terminals; L-NAME = 145 ± 3 s, n=4 slices, 67 terminals; cPTIO = 136 ± 7 s, n=3 slices, 52 terminals; $p<0.05$).

In some cases it may appear that inhibition of NO signaling with cPTIO and L-NAME affects the induction of LTP, and that the peak fEPSP magnitude immediately post-induction is increased with these inhibitors. However, a significant increase in the maximum fEPSP amplitude was measured only for LTP2 in the presence of L-NAME (Control = 86 ± 18%, n=11; L-NAME = 132 ± 21%, n=4). Given that LTP1 and LTP3 induction is not affected by the inclusion of L-NAME, and that other reports indicate that this drug does not affect peak fEPSP magnitude (Ko & Kelly, 1999), we propose that this significant result is likely to be due to experimental variation, rather than specific drug effects.

The data reported above demonstrate that only the more robust LTP2 and LTP3 require both activation of NOS and diffusion of NO in the extracellular space and that NO signalling is necessary to trigger the enhanced exocytosis associated with these more persistent forms of LTP.

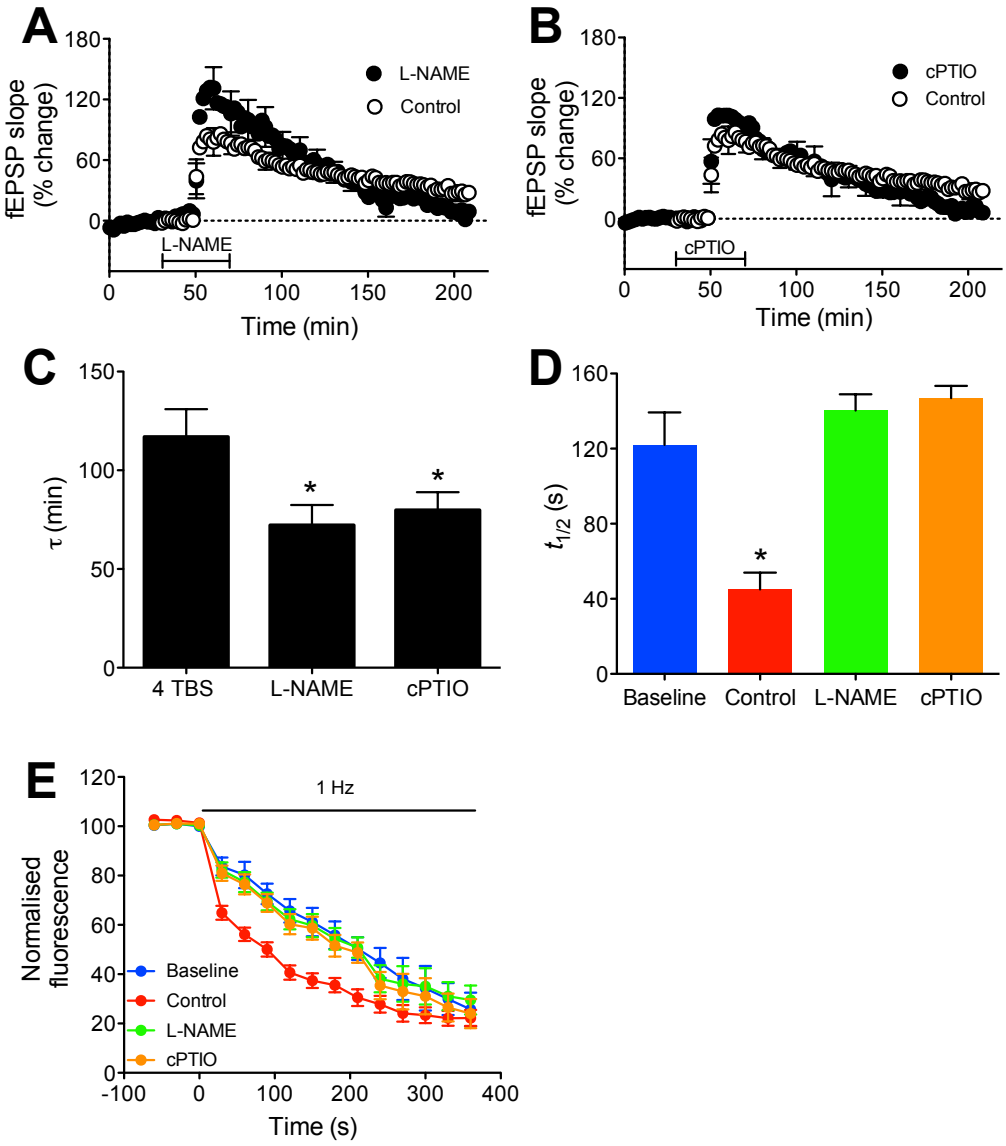

Figure 4.15. LTP2 is dependent on NO signalling.

A, Mean percent change in fEPSP slope in controls and slices incubated with L-NAME during the induction period. **B**, Mean percent change in fEPSP slope in controls and slices incubated with cPTIO during the induction period. **C**, LTP2 persistence was significantly reduced with L-NAME and cPTIO application. **D**, Histogram of FM 1-43 destaining kinetics in LTP2 experiments showing that application of L-NAME and cPTIO abolished the 160 min presynaptic enhancement (*p<0.05). **E**, Mean (±SEM) time course of FM 1-43 destaining prior to LTP induction (Baseline), 160 min post-induction (Control) and 160 min post-induction with L-NAME/cPTIO present during the induction period.

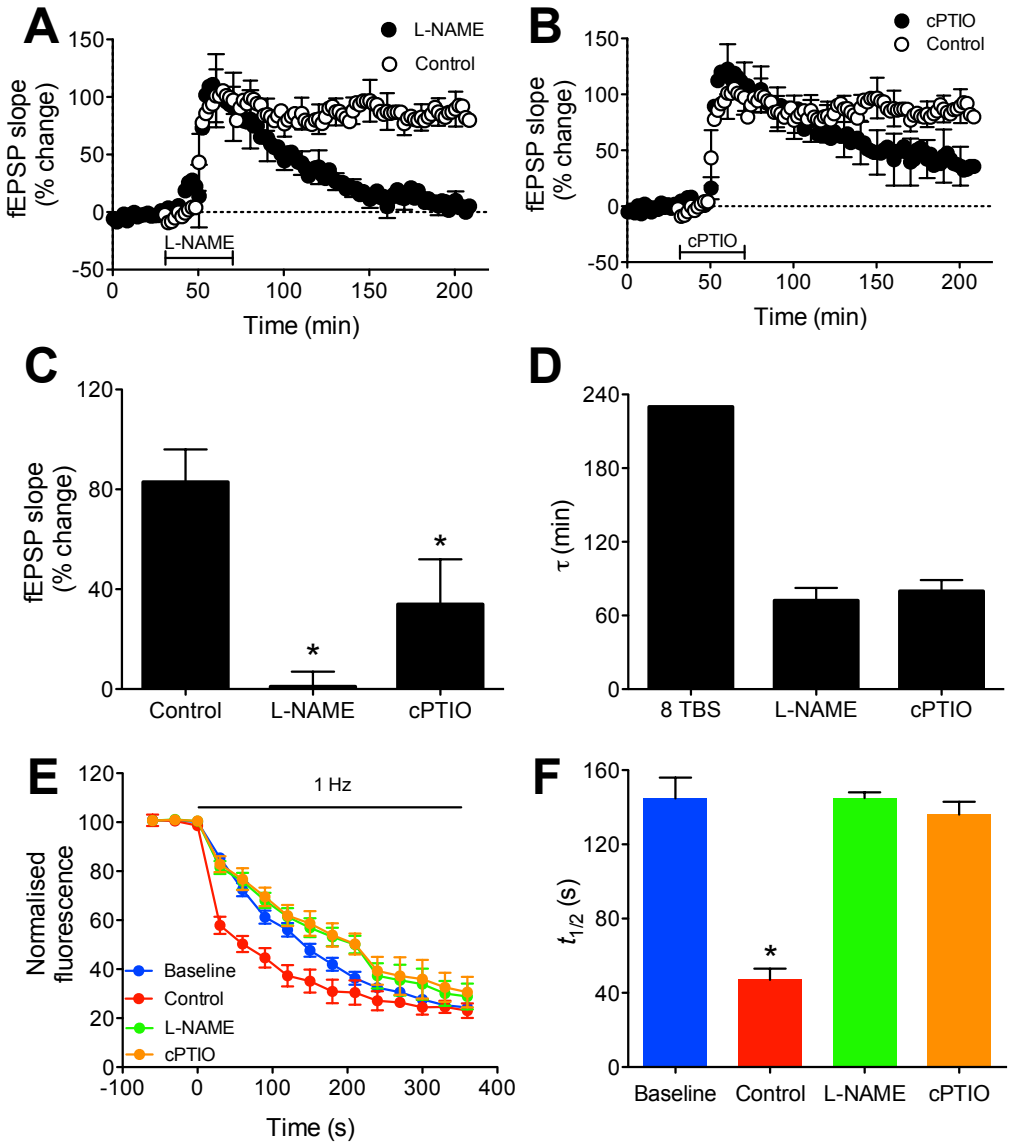

Figure 4.16. LTP3 is dependent on NO signalling.

A, Mean percent change in fEPSP slope in controls and slices incubated with L-NAME during the 8TBS induction period. **B**, Mean percent change in fEPSP slope in controls and slices incubated with cPTIO during the induction period. **C,** LTP3 magnitude in the last 5 min of recording was significantly reduced with L-NAME and cPTIO application. **D**, LTP3 persistence was dramatically reduced with L-NAME and cPTIO application. Control data reproduced from Raymond & Redman (2002). **E**, Mean (±SEM) time course of FM 1-43 destaining prior to LTP induction (Baseline), 160 min post-induction (Control) and 160 min post-induction with L-NAME/cPTIO present during the induction period. **F**, Histogram of FM 1-43 destaining kinetics in LTP3 experiments showing that application of L-NAME and cPTIO abolished the 160 min presynaptic enhancement (*p<0.05).

4.3. More persistent forms of LTP are dependent on protein synthesis

Until now LTP1, 2 and 3 have been differentiated on the basis of distinct induction and maintenance mechanisms (Raymond, 2007; Reymann & Frey, 2007). With regard to maintenance, LTP1 is proposed to involve posttranslational modifications of existing proteins and glutamate receptor trafficking (approximately equivalent to E-LTP; Lovinger *et al*., 1987; Malenka *et al*., 1989a; O'Dell *et al*., 1991b; Shi *et al*., 1999). LTP2 is dependent on protein synthesis but not gene transcription (Raymond *et al*., 2000). Finally, LTP3 is translation- and transcription-dependent and is comparable to traditional late-LTP (Nguyen *et al*., 1994; Kandel, 2001; Reymann & Frey, 2007). Importantly, this model has never been systematically tested under constant experimental conditions. We therefore applied either the translation inhibitor anisomycin (ANI, 20 μM) or the transcription inhibitor Actinomycin D (Act D, 40 μM) for 45 min, beginning 20 min prior to LTP induction. Exposure to Act D and ANI had only a minimal effect on normal synaptic transmission (approximately 10%) and had no effect on FM 1-43 release in the absence of LTP induction (Figure 4.17A, B).

Inhibition of translation with ANI or transcription with Act D had no effect on the persistence of LTP1 (Figure 4.18A, B, C; Control τ = 39 ± 12 min, n=4; ANI = 68 ± 29 min, n=4; Act D = 51 ± 16 min, n=4), consistent with this form of LTP depending only on posttranslational modifications. Unsurprisingly neither Act D nor ANI had an effect on LTP1 exocytotic rate measured at 160 min post-TBS (Figure 4.18D, E; Control $t_{1/2}$ = 160 ± 31 s, n=5 slices, 76 terminals; ANI = 135 ± 14 s, n=4 slices, 63 terminals; Act D = 127 ± 16 s, n=4 slices, 94 terminals). Together with the LTP1 decay data this supports the view that LTP1 is expressed postsynaptically and is independent of new protein synthesis.

LTP2 decayed significantly more rapidly with translation inhibited by ANI (Figure 4.19A, C; Control τ = 117 ± 14 min, n=9; ANI = 38 ± 9 min, n=3; $p<0.05$), but was unaffected by inhibition of transcription with Act D (Figure 4.19B, C; Act D τ = 130 ± 27 min, n=3). This is consistent with previously published data showing that LTP2 is dependent on local, mGluR-dependent translation, but not transcription (Raymond *et al*., 2000). ANI also prevented the increase in exocytotic rate that was observed in controls 160 min post-LTP2 induction, whereas Act D had no significant effect (Figure 4.19D, E; Control $t_{1/2}$ = 45 ± 9 s, n=6 slices, 90 terminals; ANI = 132 ± 8 s, n=6 slices, 99 terminals; Act D = 54 ± 6 s, n=5 slices, 73 terminals).

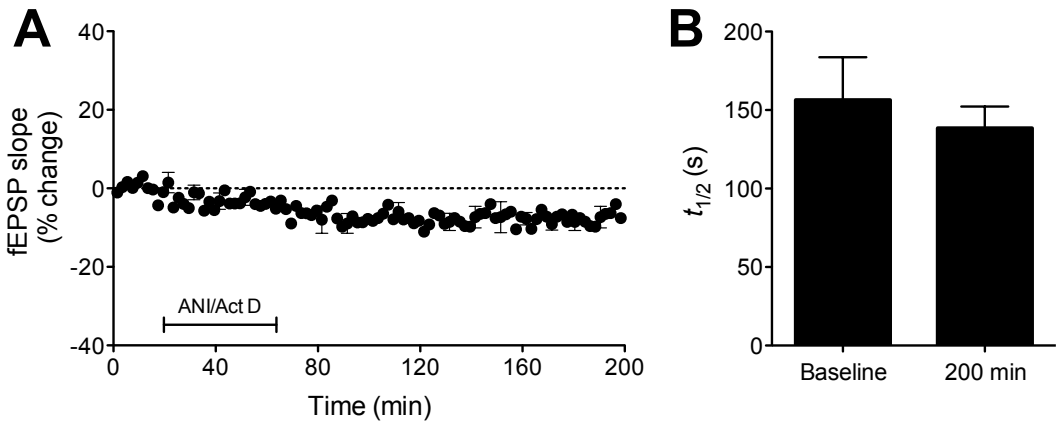

Figure 4.17. Anisomycin and Actinomycin D have only a minimal effect on fEPSPs and no effect on basal exocytotic rate.

A, Mean percent change in fEPSP slope from slices exposed to a combination of ANI and Act D over a 45 min period with no TBS. **B**, Summary histogram showing average half time of decay ($t_{1/2}$) of FM 1-43 fluorescence for the Baseline and 200 min unload.

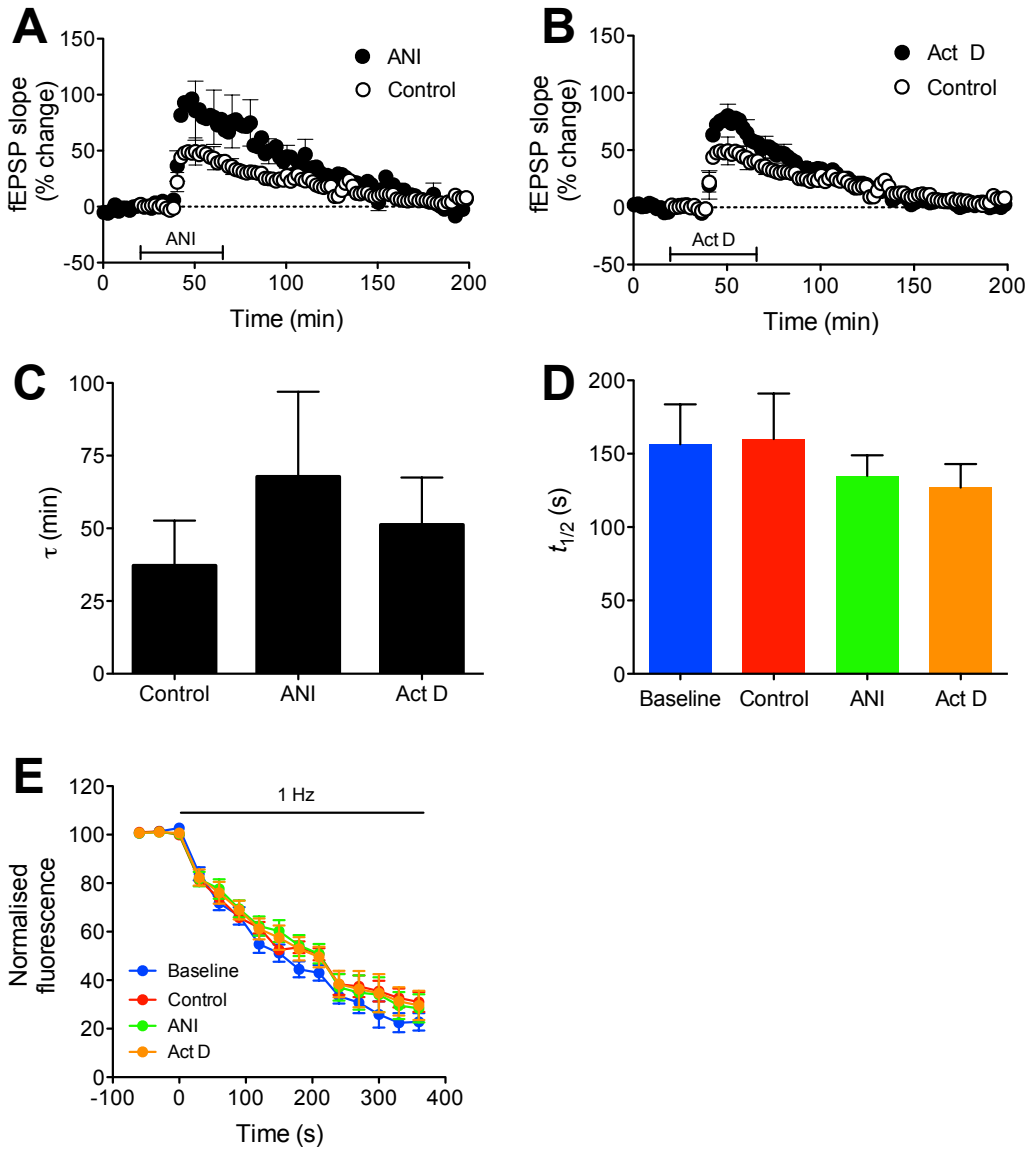

Figure 4.18. LTP1 is independent of protein translation and transcription.

A, Mean percent change in fEPSP slope in controls and slices incubated with anisomycin (ANI, 20 μM) during the 1TBS induction protocol. **B**, Mean percent change in fEPSP slope in controls and slices incubated with Actinomycin D (Act D, 40 μM) during the induction period. **C**, Application of ANI or Act D did not affect LTP1 decay. **D**, Histogram of mean FM 1-43 destaining kinetics ($t_{1/2}$) in LTP1 experiments showing that application of ANI or Act D did not affect exocytotic rate 160 min post-TBS. **E**, Mean (±SEM) time course of FM 1-43 destaining prior to LTP induction (Baseline), 160 min post-induction (Control) and 160 min post-induction with ANI/Act D present during the induction period.

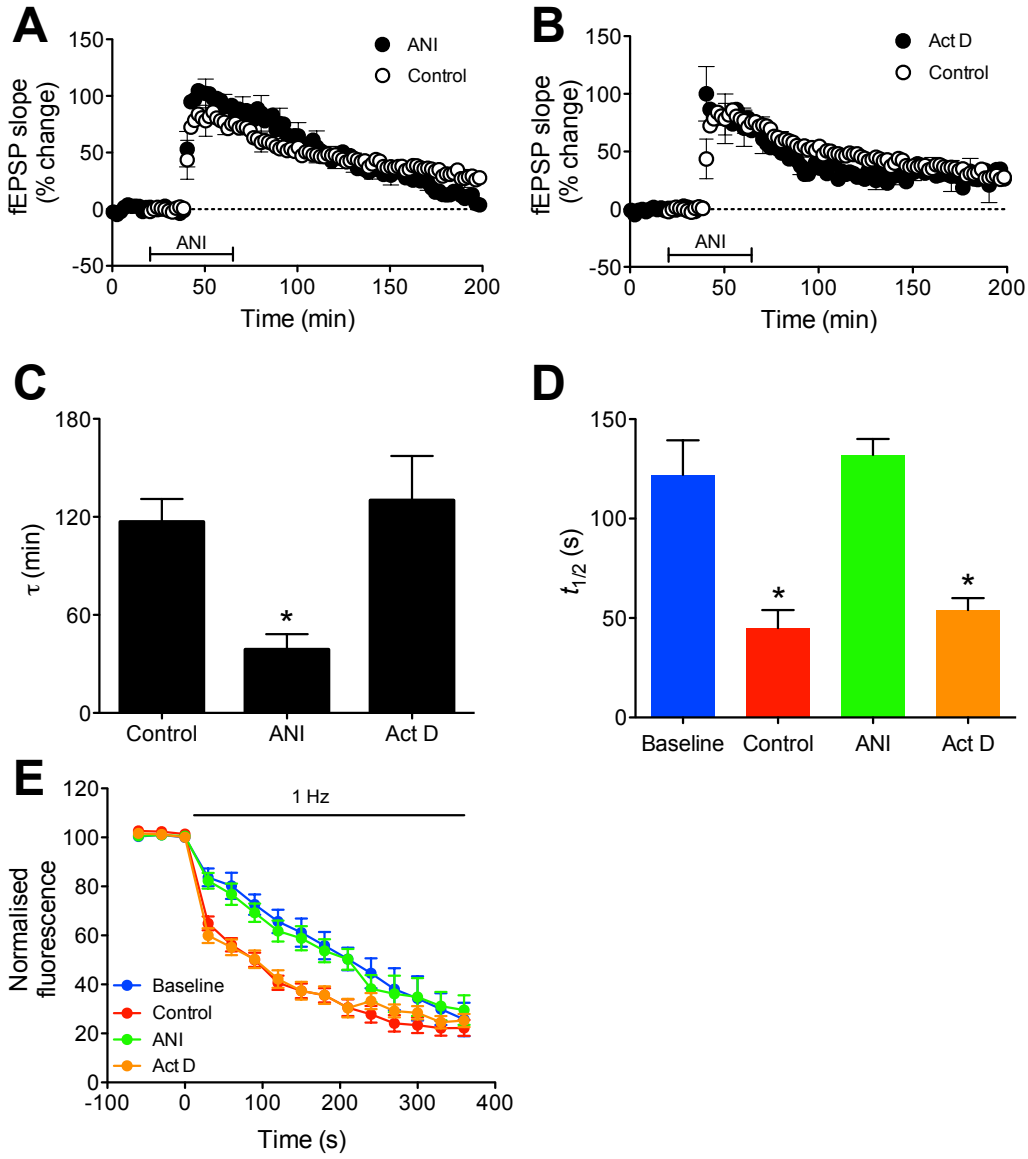

Figure 4.19. LTP2 and the associated enhanced release are dependent on protein translation, but not transcription.

A, Mean percent change in fEPSP slope in controls and slices incubated with ANI during the 4TBS induction protocol. **B**, Mean percent change in fEPSP slope in controls and slices incubated with Act D during the induction period. **C**, Application of ANI (but not Act D) increased the rate of LTP2 decay. **D**, Histogram of mean FM 1-43 destaining kinetics ($t_{1/2}$) in LTP2 experiments showing that application of ANI abolished the enhanced release, whereas Act D did not affect exocytotic rate 160 min post-TBS. **E**, Mean (±SEM) time course of FM 1-43 destaining prior to LTP induction (Baseline), 160 min post-induction (Control) and 160 min post-induction with ANI/Act D present during the induction period (*p<0.05).

Finally, LTP3 persistence was dramatically reduced by inhibitors of translation and transcription (Figure 4.20A, B, D; Control τ = 230 min [from Raymond & Redman, 2002]; ANI = 106 ± 21 min, n=6; Act D = 127 ± 19 min, n=3). As explained for the NO data, calculation of τ values for LTP3 was not possible; therefore the magnitude of LTP over the last 5 min of recording was compared. Both drugs significantly reduced LTP magnitude at 160 min post-8TBS (Figure 4.20C; Control = 83 ± 13%, n=7; ANI = 29 ± 18%, n=6; Act D = 27 ± 15%, n=3; $p < 0.05$). Intriguingly, while the enhanced exocytotic rate associated with LTP3 was also prevented by ANI, it remained unaffected by inhibition of transcription with Act D (Figure 4.20E, F; Control $t_{1/2}$ = 47 ± 6 s, n=10 slices, 148 terminals; ANI = 129 ± 13 s, n=4 slices, 57 terminals; Act D = 56 ± 7 s, n=5 slices, 74 terminals). The finding that LTP3 was significantly inhibited by Act D when the presynaptic expression component was presumably intact, suggests that LTP3 maintenance is associated with a significant postsynaptic expression mechanism. The potentiation remaining in the absence of transcription likely reflects the enhanced presynaptic component.

Importantly, Act D and ANI did not act by inhibiting the *induction* of LTP. Existing evidence suggests that inhibition of protein synthesis during the induction stimulus inhibits LTP (Okulski *et al*., 2002), however the converse was observed here. Application of the inhibitors did not affect LTP induction as evidenced by the similar initial fEPSP magnitude with the drug applied during versus immediately following the 8TBS induction protocol (Figure 4.21A; c.f. Figure 4.20A, B). Also, application of the drug immediately following the TBS protocol induced LTP that was even less persistent and therefore was perhaps a more effective means of inhibiting protein synthesis (Figure 4.21B). Nonetheless, it appears that induction mechanisms remained intact with the application protocol used in these experiments.

These data provide the first confirmation in one study that LTP1 is neither transcription nor translation dependent, LTP2 is translation but not transcription dependent and LTP3 is dependent on both transcription and translation. This is in accordance with the model proposed for LTP1, 2 and 3. These results also highlight the dependence of the enhanced release on translation. It appears that increased vesicle cycling in the presynaptic terminal is dependent only on translation from pre-existing mRNA and not gene transcription.

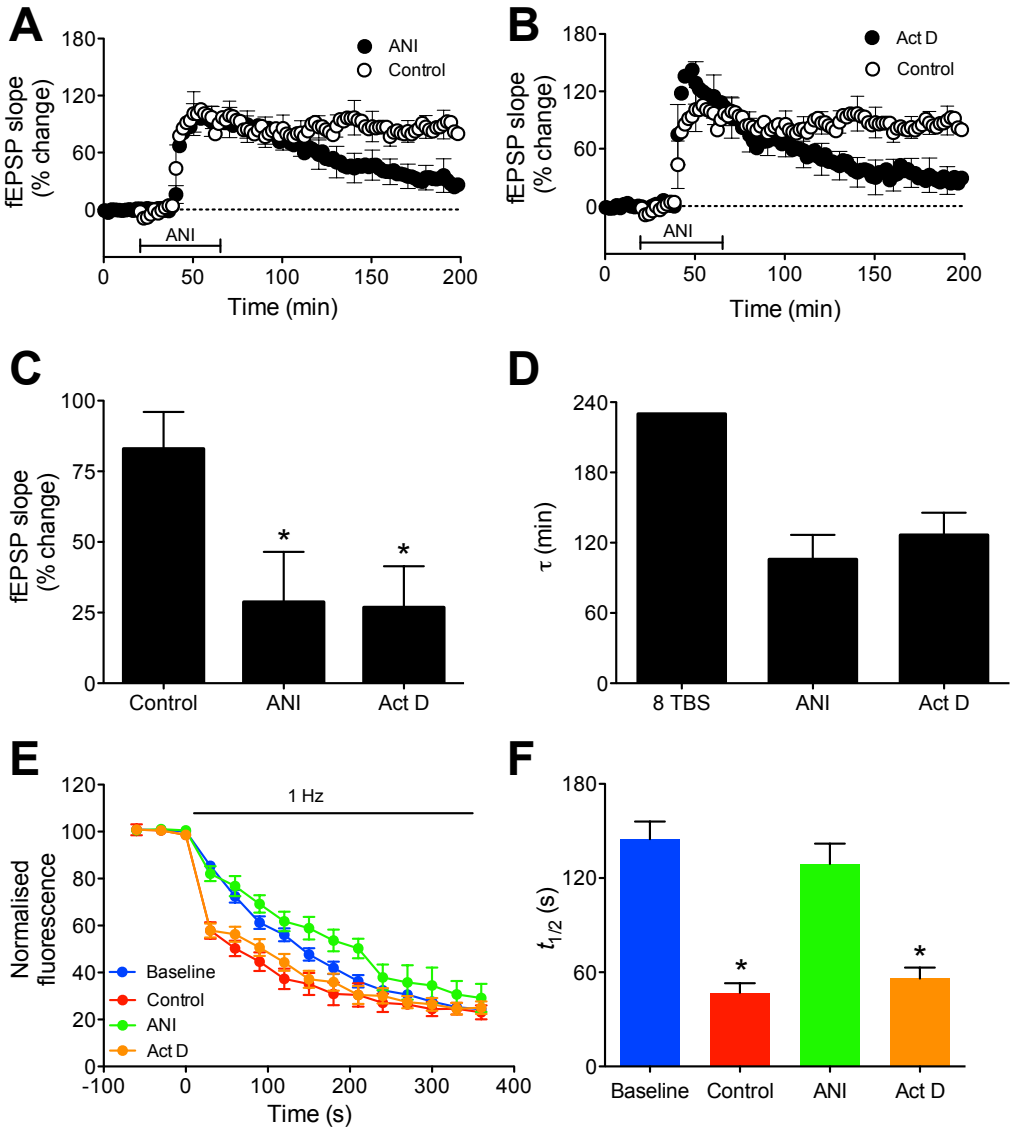

Figure 4.20. LTP3 is dependent on translation and transcription, but the associated enhanced release is dependent upon translation only.

A, Mean percent change in fEPSP slope in controls and slices incubated with ANI during the 8TBS induction protocol. **B**, Mean percent change in fEPSP slope in controls and slices incubated with Act D during the induction period. **C**, LTP3 magnitude in the last 5 min of recording was significantly reduced with ANI and Act D application. **D**, Application of ANI and Act D qualitatively increased the rate of LTP3 decay. Control data reproduced from Raymond & Redman (2002). **E**, Mean (±SEM) time course of FM 1-43 destaining prior to LTP induction (Baseline), 160 min post-induction (Control) and 160 min post-induction with ANI/Act D present during the induction period. **F**, Histogram of mean FM 1-43 destaining kinetics ($t_{1/2}$) in LTP3 experiments showing that application of ANI abolished the enhanced release, whereas Act D did not affect exocytotic rate 160 min post-TBS (*p<0.05).

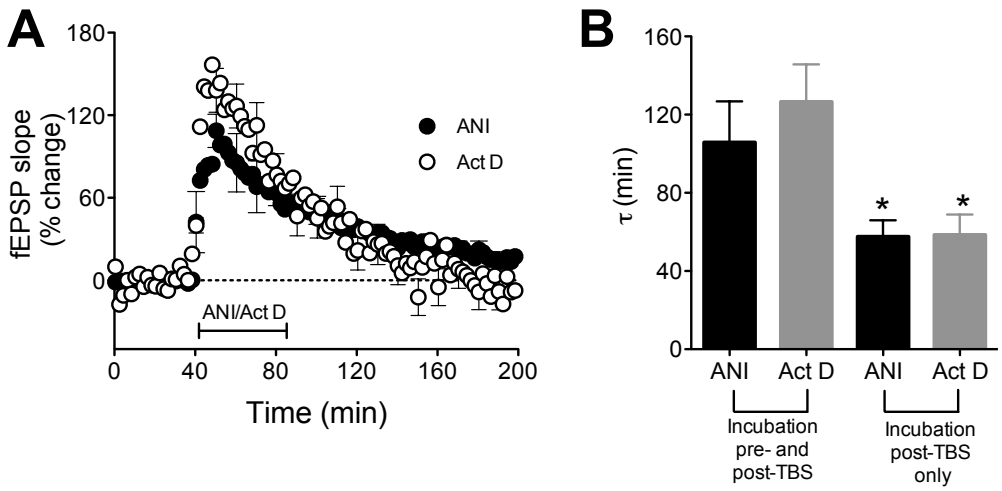

Figure 4.21. ANI and Act D do not act by interfering with LTP induction.

LTP3 persistence was not increased when application of ANI or Act D occurred immediately post-TBS. **A**, Mean percent change of fEPSP slope in slices incubated with ANI or Act D immediately following the 8TBS induction protocol. **B**, Incubation immediately post-TBS produced LTP that was significantly less persistent than LTP induced with the drugs included during the TBS protocol (*p<0.05).

4.4. The enhanced release associated with LTP2 and 3 is dependent on *postsynaptic* translation

The next subset of experiments was designed to establish the locus of translation that is required for the observed enhanced release during LTP2 and 3. There exists a wealth of evidence supporting a role for both local and somatic postsynaptic translation in LTP (Frey *et al*., 1989; Cracco *et al*., 2005) and recent evidence suggests that local presynaptic translation is also required for some forms of facilitation (Akins *et al*., 2009). It was therefore of interest to determine whether the enhanced presynaptic function was dependent upon presynaptic or postsynaptic translation.

To achieve this the whole-cell patch-clamp recording technique was utilised, allowing an individual CA1 neuron to be filled with a fluorescent dye (Alexa Fluor 488, 50 μM) and a membrane impermeable translation inhibitor (Gelonin, 3.5 μM). Gelonin inhibits protein synthesis by inactivating the 60S ribosome (Stirpe *et al*., 1980) and has previously been used to inhibit local postsynaptic protein synthesis required for learning-related facilitation in *Aplysia* (Sherff & Carew, 2004; Villareal *et al*., 2007) as well as local presynaptic translation required for synaptic plasticity in *Xenopus* nerve-muscle cultures (Zhang & Poo, 2002). Inclusion of Gelonin in the patch pipette resulted in restricted inhibition of translation in the postsynaptic cell only. FM 1-43 destaining was then measured from CA3 terminals at various distances from the filled distal dendrites of the postsynaptic cell to determine whether postsynaptic inhibition of translation affects the enhanced release observed at 160 min post-TBS. Figure 4.22 shows an example of a filled CA3 neuron with Alexa Fluor 488 (Figure 4.22A) and of FM 1-43 filled terminals surrounding filled postsynaptic distal dendritic branches (Figure 4.22B, C, D).

4.4.1. LTP2 and LTP3 are dependent on postsynaptic translation for their persistence

As an important control it was necessary to establish that EPSC amplitude remained constant in the absence of TBS with Gelonin in the patch pipette to ensure that the drug did not affect the size of postsynaptic currents. EPSC amplitude remained unchanged over the entire time period both with and without Gelonin (Figure 4.23).

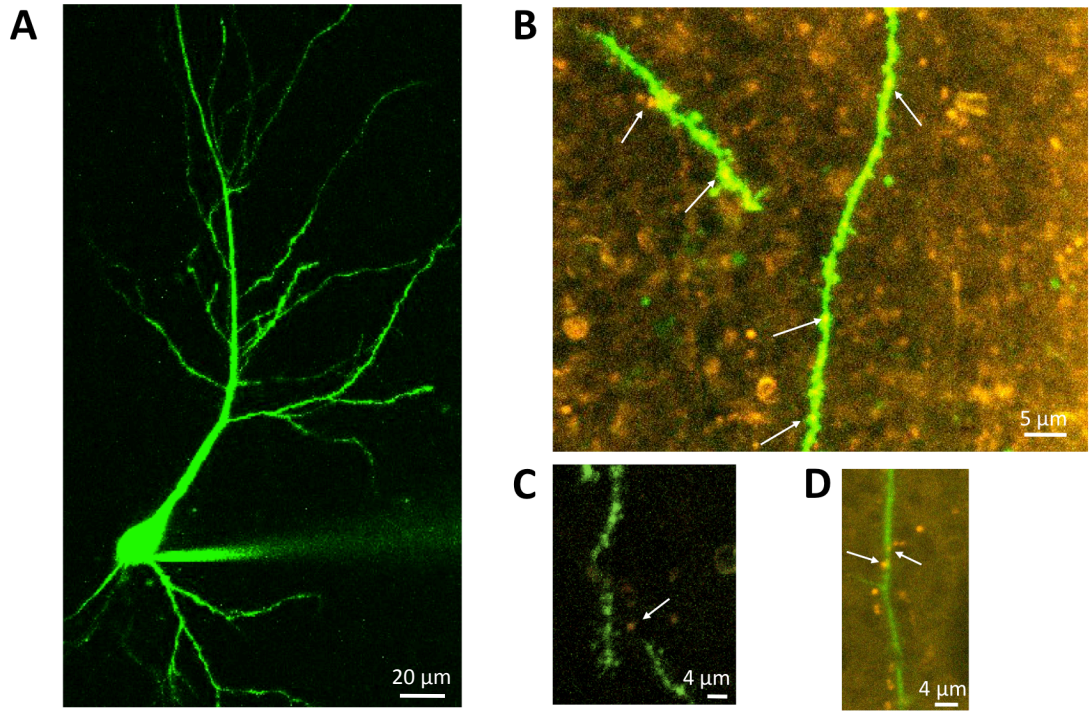

Figure 4.22. Example images showing Alexa Fluor 488 loading in a CA1 neuron and FM 1-43 loading at sites close to the distal dendrites.

A, CA1 neuron filled with Alexa Fluor 488. **B**, **C**, **D**, Examples of FM 1-43 loaded CA3 terminals (orange) in the same field of view as Alexa Fluor 488 filled CA1 dendritic branches (green). Arrows indicate putative contacts between terminals and spines/dendrites. The dendritic branch is approximately 143 µm from the soma in **B**, 183 µm from the soma in **C** and 164 µm from the soma in **D**.

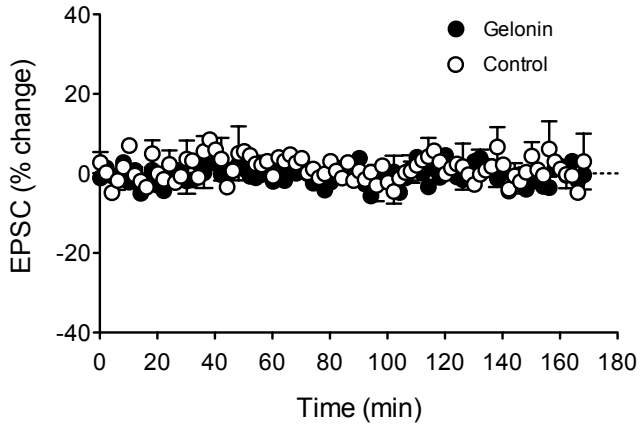

Figure 4.23. Gelonin does not interfere with basal EPSC size.

Mean percent change in EPSC amplitude over a 170 min time period in the absence of TBS. Data is shown for control cells and cells with the membrane impermeable translation inhibitor Gelonin in the pipette internal solution.

Both LTP2 persistence and final magnitude were significantly reduced with the inclusion of Gelonin in the whole-cell patch pipette (Figure 4.24). LTP2 τ was reduced from 99 ± 4 min (n=6) to 34 ± 2 min (n=9) with Gelonin in the patch pipette (Figure 4.24B; $p<0.05$). The size of the EPSC in the last 5 min of recording was also significantly reduced with the inclusion of Gelonin, from 49 ± 1% (n=6) to 17 ± 1% (n=9; Figure 4.24C; $p<0.05$). This is consistent with previously reported data demonstrating a role for translation in the persistence of LTP2.

Similarly, both LTP3 persistence and final magnitude were reduced with inhibition of translation with Gelonin (Figure 4.25; Control τ = 234 min [from Raymond & Redman, 2006]; Gelonin τ = 12 ± 23 min, n=5). As explained for earlier LTP3 decay data, calculation of τ values for LTP3 was not possible; therefore the magnitude of LTP over the last 5 min of recording was compared. Gelonin significantly reduced LTP magnitude at 160 min post-8TBS (Figure 4.25C; Control = 80 ± 6%, n=4; Gelonin = 5 ± 7%, n=5; $p<0.05$).

These results demonstrate that inhibition of postsynaptic protein synthesis is sufficient to curtail the persistence of LTP2 and LTP3.

4.4.2. LTP2 and LTP3 enhanced exocytosis is dependent on postsynaptic translation

In order to ensure that neither Gelonin nor the recording technique affected basal exocytotic rate, it was important to measure destaining rate after 170 min of recording in the absence of TBS. Under these conditions exocytotic rate from CA3 terminals remained constant both with and without Gelonin regardless of the distance to the nearest filled CA1 dendrite (Figure 4.26; one-way ANOVA, $p<0.05$). These data demonstrate that any observed changes in destaining rate are likely to be specifically related to LTP and not the experimental conditions.

The enhanced exocytosis associated with LTP2 at 160 min post-4TBS was found to be dependent on postsynaptic translation. In the control situation (without Gelonin) an increased rate of presynaptic release was measured at all terminals, regardless of distance to the dendrite (Figure 4.27A, B, D). With Gelonin in the recording pipette (and hence postsynaptic translation blocked) enhanced release was abolished only at sites very close to the postsynaptic cell (between 0 and 0.5 μm; Figure 4.27A, C, D; one-way ANOVA, $p<0.05$). Enhanced exocytosis remained intact at sites further removed from the treated postsynaptic cell.

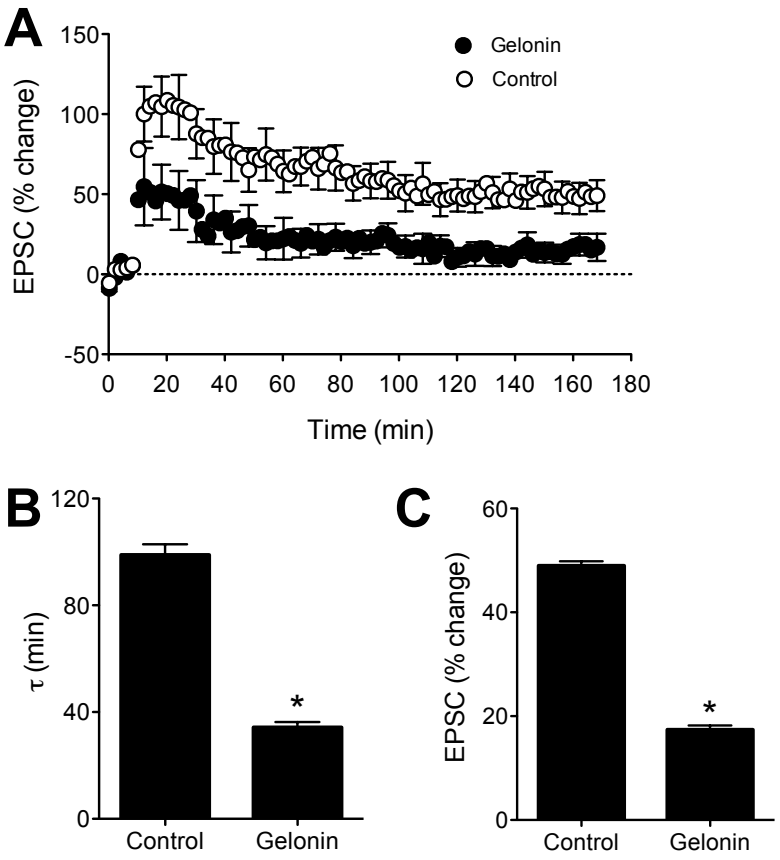

Figure 4.24. LTP2 persistence and magnitude is reduced with translation inhibition by Gelonin.

A, Mean percent change in EPSC amplitude following 4TBS stimulation in control cells and cells filled with the membrane impermeable translation inhibitor Gelonin. **B**, Inclusion of Gelonin in the patch pipette significantly increased the rate of LTP2 decay. **C**, Inclusion of Gelonin in the patch pipette significantly reduced the magnitude of the fEPSP at 160 min-post TBS (*p<0.05).

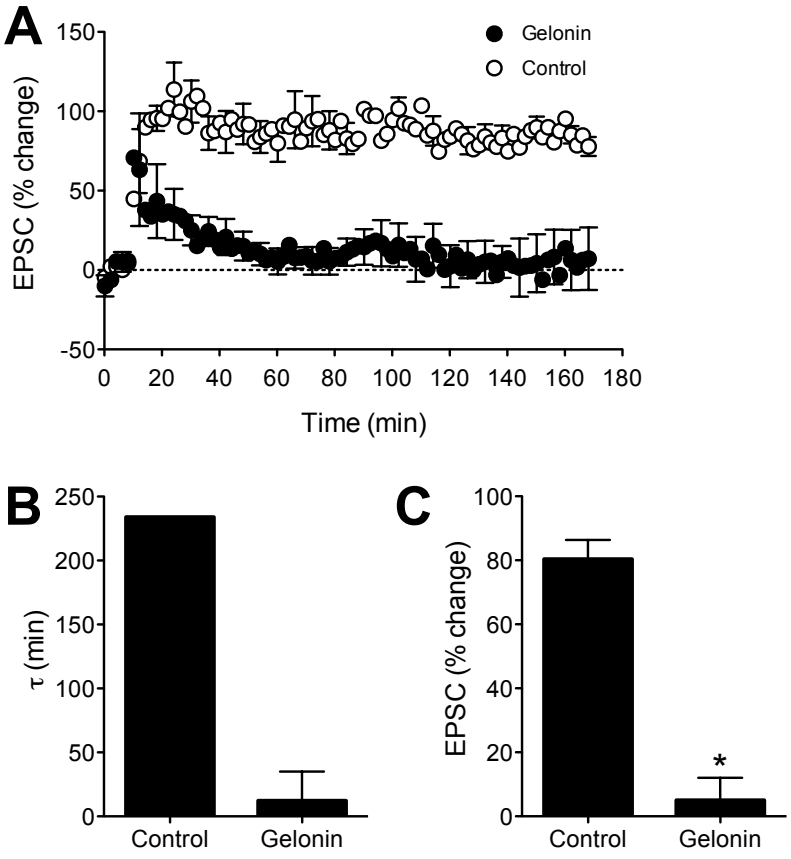

Figure 4.25. LTP3 persistence and magnitude is reduced with translation inhibition by Gelonin.

A, Mean percent change in EPSC amplitude following 8TBS stimulation in control cells and cells filled with Gelonin. **B**, Inclusion of Gelonin in the patch pipette qualitatively increased the rate of LTP3 decay. Control data reproduced from Raymond & Redman (2002). **C**, LTP3 magnitude in the last 5 min of recording was significantly reduced with Gelonin in the patch pipette (*p<0.05).

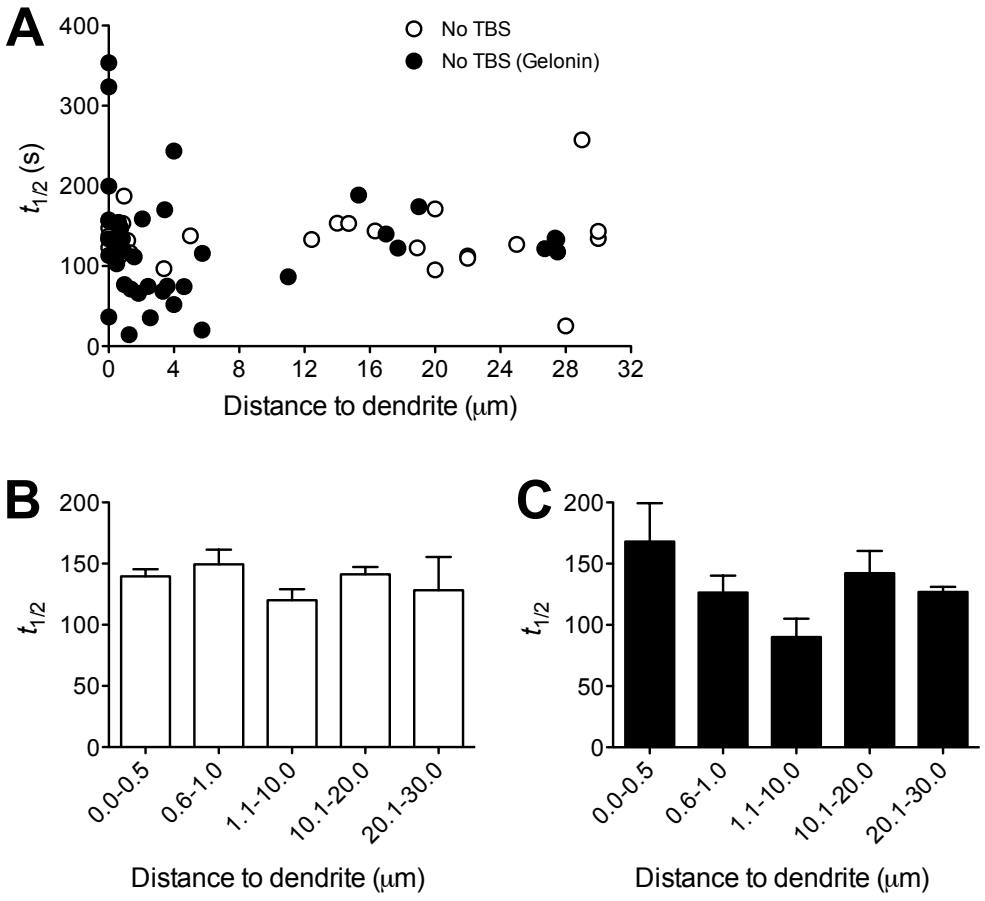

Figure 4.26. Basal release rate is not affected by Gelonin or the whole-cell recording technique.

A, Scatter plot showing the half-life of destaining versus distance to the nearest filled dendrite for individual terminals. **B**, Bar graph with data obtained from the 'No TBS' group in **A**, except with distances binned. There was no difference in mean half-life of destaining between each distance group. **C**, Bar graph with data obtained from 'No TBS (Gelonin)' group in **A**, except with distances binned. Inclusion of Gelonin in the patch pipette did not affect terminal basal exocytotic rate (one-way ANOVA, $p<0.05$).

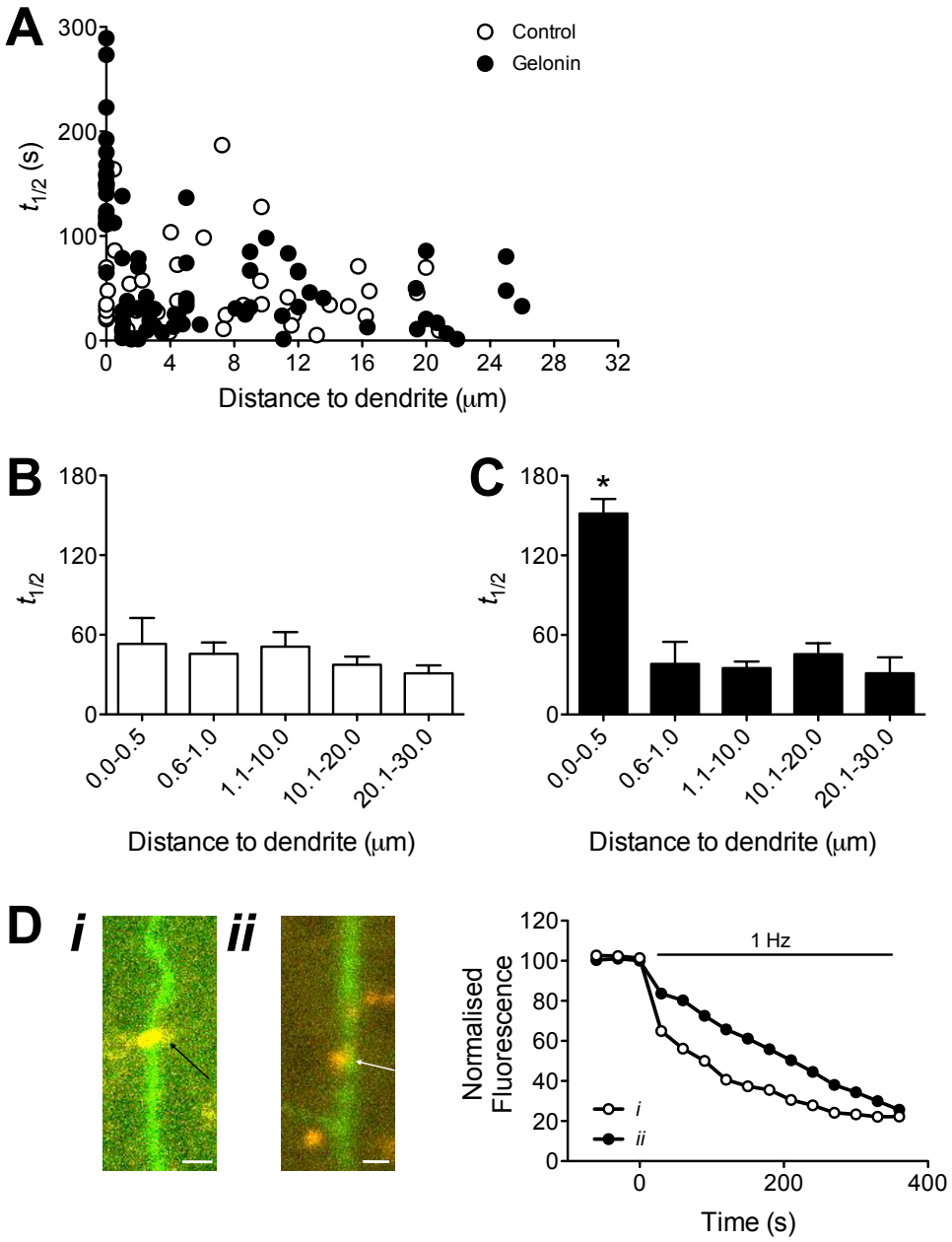

Figure 4.27. LTP2 enhanced presynaptic function is dependent on postsynaptic translation.

A, Scatter plot showing the half-life of destaining versus distance to filled dendrite for individual terminals in slices stimulated with 4TBS with and without Gelonin in the patch pipette. **B**, Bar graph with data obtained from the 'Control' group in **A**, except with distances binned. There was no difference in mean half-life of destaining between each distance group. **C**, Bar graph with data obtained from 'Gelonin' group in **A**, except with distances binned. Inclusion of Gelonin in the patch pipette abolished enhanced release only at sites close to the filled postsynaptic cell (one-way ANOVA, $*p<0.05$). **D**, Example of destaining from single terminals (arrow) in the 0.0-0.5 μm distance group. ***i***, control terminal with no Gelonin in the pipette. Destaining plot on the right shows enhanced destaining. ***ii***, terminal with Gelonin in the postsynaptic cell. Destaining plot shows no enhanced destaining. Scale bar represents 1 μm.

Similarly, the enhanced exocytosis associated with LTP3 was found to be dependent on postsynaptic translation. In control slices enhanced release was observed regardless of distance to the nearest filled dendrite (Figure 4.28A, B, D). However inhibition of postsynaptic translation with Gelonin blocked enhanced release at 160 min post-8TBS only at distances between 0 and 0.5 μm to the nearest filled dendrite (Figure 4.28A, C, D; one-way ANOVA, $p<0.05$). As for LTP2, enhanced release remained unaffected at sites further removed from the treated postsynaptic cell.

These data suggest that translation in the postsynaptic cell is required for the enhanced exocytosis associated with LTP2 and LTP3.

4.5. PPF data support a role for presynaptic expression in LTP2 and LTP3

As a complementary experiment, levels of paired-pulse facilitation (PPF) were recorded with and without Gelonin using the whole-cell recording technique before and at various time points following induction of LTP2 and 3. A reduction in the paired-pulse ratio (PPR) is thought to be indicative of increased P_r (Schulz *et al*., 1994, 1995; Sokolov *et al*., 1998; Sokolov *et al*., 2002), therefore this analysis was valuable as it allowed for comparison of release probability with FM 1-43 destaining at 80 min and 160 min post-TBS.

LTP2 was associated with a reduction in PPR at 160 min after induction (PPR Baseline = 2.13 ± 0.07, n=5 cells; PPR 160 min = 1.26 ± 0.05, n=5 cells; $p<0.05$), but not at 80 min (PPR 80 min = 1.82 ± 0.28, n=5 cells; Figure 4.29A, C). LTP3 was associated with a reduction in PPR at both 80 min (PPR Baseline = 1.85 ± 0.08, n=4 cells; PPR 80 min = 1.28 ± 0.01, n=4 cells) and 160 min post-TBS (PPR 160 min = 1.28 ± 0.005, n=4 cells; Figure 4.29A, D; $p<0.05$). Importantly, the PPR remained stable over the entire recoding period in the absence of TBS (Figure 4.29B). These data are consistent with the FM 1-43 destaining data and support the finding that LTP2 involves a presynaptic component that is not expressed until 160 min post-TBS, whereas LTP3 involves a presynaptic component that may be recruited earlier and is apparent at 80 min post-TBS.

Interestingly, inclusion of Gelonin in the patch pipette internal solution abolished the elevated P_r that was associated with LTP2 (PPR Baseline = 1.60 ± 0.07, n=9 cells; PPR 80 min = 1.56 ± 0.05, n=9 cells; PPR 160 min = 1.58 ± 0.05, n=9 cells) and LTP3 (PPR Baseline = 1.57 ± 0.02, n=5 cells; PPR 80 min = 1.57 ± 0.10, n=5 cells; PPR 160 min = 1.45 ± 0.14, n=5 cells; Figure 4.30).

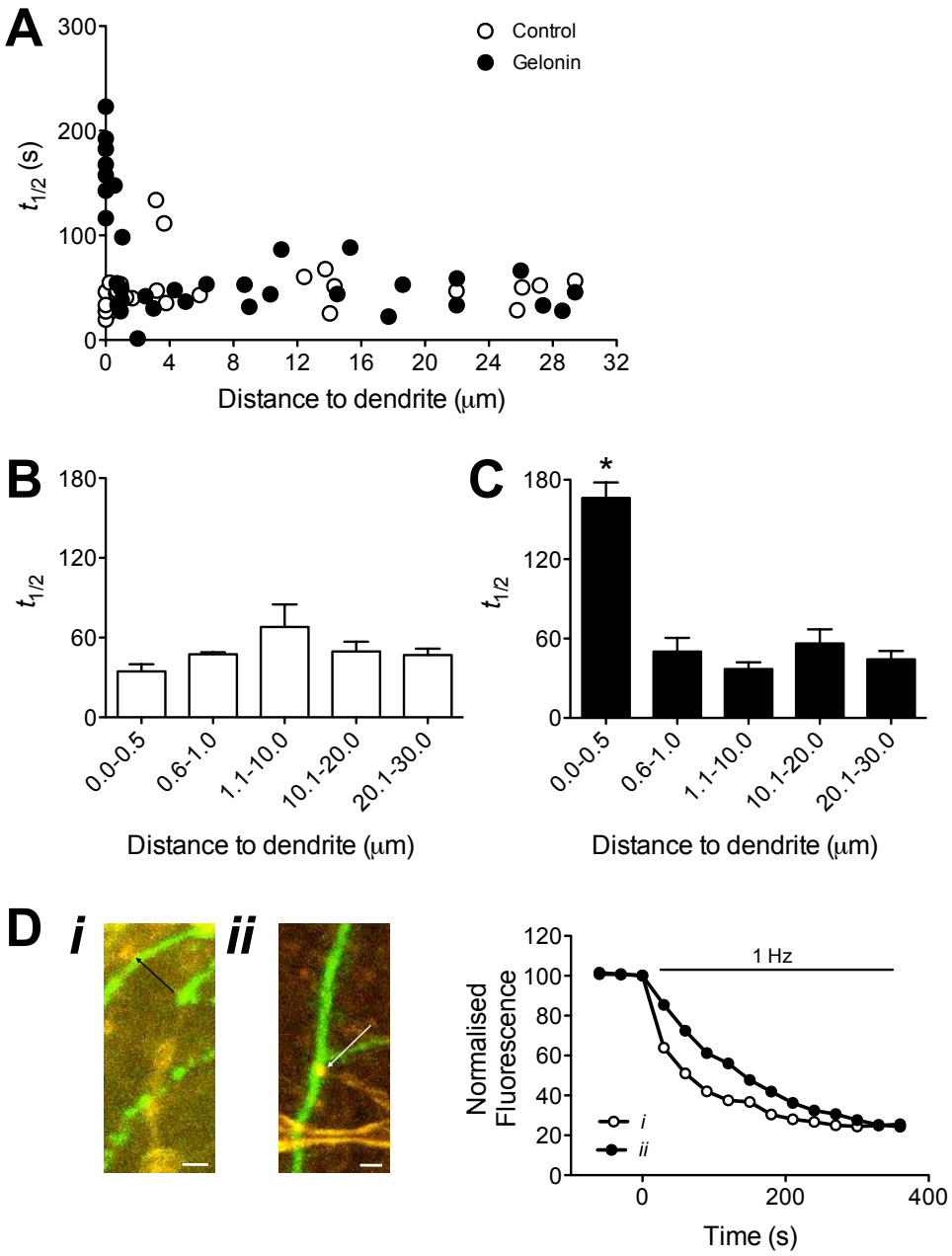

Figure 4.28. LTP3 enhanced presynaptic function is dependent on postsynaptic translation.

A, Scatter plot showing the half-life of destaining versus distance to filled dendrite for individual terminals in slices stimulated with 8TBS with and without Gelonin in the patch pipette. **B**, Bar graph with data obtained from the 'Control' group in **A**, except with distances binned. There was no difference in mean half-life of destaining between each distance group. **C**, Bar graph with data obtained from 'Gelonin' group in **A**, except with distances binned. Inclusion of Gelonin in the patch pipette abolished enhanced release only at sites close to the filled postsynaptic cell (one-way ANOVA, *p<0.05). **D**, Example of destaining from single puncta (arrow) in the 0.0-0.5 μm distance group. ***i***, control puncta with no Gelonin in the pipette. Destaining plot on the right shows enhanced destaining. ***ii***, puncta with Gelonin in the postsynaptic cell. Destaining plot shows no enhanced destaining. Scale bar represents 2 μm.

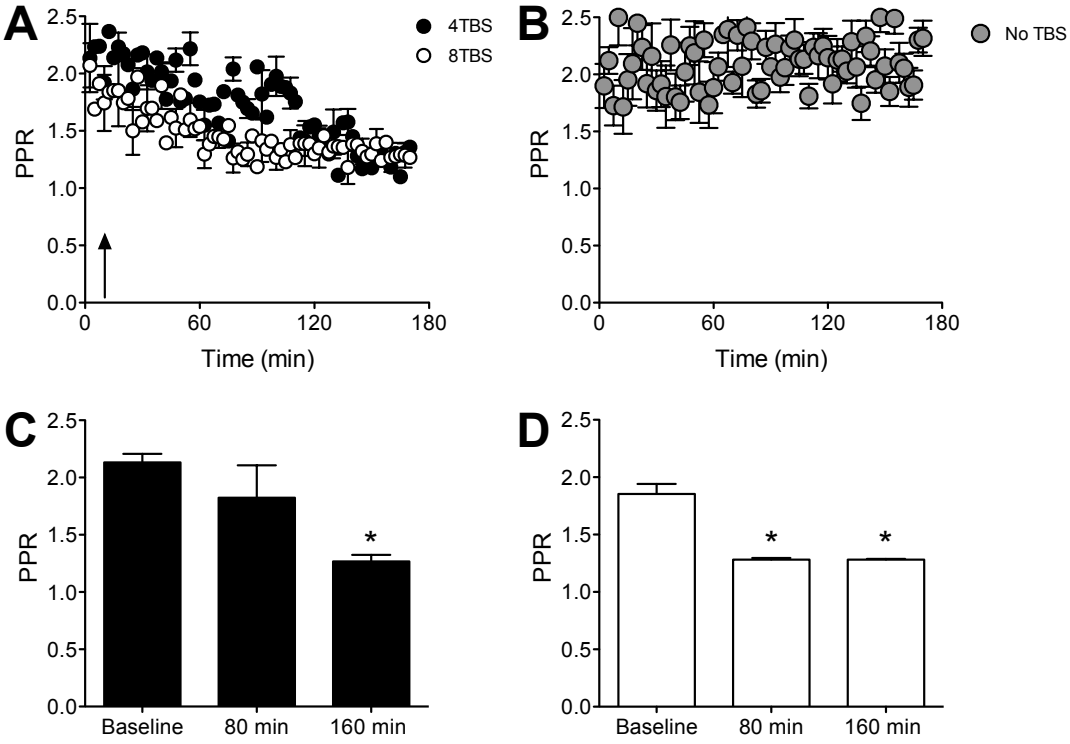

Figure 4.29. PPR measurements demonstrate increased release probability with LTP2 and LTP3.

A, Paired-pulse ratio (PPR) in slices stimulated with 4TBS and 8TBS (at time point indicated by arrow) showing a reduction in the ratio over time for both groups. **B**, PPR was stable in the absence of TBS. **C**, Bar graph showing mean PPR during the first 10 min of recording (Baseline) and at 80 min and 160 min post-4TBS. PPR was significantly reduced at 160 min post-4TBS, consistent with the FM 1-43 destaining data for LTP2. **D**, Bar graph as for **C**, except for LTP3 data induced with 8TBS. PPR was significantly reduced at both 80 min and 160 min post-TBS, consistent with the FM 1-43 destaining data for LTP3

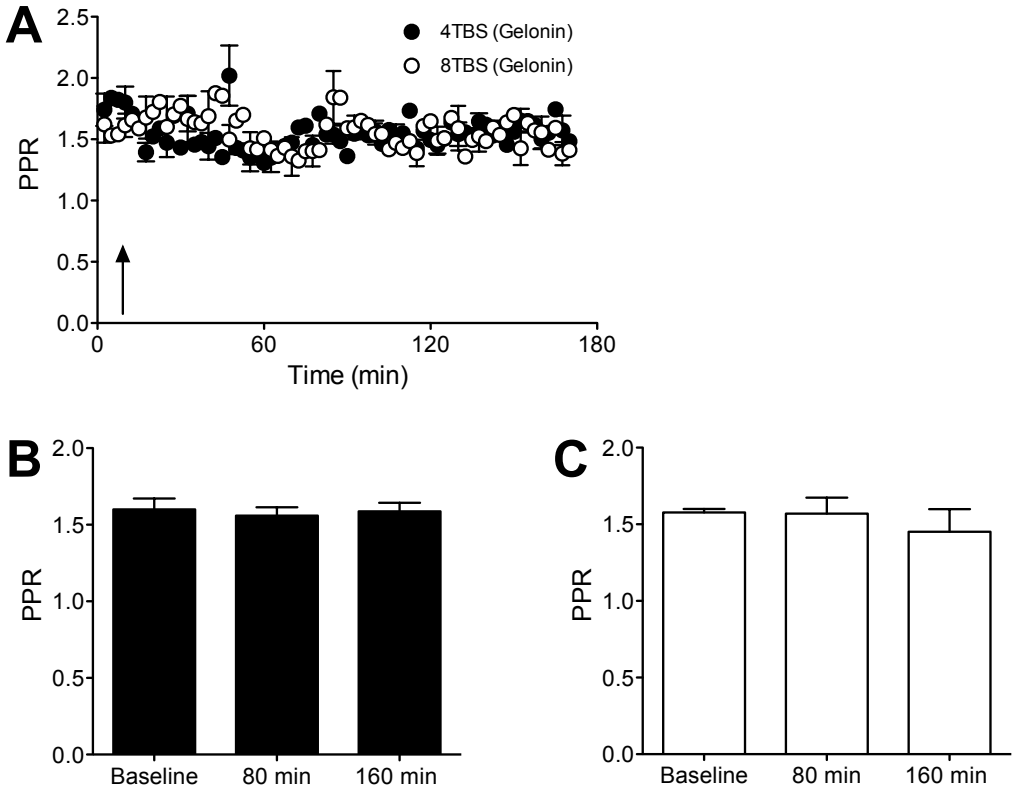

Figure 4.30. No change in PPR with inhibition of postsynaptic translation.

A, PPR data in slices stimulated with 4TBS and 8TBS (at time point indicated by arrow) with Gelonin in the patch pipette internal solution. **B**, Bar graph showing mean PPR during the first 10 min of recording (Baseline) and at 80 min and 160 min post-4TBS. PPR remained unchanged for the duration of the recording period. **C**, Bar graph as for **B**, except for LTP3 data induced with 8TBS. PPR did not change over the entire recording period.

This finding was in accordance with previous data demonstrating the abolition of enhanced exocytosis with translation inhibition by ANI.

These results support earlier FM 1-43 destaining data suggesting that persistent forms of LTP are associated with a presynaptic mode of expression and that this expression component may be recruited earlier for more persistent forms of LTP such as LTP3. The data also suggests that translation in the postsynaptic cell is critical for this presynaptic expression.

5. Discussion

5.1. Overview

The broad aim of this research was to examine the expression locus of LTP1, 2 and 3, specifically, whether each form was associated with enhanced presynaptic function. The dependence of each form of LTP on new protein synthesis and NO signalling was also investigated and the locus of translation required for LTP2 and 3 was determined. This chapter will critically examine the data presented, as well as offer a more general discussion and interpretation of the results.

5.2 Validation of methods

5.2.1. Stimulation with 1, 4 and 8TBS to induce LTP1, 2 and 3

It was first of critical importance to ensure that stimulation with 1, 4 and 8TBS reliably induced LTP1, 2 and 3 so that legitimate comparisons between the current data and previously published data could be made. The τ values for LTP1 and LTP2 reported here do not differ substantially from those previously published (Raymond & Redman, 2002). The present LTP3 data was more persistent than has been previously reported and this may in part result from differences in recording technique and apparatus between the two sets of data. The remarkable persistence of LTP induced by 8TBS is consistent with the broad concept of LTP3 suggested by the model, in which changes in gene expression help to establish robust forms of LTP. Also, the common requirement for translation and transcription with 8TBS-induced LTP reported here and LTP3 *in vivo* (Abraham & Otani, 1991; Abraham *et al*., 1993) support the conclusion that 8TBS under these conditions induces LTP3.

5.2.2. Use of FM 1-43 as a faithful reporter of presynaptic function

In order to assess changes in presynaptic function associated with LTP1, 2 and 3, the destaining of FM 1-43 was used to measure vesicle exocytosis at CA3 terminals. Whilst other groups have successfully used this method as an indicator of release rate, it was important to verify this using our setup and experimental paradigm. We first aimed to determine the optimal stimulation frequency for unloading and found there to be a negative exponential relationship between stimulation frequency and half-life of destaining. This likely reflects the first order kinetics of neurotransmitter release (Ryan *et al*., 1993; Liu & Tsien, 1995) and is consistent with the view that each action potential initially releases a constant fraction of dye-filled vesicles until a maximal rate is reached (Zakharenko *et al*., 2001). For all subsequent experiments a stimulation frequency of 1 Hz was used, as this produced an intermediate rate of unloading that allowed for bidirectional changes in release kinetics to be detected. This is in agreement with published data demonstrating frequencies of approximately 1 Hz to be optimal at hippocampal synapses (Ryan *et al*., 1996; Zakharenko *et al*., 2001; Sara *et al*., 2002; Zakharenko *et al*., 2002; Zakharenko *et al*., 2003; Kavalali, 2007). It must be noted however that other data suggests that higher frequency 10 Hz bursts at intermittent intervals also produce optimal unloading (Stanton *et al*., 2003; Stanton *et al*., 2005), however a lower frequency train of unloading stimulation was the preferred

method for our purposes in order to minimise any plasticity associated with the unloading protocol.

Using a stimulation frequency of 1 Hz, our measurements indicate that a single action potential released 0.4-0.6% of the total fluorescence associated with a fully loaded vesicle pool. Assuming that a typical terminal contains approximately 100-200 synaptic vesicles (Schikorski & Stevens, 2001; Micheva & Smith, 2005), this equates to the release of 0.4-1.2 vesicles per action potential. This is consistent with previous studies suggesting that an action potential results in the release of a single vesicle at hippocampal synapses (Stevens & Wang, 1994; Bolshakov & Siegelbaum, 1995; Ryan & Smith, 1995; Rosenmund & Stevens, 1996; Murthy *et al*., 1997).

To ensure that the rate of FM 1-43 unloading provided a reliable index of presynaptic function, the effect of altering the presynaptic probability of exocytosis was examined. Increasing P_r by elevating the Ca^{2+} concentration in the external solution predictably resulted in enhanced destaining. Conversely, lowering recording temperature resulted in a reduced rate of destaining as predicted by the slowed vesicle cycling kinetics that exist at lower temperatures (Gaffield & Betz, 2007; Ruiz *et al*., 2011). These results demonstrate that measurements of FM 1-43 destaining can reliably be used to make inferences about changes in presynaptic release.

5.2.3. Synaptic plasticity associated with load/unload protocols

The NMDAR antagonist D-APV was present during all FM 1-43 loading and unloading in order to minimise plasticity associated with the stimulation protocols. Despite a long application time and high drug concentration (100 μM), some short-term plasticity was observed with the stimulation protocols used to load and unload FM 1-43. This plasticity involved an initial potentiation, followed by a depression and then a subsequent potentiation phase. Regardless of this, field potentials returned to baseline well before the next load/unload protocol and these plastic events did not significantly affect the overall decay of LTP. Nevertheless, there was some potentiation remaining at the time of TBS that could complicate interpretation. However, importantly for our purposes, the basal exocytotic rate remained unchanged throughout the entire experimental period in the absence of any TBS (Figure 4.7), suggesting that the plasticity associated with loading and unloading of the dye was not substantial enough to affect release rate. These findings demonstrate that any changes in release rate observed during LTP experiments were due to the LTP itself and not a secondary effect of the load/unload protocols.

5.3 Expression mechanisms associated with LTP1, 2 and 3

5.3.1. LTP1 is exclusively postsynaptic

The data presented here demonstrate that LTP2 and LTP3 involve a presynaptic component to their expression, whereas LTP1 does not. It therefore seems likely that LTP1 is exclusively postsynaptic, requiring activation of biochemical signalling cascades only within the postsynaptic neuron for its persistence. It was predicted that changes in release rate would not be observed at 160 min post-1TBS given that the size of the postsynaptic response had returned to baseline values by this time point and there was no potentiation remaining. That there was no presynaptic enhancement observed at the earlier 80 min time point is also unsurprising and is consistent with published data showing that weaker forms of LTP are predominantly postsynaptic (Zakharenko *et al*., 2001; Bayazitov *et al*., 2007).

There are three possible interpretations underlying the observation that LTP1 does not involve enhanced release and rather is expressed postsynaptically. First, the FM 1-43 destaining measurements may not be sensitive enough to detect changes in presynaptic release associated with weaker forms of LTP. We deem this unlikely given that changes in release rate appear to be an 'all-or-none' phenomenon and that there are not varying degrees to which presynaptic release is enhanced (Figure 4.12). Second, there may be distinct Ca^{2+} thresholds that must be surpassed for inducing the presynaptic and postsynaptic components of LTP. Given the LTP1 requirement for Ca^{2+} signalling through NMDARs and RyRs (Raymond & Redman, 2006), it is possible that these pathways would play a fundamental role in determining whether this threshold is surpassed. For example, Ca^{2+} signalling via RyRs and NMDARs during the 1TBS protocol may produce sufficient Ca^{2+} to induce the postsynaptic component of expression, however be insufficient to induce the presynaptic aspect. In this model the presynaptic expression component would have a higher Ca^{2+} threshold for its recruitment. A third more likely interpretation may be that the presynaptic and postsynaptic components exhibit a similar postsynaptic Ca^{2+} threshold and that presynaptic expression is instead determined by the spatial location of Ca^{2+}. Indeed, this is supported by data from Raymond and Redman (2006) who demonstrated that as well as requiring unique sources of Ca^{2+} for their induction, LTP1, 2 and 3 Ca^{2+} signals are spatially segregated. Therefore, based on the published model it seems most likely that the presynaptic expression component will only be activated if Ca^{2+} signalling occurs within a particular postsynaptic compartment, or even a microdomain.

Consequently, the effector mechanisms underlying presynaptic expression might be colocalised with a particular Ca^{2+} source. Given the LTP1 requirement for Ca^{2+} signalling in the spine, it is likely that the cascade that triggers presynaptic expression lies beyond this postsynaptic region.

5.3.2. LTP2 and LTP3 are expressed presynaptically and postsynaptically

In contrast to LTP1, the more persistent LTP2 and LTP3 are associated with a presynaptic enhancement and therefore have a duality to their expression involving both presynaptic and postsynaptic signalling mechanisms. This is consistent with other reports showing that enhanced FM 1-43 destaining is only apparent following LTP induction by 200 Hz or strong TBS protocols, but not with lower frequency stimulation (Zakharenko *et al*., 2001; Bayazitov *et al*., 2007). Strong protocols such as 200 Hz are known to induce compound LTP, consisting of both NMDAR-dependent and VGCC-dependent components (Grover & Teyler, 1990), as well as presynaptic expression (Zakharenko *et al*., 2001; Bayazitov *et al*., 2007). Considering the LTP3 induction requirement for Ca^{2+} via L-type VGCCs and NMDARs (Raymond & Redman, 2002, 2006) it is perhaps unsurprising that a presynaptic component to LTP3 expression was observed. As considered previously for LTP1 expression, it seems likely that 4 and 8TBS produce Ca^{2+} signalling in the necessary postsynaptic compartment to trigger presynaptic expression. As discussed previously, LTP2 requires Ca^{2+} release via dendritic IP_3Rs and LTP3 requires somatic signalling via L-type VGCCs for their selective induction (Raymond & Redman, 2006). Given the requirement for transcription-independent protein synthesis in the presynaptic expression, it seems likely that dendritic Ca^{2+} signalling, triggering local protein synthesis might be an important step. In order for signalling external to the potentiated spine to trigger enhanced release in an input specific manner, there might be a synaptic tag that is established (Frey & Morris, 1997; Frey & Frey, 2008), limiting changes in presynaptic expression only to potentiated synapses.

An interesting aspect of the expression mechanisms associated with LTP2 and LTP3 is the difference in onset timing of the presynaptic expression. Whilst enhanced presynaptic function was only apparent for LTP2 at the 160 min post-TBS unload, enhanced release was observed for LTP3 as early as 80 min post induction. Other studies have reported a slow onset for the presynaptic expression associated with more persistent forms of LTP (Bayazitov *et al*., 2007) as is also observed here, however this is the first demonstration that different forms of LTP may recruit changes in presynaptic

expression at different times. Based on the data presented here, it seems likely that 8TBS stimulation may more rapidly trigger the translation that is required for enhanced release. Indeed, previous studies have suggested that 8TBS produces a stronger Ca^{2+} signal than 4TBS (Raymond & Redman, 2006) and that dendritic translation is Ca^{2+}-dependent (Atkins *et al*., 2004). This suggests that the Ca^{2+}-dependent signalling cascades initiated during the induction phase of LTP3 may more rapidly activate pathways that lead to presynaptic expression changes. Such temporal coordination of expression mechanisms may act to more effectively enhance synaptic strength, as the earlier onset of presynaptic changes reinforces the postsynaptic signalling events.

The temporality of LTP expression is confirmed by PPF data showing that LTP3 recruits a presynaptic component to its expression earlier than LTP2. PPF measurements were made using the whole-cell recording configuration and changes in PPR were used as indications of changes in P_r. An increased P_r was observed for LTP2 only at 160 min post-TBS, however was apparent at 80 min-post TBS for LTP3. This is consistent with the destaining data described above. The increase in P_r associated with LTP2 and LTP3 also provides insight into the mechanisms underlying the enhanced release and suggest that changes in the probability of vesicle fusion must (at least in part) play a role.

5.3.3. LTP3 involves loading into the reserve pool

The loading protocol used here (10 Hz, 2 min) is known to induce turnover of the functional vesicular pool (i.e. the RRP and recycling pool) at hippocampal synapses (Harata *et al*., 2001). Destaining data from the present study suggests that following LTP3 the loading stimulation results in uptake of dye into the functional vesicular pool as well as the reserve pool of vesicles in a significant fraction of CA3 terminals. This dye is then sequestered in the reserve pool and cannot be released by either the standard 1 Hz unloading stimulation nor higher frequency patterns of activity. Only upon depletion of the RRP with sucrose are the FM-loaded reserve pool vesicles once again recruited to the active zone. This suggests that LTP3 induces dramatic changes in the cycling kinetics in the presynaptic terminal that initially requires the recruitment of reserve pool vesicles to aid in transmission during the loading protocol, however this recruitment is not required upon subsequent 10 Hz stimulation.

It is unlikely that these changes in vesicle cycling kinetics are a direct result of the load/unload protocol given that basal release remains unchanged with sequential load/unloads over the experimental time frame. It is however possible that the RRP

vesicles undergo a change in their mode of transmitter release from full fusion to a kiss-and-run type release that would allow for them to sustain transmission even at frequencies as high as 10 Hz. In this scenario during the 1 Hz unload they would initially undergo classical fusion and then switch to a mode of fast release (hence the only dye remaining in the terminal is trapped in the reserve pool). Therefore only when the RRP is depleted do the reserve pool vesicles take part in transmission and destaining occurs. This finding suggests that persistent LTP may involve dynamic changes in the cycling and exchange between the various vesicle pools in conjunction with changes in exocytosis. Consequently the mechanisms underlying the presynaptic enhancement associated with persistent forms of LTP may differ. In this case, LTP3 involves changes in release rate as well as changes in the interaction between vesicle pools, whereas LTP2 involves only changes in exocytosis.

5.3.4. LTP1, 2 and 3 do not evoke unsilencing of presynaptic terminals

Synaptic connections between neurons can be either postsynaptically silent due to lack of functional AMPARs (Isaac *et al*., 1995; Kullmann & Siegelbaum, 1995; Liao *et al*., 1995) or presynaptically silent due to an insufficient amount of transmitter release to produce a measurable EPSC (Voronin & Cherubini, 2003). Presynaptically silent synapses have been described in various regions of the CNS, accounting for approximately 16-30% of inputs tested in the CA1 region of the hippocampus (Voronin *et al*., 1988; Hanse & Gustafsson, 2001) and are argued to be an important mechanism underlying certain types of LTP (Voronin & Cherubini, 2003; Voronin *et al*., 2004).

We observed that there was no change in the mean number of release sites measured before and after LTP induction. It seems then that presynaptic unsilencing is not an important process underlying LTP1, 2 or 3 under these experimental conditions and that changes in the postsynaptic cell that trigger the enhanced release do not also act to increase the reliability of transmission from nearby synapses with low (or zero) P_r. Therefore only functional terminals that participate in transmission during the induction process are associated with enhanced exocytosis in accordance with the property of input-specificity dictated by a Hebbian form of synaptic strengthening.

5.3.5. Enhanced exocytosis is an 'all-or-none' phenomenon

Enhanced exocytosis associated with LTP2 and LTP3 does not occur in a graded manner such that the enhancement is less pronounced with weaker LTP2 than for more persistent LTP3 (Figure 4.12). Rather, enhanced release seems to be an 'all-or-none' phenomenon. This is an important concept that provides valuable insight into the

mechanisms underlying the enhanced presynaptic release. It seems that the enhancement appears to affect terminals in a global manner and that there may be two predetermined exocytotic set-points within terminals, one that provides low levels of release and one that allows for elevated rates of transmission.

It is also important to note that regardless of the stimulation used to induce LTP, it is the persistence of the LTP that dictates whether enhanced release occurs or not. For instance, some of the slices stimulated with 4TBS are less persistent and have decay characteristics similar to LTP1. In these slices release is not enhanced. Under certain circumstances 4TBS may trigger insufficient dendritic Ca^{2+} entry (either via the NMDAR or the IP_3R pathway) to activate the cascades responsible for initiating the maintenance mechanisms that allow for the persistence of LTP2. Under these conditions the mechanisms required to initiate presynaptic changes might also not be activated, therefore expression remains solely postsynaptic and LTP resembles LTP1.

5.3.6. Disparities in LTP expression data

The changes in presynaptic function associated with LTP2 and LTP3 described here may explain some of the discrepancies in the literature with regard to expression locus of LTP (Frey & Morris, 1997; Frey & Frey, 2008). Different forms of LTP exhibit varying degrees of presynaptic expression in a temporally controlled manner; therefore testing roles of presynaptic and postsynaptic function at different times (for instance 30 mins vs. 180 mins) will produce profoundly different results. This is particularly important considering that many of the studies refuting presynaptic LTP expression terminated recordings well before a presynaptic component would be recruited, based on the timeline of presynaptic expression reported here. Moreover, weaker patterns of stimulation (such as 1TBS or brief 50 Hz and 100 Hz trains; Zakharenko *et al*., 2001) induce forms of LTP that are entirely independent of presynaptic changes. Therefore some of the confusion in the literature may arise from the use of different induction protocols and assessments of presynaptic function at different times post-induction.

5.4 Role of NO signalling in LTP1, 2 and 3

This study has identified a role for NO signalling in the persistence of LTP2 and LTP3 and the associated enhanced presynaptic function. Whilst a number of studies have reported that LTP persistence is dependent on NO signalling (Schuman & Madison, 1991; Haley *et al*., 1992; Arancio *et al*., 1996a; Arancio *et al*., 1996b; Ko & Kelly, 1999; Bon & Garthwaite, 2001b, a), this is the first study in which unequivocal NO-dependent effects on both LTP and presynaptic function have been directly and simultaneously measured.

5.4.1. LTP2 and LTP3 are NO-dependent

Our results demonstrate that LTP1 is independent of NO signalling. Neither the NOS inhibitor L-NAME nor the NO scavenger cPTIO affected the decay of LTP1 or exocytotic rate at 160 min post-induction. This result contradicts those from a previous study by Haley *et al*. (1993) who reported a dependence on NO signalling only for weaker forms of hippocampal LTP. This difference is perhaps explained by the different stimulation protocols used to induce LTP – in the aforementioned study 100 Hz trains with varying duration and stimulus strength were tested. This renders comparison of the two sets of data somewhat difficult. Given our finding that LTP1 is expressed entirely postsynaptically it is consistent that it should be independent of NO signalling.

In contrast both LTP2 and LTP3 are dependent on NOS activity and the presence of NO in the extracellular space for their persistence and associated enhanced presynaptic function. One previous study has demonstrated the effects of NO antagonism on presynaptic LTP that was induced by 100 Hz stimulation and found a partial abolition of enhanced FM 1-43 release (Stanton *et al*., 2005). Interestingly, in this study no change in the persistence of LTP was reported, suggesting that the reduced rate of cycling in the presynaptic terminal had no effect on the postsynaptic response. Therefore although 100 Hz-induced LTP involves a presynaptic component, postsynaptic expression changes appear to be most crucial in dictating persistence for this form of LTP. This is in contrast to LTP2 and LTP3 as defined here, in which persistence is significantly hindered with presynaptic expression disrupted, demonstrating that enhanced release is required for the long-term elevation in the size of the postsynaptic response.

Interestingly, cPTIO appears to be slightly less effective at inhibiting LTP3 persistence than L-NAME, perhaps resulting from its action later in the NO signalling

pathway. Therefore even despite the inclusion of the scavenger there may still be freely diffusing NO available to exert effects. This does not seem to be the case for LTP2 however, in which the persistence of LTP is equally hindered by L-NAME and cPTIO. It is possible that 8TBS results in more NO being liberated from the postsynaptic cell in comparison to 4TBS, therefore the scavenger is less effective at preventing extracellular NO diffusion. This is supported by the observation that a small degree of potentiation remains at the conclusion of the experiment, which possibly results from a combination of both pre and postsynaptic effects. Enhanced release may be maintained within a small proportion of presynaptic terminals, allowing for the potentiated postsynaptic response. Alternatively, the NO may also exert its actions via the postsynaptic cell during LTP3, therefore a component of LTP remains that is independent of diffusing NO.

5.4.2. Presynaptic changes triggered by NO signalling

The general cascade for NO action in hippocampal LTP is that Ca^{2+} influx via NMDARs activates NOS, which is tightly regulated by NMDARs via their close physical proximity (Christopherson *et al*., 1999; Sattler *et al*., 1999; Mungrue & Bredt, 2004). The NOS enzyme synthesizes NO from L-arginine (Boehning & Snyder, 2003; Garthwaite, 2008), which diffuses out of the postsynaptic cell and acts on soluble guanylyl cyclase in the presynaptic neuron. Activation of guanylyl cyclase switches on a variety of cGMP-dependent protein kinases, of which cGMP-dependent protein kinase (PKG) seems to be particularly critical.

A number of studies have highlighted a role for various biochemical intermediates involved in the NO signalling cascade in the presynaptic expression of LTP. Activity of guanylyl cyclase is known to be vital for the regulation of transmitter release in the CA1 region. Genetic deletion of guanylyl cyclase in mice reduces glutamate release under both basal and stimulated conditions and release can be restored to wild-type levels with a cGMP analog (Neitz *et al*., 2011). LTP was also impaired in these animals yet could be restored with the cGMP analog (Taqatqeh *et al*., 2009). PKG activity is also known to be important for particular forms of presynaptic LTP. Intracellular injection of a PKG blocker inhibits LTP when injected presynaptically but not postsynaptically, conversely injection of a PKG isozyme produced LTP when injected into the presynaptic, but not the postsynaptic cell (Arancio *et al*., 2001). Another study argues strongly against a role for PKG in LTP, reporting no change in LTP in a PKG knockout mouse (Kleppisch *et al*., 1999). This study is of particular interest as the LTP was found to be PKG-independent yet susceptible to

inhibition of NOS. This suggests that NO may have actions crucial for LTP that are independent of the guanylyl cyclase pathway.

It is possible that NO could also act on an ADP ribosyltransferase in the presynaptic terminal (Schuman *et al*., 1994; Kleppisch *et al*., 1999). ADP ribosyltransferases are enzymes responsible for the covalent modification of protein substrates by attaching ADP-ribose moieties to specific residues (Brüne & Lapetina, 1990). Common targets for ribosylation include GTP-binding proteins and the growth-associated protein GAP-43/B-50 (Coggins *et al*., 1993), both of which are thought to modulate exocytosis (Dekker *et al*., 1989; Fischer von Mollard *et al*., 1991). Therefore NO-dependent ribosylation of these proteins provides a potential mechanism for the increased exocytosis associated with LTP2 and LTP3.

NO may also act directly on a wide variety of proteins via S-nitrosylation of cysteine residues (Hess *et al*., 2001; Jaffrey *et al*., 2001; Stamler *et al*., 2001; Huang *et al*., 2005a). One study suggests that NO directly nitrosylates dynamin, a scaffolding protein required for the endocytosis of synaptic vesicles (Wang *et al*., 2006). This nitrosylation enhances the activity of dynamin and increases the rate of vesicle cycling. However the physiological credibility of experiments supportive of a role for direct S-nitrosylation by NO has been called into question (Garthwaite, 2008) and it seems more likely that S-nitrosylation may only be important in pathological states in which the redox environment is favourable for nitrosylation reactions (Zhang & Hogg, 2005).

Alternatively, NO may act on astrocytes, or other intermediary cells that then initiate signalling to the presynaptic neuron. Indeed there is increasing evidence to support a role for astrocytes in the regulation of LTP in culture and slice preparations (Filosa *et al*., 2009; Bélair *et al*., 2010; Henneberger *et al*., 2010). An indirect pathway such as this would likely be dependent upon release of some other critical factor that would act on the presynaptic neuron.

NO could also act postsynaptically to prolong LTP (Ko & Kelly, 1999), however this seems unlikely in our experiments given the concomitant inhibition of the LTP-associated presynaptic enhancement by NO antagonists. Also, if this were the case we might not see an inhibition of LTP persistence with antagonism of extracellular NO with cPTIO, as the postsynaptic actions of NO may not require its presence extracellularly.

5.4.3. Maintenance of input specificity with NO signalling

The dependence of LTP2 and LTP3 on NO signalling raises the interesting question of how the Hebbian property of input specificity is maintained with release of a readily

diffusible gaseous molecule such as NO. NO diffuses extremely rapidly from its source cell, with a tissue diffusion coefficient of 848 $\mu m^2/s$ (Liu *et al*., 2008). However despite its fast diffusion, the highly labile nature of NO results in rapid decay upon release (Ogasawara *et al*., 2007). Nonetheless it seems prudent to assume that NO release from a single potentiated postsynaptic spine will likely trigger signalling cascades within terminals in the near vicinity.

How then are presynaptic changes restricted only to terminals that communicate with potentiated spines? It is possible that a relatively high concentration of NO is required to trigger activation of the signalling pathway that initiates the enhanced release, such that although nearby terminals may also be exposed to a NO signal, it is at too low a concentration to trigger the presynaptic changes. This seems unlikely given the dramatic amplification of NO signals that is known to occur. For example, even NO at a concentration as small as 0.3 nM can evoke an approximate 0.4 μM cGMP signal, a striking 1000-fold amplification occurring within approximately 1 second (Garthwaite, 2008).

Alternatively, some evidence exists for the involvement of other retrograde signalling molecules in LTP, such as arachidonic acid (Williams *et al*., 1989), BDNF (Ying *et al*., 2002; Pang *et al*., 2004) and cell adhesion molecules (Murase & Schuman, 1999). One of more of these pathways may work in conjunction with NO to generate changes in the presynaptic terminal. The importance of BDNF is of particular relevance to this study, as restricted genetic deletion of BDNF is known to diminish enhanced FM 1-43 release associated with TBS-induced LTP (Zakharenko *et al*., 2003). Input specificity would be preserved with a messenging molecule such as BDNF, as the rate of diffusion is markedly slower than with NO, therefore only presynaptic cells in close proximity to a BDNF-releasing cell will be exposed to the signal. Conceivably NO signalling alone may be too weak to trigger the presynaptic changes, however its release in parallel with that of BDNF (for example) may be required to induce enhanced release. In this scenario presynaptic signalling cascades activated by BDNF and NO may converge upon a common downstream signalling pathway, which would ultimately result in the amplification of this pathway and an enhancement of presynaptic output. Alternatively, the signalling cascades may act upon different aspects of the release apparatus. For example, BDNF application in culture leads to increased levels of intracellular Ca^{2+}, which seems to be primarily due to release from internal Ca^{2+} stores (Berninger *et al*., 1993). Such an elevation of intracellular Ca^{2+} would lead to increased probability of transmitter release. Additionally, BDNF has been shown to induce a

MAPK-dependent phosphorylation of synapsin I (a membrane associated synaptic vesicle protein) in cortical neurons (Knipper *et al*., 1994; Jovanovic *et al*., 1996), however it remains unclear how synapsin I phosphorylation may impact glutamate release. Nevertheless, activation of either of these pathways in concert with those initiated by NO could potentially increase the rate of release, with activity of either pathway on its own being insufficient.

The preservation of input specificity may also be explained if NO were to act indirectly via an intermediary cell such as a glial cell. NO could induce release of a second messenger (such as BDNF) from such a cell, which would then exert its effects directly and specifically on the presynaptic neuron. However this also leads to a requirement for potentiated synapses to be closely apposed with a glial cell. This is not uncommon and astrocytes are known to ensheath synapses where they play a role in regulation of ionic and neurotransmitter concentration in the synaptic cleft (Barres, 2008). Although a single astrocyte can envelop many synapses, the existence of astrocytic signalling microdomains provides a means of autonomous glial interaction with a single synapse which would allow for input specificity to be preserved (Grosche *et al*., 1999). Additionally, a number of studies indicate that astrocytes are a predominant source of BDNF in various brain regions (Taylor *et al*., 2003; Wu *et al*., 2004; Jean *et al*., 2008) and astrocytes are known to mediate NO signalling via cGMP mechanisms (de Vente & Steinbusch, 1992). Therefore NO could induce astrocytic release of BDNF, which would then initiate changes in the presynaptic neuron that establish the increased rate of exocytosis associated with LTP2 and LTP3.

5.5 Role of new protein synthesis in LTP2 and LTP3

5.5.1. Overview

Another major finding of this study is that LTP1, 2 and 3 have differing dependencies on new protein synthesis. This confirms the LTP1, 2 and 3 model and supports the hypothesis that each form of LTP is mechanistically discrete, requiring unique sources and spatial location of Ca^{2+} for their induction, as well as exhibiting differing requirements for protein synthesis for their maintenance. Here we show that the maintenance of LTP3 is both transcription- and translation-dependent, whereas LTP2 maintenance requires only translation and LTP1 is protein synthesis-independent. Additionally, the enhanced exocytosis associated with LTP2 and LTP3 is dependent on translation (but not gene transcription) that is initiated in the postsynaptic cell.

5.5.2. Validity of Act D/ANI methodology

In this study, both Act D and ANI were applied using concentrations and application durations that have been widely used by others to induce substantial inhibition of transcription and translation. Here we applied the transcription inhibitor Act D for 45 mins, beginning 20 min pre-induction, which is similar to other studies (Huber *et al*., 2000; Kelleher *et al*., 2004b; Levenson *et al*., 2004) and is sufficient to achieve substantial reduction of transcription in culture preparations (Perry & Kelley, 1970). The translation inhibitor ANI was also applied for a similar duration to that used in other studies (Stanton & Sarvey, 1984; Huang & Kandel, 1994; Hardingham *et al*., 1999; Gelinas & Nguyen, 2005) and at a concentration known to block protein synthesis by greater than 80 percent in hippocampal slices (Stanton & Sarvey, 1984). In addition to its role in protein synthesis inhibition, ANI is also known to activate the p38/MAPK pathway under some conditions (Shifrin & Anderson, 1999) and can elicit synaptic depression at specific frequencies (Xiong *et al*., 2006). It seems unlikely that these or other nonspecific effects of ANI and Act D are significant in our experiments, as inclusion of both drugs in the bath did not appreciably affect the magnitude of the fEPSP.

Other evidence suggests that inhibition of protein synthesis during the induction stimulus inhibits LTP *induction* by means unrelated to transcription/translation inhibition (Okulski *et al*., 2002). The converse was observed in these experiments; application of both inhibitors during induction did not affect the magnitude of LTP immediately post-induction and application of the drugs immediately post-TBS was a

more effective method of reducing LTP persistence. This suggests that the inhibition of LTP2 and LTP3 persistence observed with the use of these inhibitors was a direct result of their repression of translation and transcription and not due to nonspecific effects during the TBS induction.

5.5.3. LTP1 is independent of new protein synthesis

Results from this study demonstrate that LTP1 maintenance is independent of protein synthesis. This is in accordance with it being a weak form of LTP that is expressed entirely postsynaptically. It is likely that LTP1 is therefore similar to E-LTP, requiring posttranslational modifications of pre-existing proteins and glutamate receptor trafficking for its maintenance (Malinow *et al*., 1988; Malenka *et al*., 1989a; Sweatt, 1999; Malinow & Malenka, 2002).

Based on the results presented here it is difficult to ascertain the exact mechanisms underlying the maintenance of LTP1, nevertheless it is interesting to speculate about the processes that may be involved. It is likely that posttranslational modifications of key proteins are predominantly mediated by CaMKII (Raymond, 2007) which is a major component of the postsynaptic density (Kennedy, 2000) and preferentially activated by pulses of Ca^{2+} (De Koninck & Schulman, 1998; Dupont & Goldbeter, 1998). Both of these properties make it a likely target for the localised spine Ca^{2+} signal generated via NMDAR and RyR conductance associated with LTP1 induction (Raymond & Redman, 2006). Most of the CaMKII-dependent posttranslational modifications act to increase AMPAR-mediated transmission (Lisman *et al*., 2002). CaMKII is responsible for phosphorylation of the GluR1 subunit of the AMPAR (Barria *et al*., 1997a; Barria *et al*., 1997b; Mammen *et al*., 1997b) which allows for increased AMPAR conductance during LTP (Benke *et al*., 1998) and may also be important for the trafficking of AMPARs to potentiated spines (Barria *et al*., 1997a; Mammen *et al*., 1997b). This is largely achieved via CaMKII direct binding to the NMDAR which facilitates the creation and filling of new AMPAR anchoring sites (Lisman & Zhabotinsky, 2001). These mechanisms could provide a means for the short-term persistence of LTP1.

5.5.4. LTP2 is dependent on translation but not transcription

LTP2 was found to be dependent on translation, but not on gene transcription, suggesting that protein synthesis from pre-existing mRNA may be critical for its persistence. A form of LTP requiring transcription-independent protein synthesis was first demonstrated in the dentate gyrus *in vivo* (Otani *et al*., 1989) and was later also identified in CA1 *in vitro* (Raymond *et al*., 2000; Gelinas & Nguyen, 2005). In these

studies LTP persistence was dependent on local protein synthesis, presumably from a constitutive pool of dendritic mRNA, triggered by activation of either Group 1 mGluRs or β-adrenergic receptors.

Such translation could either occur locally within the potentiated spine, or somatically with the newly synthesised proteins being trafficked to the dendrites to be utilised by 'tagged' synapses (Frey & Frey, 2008). Based on several contributing factors, we suggest that LTP2 is more likely to involve local rather than somatic translation. A study by Raymond *et al*. (2000) reported that Group I mGluR activation during LTP2 triggered new protein synthesis in a homosynaptic manner. This suggests that the newly synthesised proteins are in close proximity to the sites of mGluR activation. Other results suggest that activation of Group I mGluRs is a major trigger for dendritic protein synthesis (Sutton & Schuman, 2005) and a number of specific proteins have been identified as targets of mGluR translational regulation. These include the synaptic scaffolding protein PSD-95 (Todd *et al*., 2003), Fragile X Mental Retardation protein (Weiler *et al*., 1997) and CaMKII (Kao *et al*., 2010). Additionally mGluR-mediated translation is required for synaptic plasticity in isolated dendritic slice preparations (Huber *et al*., 2000). A concomitant requirement for IP_3R-mediated Ca^{2+} release during LTP2 induction also supports a role for local dendritic translation. Several components of the dendritic translational machinery are modulated by Ca^{2+} and are therefore potential targets for IP_3R-mediated Ca^{2+} release. Both IP_3R and NMDAR-mediated Ca^{2+} release can activate PKC by working in concert with diacylglycerol generated by the activity of mGluRs (Codazzi *et al*., 2006). Additionally, the Erk/MAPK pathway is a downstream target of PKC and is a known regulator of dendritic protein synthesis (Kelleher *et al*., 2004a; Sutton & Schuman, 2005). In this way coordinated activity of Group I mGluRs, IP_3Rs and NMDARs during LTP2 induction could result in the dendritic translation of key proteins that allow for its persistence beyond that of LTP1.

5.5.5. LTP3 is both translation- and transcription-dependent

LTP3 was found to be dependent on both mRNA translation and gene transcription for its persistence. Protein synthesis-dependent LTP (historically known as late-LTP) has been extensively characterised in the hippocampus, generally involving CREB-mediated transcription of key plasticity-related gene products (Abraham, 2003; Pittenger & Kandel, 2003; Benito & Barco, 2010). Similar forms of transcription-dependent LTP have also been associated with activation of L-type VGCCs (Morgan &

Teyler, 2001) and inhibition of L-type VGCCs is reported to selectively impair transcription and translation dependent LTP, without affecting LTP1 (Impey *et al*., 1996; Moosmang *et al*., 2005).

It is therefore likely that LTP3, with its dependence on L-type VGCCs (Raymond & Redman, 2002, 2006), is dependent on CREB-mediated transcription, which is activated by various kinase pathways and has a well-established role in the persistence of L-type VGCC-dependent forms of LTP (Impey *et al*., 1996; Moosmang *et al*., 2005). Although both L-type VGCCs and NMDARs provide the necessary Ca^{2+} signal to activate CREB-mediated transcription, only L-type VGCC activation provides sufficient signal to recruit CBP and trigger gene expression (Hardingham *et al*., 1999). NMDAR-mediated Ca^{2+} may be important for prolongation of CREB-phosphorylation, maximising the time for CREB-CBP interaction. Alternatively, NMDARs are potent activators of serum-responsive element (SRE)-dependent transcription (Bading et al 1993), which could be critical for the transcription of other, non-CRE-regulated genes. Therefore for LTP3 it seems likely that transcription is initiated by the somatic L-type VGCC Ca^{2+} signal, leading to CREB-dependent transcription of key genes. This transcription may act in concert with NMDAR-mediated SRE-dependent transcription during the late phase of LTP3.

5.5.6. Temporal changes in gene expression

Two questions of particular importance in this study relate to the LTP3 transcription dependence. Is it reasonable to expect changes in gene expression during the 160 min recording time frame? And how would products from these transcripts exert their effects over such a short period? There are several cascades of genes that are activated following LTP, each being initiated at different time points. The initial transient activation of so-called immediate early genes (IEGs) largely encodes for transcription factors and is thought to be critical in mediating subsequent waves of gene expression (Walton *et al*., 1999). Once translated, these transcription factors re-enter the nucleus and stimulate transcription of late-response genes that are important for persistence of LTP beyond several hours. The most widely studied of the IEGs are jun (c-jun, jun-B, jun-D), fos (c-fos, fos-B, fra-1, fra-2) and Krox (Krox-24, also known as zif268; Krox-20) gene families (Walton *et al*., 1999). The transcription of IEGs occurs rapidly following the induction of LTP – in the case of c-fos within approximately 5 min (Flavell & Greenberg, 2008) and between 10 minutes and 2 hours for Krox-24 (Cole *et al*., 1989; Wisden *et al*., 1990; Richardson *et al*., 1992).

Aside from the upregulation of transcription factors, LTP induction also results in the transcription of genes encoding proteins. Gene array data suggests that proteins synthesised as early as 20 mins post-LTP induction at perforant path synapses may be important for stabilising LTP, as well as replenishing proteins depleted during the induction stimulation (Ryan *et al*., 2011). In this study upregulated expression of Erk and dual-specificity phosphatases (DUSP) was reported (Ryan *et al*., 2011), both of which have known roles in modulating MAPK signalling (Patterson *et al*., 2009). Additionally, time course microarray analyses (also at perforant path synapses) demonstrated the upregulation of a large number of protein-encoding genes at 30, 60, 90 and 120 min post LTP induction (Park *et al*., 2006). These include proteins important for structural changes at the synapse (required for cytoskeleton regulation and cell-cell/cell-extracellular matrix interactions) and an upregulation of Wnt signalling-related genes (Park *et al*., 2006). Wnts are secreted glycoproteins (Freese *et al*., 2010) that have a known role in regulating the density of the postsynaptic glutamate receptor GLR1 in *C.elegans* (Dreier *et al*., 2005) and in governing the function of cadherin-containing adhesion complexes, important for synaptic remodelling (Goda, 2002; Togashi *et al*., 2002). Several studies have demonstrated a role for Wnt signalling in LTP (Chen *et al*., 2006; Beaumont *et al*., 2007) and the cascade is thought to be particularly important for presynaptic expression of certain forms of hippocampal LTP by increasing neurotransmitter release (Cerpa *et al*., 2008). It is unlikely that Wnt-dependent transcription of gene products is important for the presynaptic expression of LTP3 described here however, given the finding that enhanced exocytosis associated with LTP3 is independent of transcription.

Nevertheless, the above results suggest that changes in gene expression occur very rapidly following LTP induction, involving the transcription of genes encoding both transcription factors as well as proteins important for the maintenance of LTP. These changes occur over a period relevant to the recording time frame tested here, hence it is perhaps unsurprising that inhibition of this transcription affects LTP3 persistence. The effects of these changes in gene expression during LTP3 are presumably limited to the postsynaptic cell and do not appear to be important for the observed changes in presynaptic neurotransmitter release. Rather, this early gene expression appears important for mediating remodelling of synapses and postsynaptic receptor densities, as well as protein replenishment.

5.5.7. Postsynaptic translation is required for enhanced exocytosis

An interesting finding from this study is that the enhanced exocytosis associated with LTP2 and LTP3 is translation-dependent. Until now there has been little to link the protein synthesis requirements of LTP2 and LTP3 to a presynaptic expression mechanism. Our data show that in the absence of translation the enhanced presynaptic function associated with the maintenance of both LTP2 and LTP3 is eliminated. Interestingly, although LTP3 as measured via fEPSPs is transcription-dependent, the presynaptic component is not. Thus, the transcription underlying the greater persistence of LTP3 over LTP2 appears to support only postsynaptic expression mechanisms. It should be noted however, that since the CA3 cell bodies containing the presynaptic transcriptional machinery are removed during our hippocampal slicing protocol, it remains possible that presynaptic transcription may contribute to LTP persistence *in vivo*. Indeed, changes in the levels of several synapse-related transcripts have been observed in area CA3 following LTP induction at Schaffer collateral synapses (reviewed by Abraham & Williams, 2003).

The translation required for the presynaptic component of both forms of LTP could conceivably either occur postsynaptically, (either upstream, downstream or in parallel with NO signalling), or presynaptically in response to NO. Local postsynaptic protein synthesis has been extensively characterised (Steward & Levy, 1982; Sutton & Schuman, 2006; Bramham & Wells, 2007) and is often associated with LTP. There is also mounting evidence to support a role for translation in presynaptic terminals (reviewed by Akins *et al*., 2009). Local presynaptic protein synthesis has been established in both immature and mature rat hippocampal cultures (Sebeo *et al*., 2009), in forms of LTP and long-term depression in *Xenopus* nerve-muscle cultures (Zhang & Poo, 2002), in corticostriatal fibres (Yin *et al*., 2006) and in hippocampal mossy fibre-CA3 synapses (Huang & Hsu, 2004).

In this study the enhanced exocytosis associated with LTP2 and LTP3 was found to be dependent on postsynaptic translation, being eliminated upon selective inhibition of postsynaptic translation with Gelonin. Enhanced release was only abolished at terminals very closely apposed to postsynaptic spines – between 0 and 0.5 μm. Although a distance of 0.5 μm is substantially larger than that of a typical synaptic cleft (approximately 20 nm; Schikorski & Stevens, 2001), due to the limitations of light microscopy accurate resolution of distances can only be achieved at distances such as these. Nevertheless it appears that only terminals making putative contact with dendritic spines in which translation is inhibited exhibit reduced release rates that

approximate basal pre-LTP levels. Conversely, at sites removed from the treated dendrite enhanced release remains unimpaired. This finding strengthens the hypothesis that the property of input specificity remains intact with NO-dependent enhanced release and that other messengers (such as BDNF) may be required in conjunction with NO to elicit enhanced release.

The dependence of presynaptic expression on postsynaptic translation is further highlighted by the unchanged PPR with postsynaptic translation inhibition by Gelonin. In the absence of Gelonin a reduced PPR (and therefore enhanced P_r) was measured at 160 min post-TBS for LTP2 and at 80 min and 160 min post-TBS for LTP3, consistent with the presynaptic expression observed with FM 1-43 destaining. However with postsynaptic translation inhibited, no change in PPR was observed and release probability remained similar to that of basal levels. This reinforces the finding that postsynaptic translation is required for the enhanced presynaptic function associated with LTP2 and LTP3.

It must be noted that the results presented here do not conclusively exclude a role for presynaptic translation in the expression of LTP2 and LTP3. It is possible that translation in the postsynaptic cell may trigger changes in the presynaptic terminal leading to further presynaptic translation of key proteins. Additional work is required to elucidate the dependence of LTP2 and LTP3 presynaptic expression on presynaptic translation.

5.5.8. Identity of newly synthesised proteins required for enhanced exocytosis

There are a number of candidates for newly translated proteins in the postsynaptic cell that might be important for the observed enhanced presynaptic function. These could include cell-cell adhesion molecules, importins and molecules responsible for retrograde signalling.

Changes in translation of cell-cell adhesion molecules may alter signal communication between the postsynaptic and presynaptic neuron, and this altered signalling could trigger facilitated exocytosis. Indeed, expression of such adhesion molecules are known to be crucial for some forms of LTP (Dityatev *et al*., 2008) and are important for regulating exocytosis (Saghatelyan *et al*., 2004; Dityatev *et al*., 2008).

Alternatively, dendritic translation of importins may play a role. Importin proteins provide a physical porous link between presynaptic and postsynaptic cells, allowing for intercellular shuttling of signalling molecules (Donnelly *et al*., 2010). These proteins have been detected in cultured hippocampal neurons (Thompson *et al*., 2004; Lai *et al*.,

2008) and could provide a means for triggering enhanced release within the presynaptic terminal.

Translation could also potentially upregulate expression of proteins required for the production of a retrograde signal. Given that NO is thought to exert its influence only during induction of LTP (Schuman & Madison, 1991), this indicates that at a later time point, a second translation-dependent retrograde messenger may act to reinforce presynaptic changes that are initiated earlier by NO. As discussed previously, BDNF may help preserve input specificity by acting in concert with NO to trigger presynaptic expression. Therefore it seems reasonable that translation of BDNF might be a key step in establishing enhanced presynaptic release. A great deal of evidence exists for the presence of BDNF mRNA at dendritic sites (Tongiorgi *et al*., 1997; An *et al*., 2008) and enhanced release associated with TBS-induced LTP is BDNF dependent (Zakharenko *et al*., 2003). Therefore it is possible that dendritic translation of BDNF may be required to fortify the initial presynaptic changes that are initiated by NO.

5.6 General discussion

5.6.1. Confirmation of the LTP1, 2 and 3 model

The LTP1, 2 and 3 model is considerably strengthened by the addition of our current findings. It is now abundantly clear that CA3-CA1 synapses can support at least 3 mechanistically discrete forms of LTP. LTP1 is dependent entirely on local postsynaptic mechanisms, requiring spine Ca^{2+} signals via NMDARs and RyRs for its induction. Its persistence is dictated by biochemical processes within the postsynaptic cell only, and this is likely mediated by kinase-dependent posttranslational modification of key proteins. LTP2 induction involves dendritic Ca^{2+} signalling via NMDARs and IP_3Rs and its persistence is dependent on dendritic protein synthesis (which is likely initiated by Group I mGluR activity) and this synthesis is important for the late onset of a presynaptic expression component. Finally, LTP3 involves cell-wide mechanisms, requiring both NMDAR and L-type VGCC-dependent somatic Ca^{2+} signalling during induction, a postsynaptic expression component that is maintained by postsynaptic gene transcription and protein synthesis and a presynaptic expression component dependent only on postsynaptic protein synthesis. In contrast to LTP2, the presynaptic expression associated with LTP3 is recruited earlier, suggesting that the relative weighting of presynaptic and postsynaptic expression contributions may govern the long-term persistence of LTP.

We contend that many of the controversies in CA1 LTP research have arisen from the co-existence of LTP1, 2 and 3. Detailed investigations into the mechanisms underlying LTP in this region have produced many contradictory results, which is unsurprising given the heterogeneity in induction, expression and maintenance mechanisms associated with LTP1, 2 and 3 described here. Appreciation of the individual characteristics associated with individual forms of LTP should assist in the design and interpretation of future investigations.

5.6.2. Extension of the LTP1, 2 and 3 model

The current study has shed new light on the LTP1, 2 and 3 model by providing evidence for unique expression mechanisms and dependence on NO signalling and protein synthesis. The existing LTP1, 2 and 3 model can now be revised to include these new properties.

As already discussed, LTP1 is a weak form of LTP that is expressed entirely postsynaptically and is independent of NO signalling and gene transcription and protein synthesis. RyR-mediated Ca^{2+} signals during the 1TBS induction protocol are likely to

activate various kinase pathways that result in phosphorylation of key plasticity-related proteins. The outcome of such modifications might result in increased AMPAR conductance and density at potentiated dendritic spines. Such increases would only be transient however; hence LTP1 decays rapidly in the absence of any presynaptic changes or new protein synthesis.

In contrast LTP2 and LTP3 involve a presynaptic component to their expression that is maintained by NO signalling and dendritic protein synthesis. This presynaptic expression may be temporally controlled, being recruited earlier for more persistent LTP3. This difference in expression onset is unlikely to be explained by the different induction requirements for LTP2 and LTP3. Previous studies using FM 1-43 or synaptopHlourin-expressing mice suggest that L-type VGCC activation is necessary for LTP involving a presynaptic component (Zakharenko *et al*., 2001; Bayazitov *et al*., 2007). Certainly, with respect to LTP3 our data are consistent with this since LTP3 is dependent on somatic Ca^{2+} signalling via L-type VGCCs (Raymond & Redman, 2006). However, our model suggests that L-type VGCCs are not obligatory for activating a presynaptic LTP expression mechanism since LTP2, which is independent of L-type VGCCs (Raymond & Redman, 2002; 2006), also involves a presynaptic enhancement. Rather, our data suggest that L-type VGCCs are upstream of a crucial common effector of presynaptic change – protein synthesis – and that different signalling pathways (e.g., internal Ca^{2+} release via IP_3 receptors) activated by weaker stimulation protocols may also suffice.

Dendritic translation initiated by both 4TBS and 8TBS Ca^{2+} signals is likely to result in the synthesis of many plasticity related proteins, one or more of which is required for the presynaptic enhancement. Considering the dependence of presynaptic expression on NO signalling, it is logical to suggest that translation of NO-related signalling proteins, such as NOS, could be important. NOS expression is known to be upregulated under certain stress conditions (de Oliveira *et al*., 2000; Yao *et al*., 2007), although has yet to be demonstrated during hippocampal synaptic plasticity. Furthermore, NOS mRNA has not been detected in CA1 dendrites and to date has only been observed in developing olfactory (Gibson & Nighorn, 2000) and optic (Koriyama *et al*., 2009) axons. Despite this, it remains a possibility that local NOS translation may allow for NO-dependent consolidation of initial NO changes within the terminal.

Given the existing data reporting a role for BDNF in TBS-induced LTP (Zakharenko *et al*., 2003), the preponderance of BDNF transcripts in hippocampal dendrites (Tongiorgi *et al*., 1997; An *et al*., 2008) and the observation that translation-dependent forms of LTP require BDNF (Bramham & Messaoudi, 2005; Lynch *et al*., 2007) it seems likely that

BDNF might also play an important role. If this were the case, how would BDNF exert its effects relative to those of NO? It is likely that NO signalling to the presynaptic neuron is important during the induction phase of LTP2 and LTP3 (as is reported for other forms of LTP; Schuman & Madison, 1991) and this could potentially serve to 'prime' the terminal for subsequent BDNF signalling. For example, NO could act to ribosylate synaptic vesicle proteins (Schuman *et al*., 1994; Kleppisch *et al*., 1999), which would only lead to increased transmitter release with subsequent BDNF-dependent increases in intracellular Ca^{2+} via release from internal stores (Berninger *et al*., 1993). In this manner BDNF and NO could act cooperatively to produce enhanced transmitter release, with both messengers being necessary but not sufficient on their own to achieve this function.

5.6.3. Physiological relevance of LTP1, 2 and 3

Here the existence of three mechanistically discrete forms of LTP that can co-exist at CA3-CA1 synapses *in vitro* is reported. An important question raised from this study is whether LTP1, 2 and 3 are also functionally discrete, i.e. whether each form subserves a different function in learning and memory processing. The characteristics of LTP1, 2 and 3 described here seem to mimic the LTP features observed in the dentate gyrus *in vivo* (Abraham & Otani, 1991), at least in terms of maintenance mechanisms (Otani & Abraham, 1989; Otani *et al*., 1989; Raymond, 2007). Therefore it is reasonable to postulate that LTP1, 2 and 3 may also be inducible at other synapses in the hippocampal circuit and perhaps even in different brain regions that are also known to exhibit synaptic plasticity. If this were the case, LTP of differing persistence could be induced at various synapses depending on the pattern of afferent input,

Behavioural studies provide insight into the role that LTP1, 2 and 3 may play *in vivo*. In relation to LTP1, stimulation of RyRs is reported to improve spatial learning and memory and enhance memory consolidation (Adasme *et al*., 2011). Also, animals in which RyRs have been genetically deleted exhibit intact spatial long-term memory, but interestingly demonstrate reduced potential to subsequently learn a new arrangement of the same task (Balschun *et al*., 1999; Futatsugi *et al*., 1999).

With respect to LTP2, inhibition of Group I mGluRs selectively impairs reference memory over the course of two days (Naie & Manahan-Vaughan, 2004; Naie & Manahan-Vaughan, 2005). Additionally, Group I mGluR knockout mice display long-term memory deficits for spatial water maze learning and exhibit impaired fear conditioning memory

(Aiba *et al*., 1994; Conquet *et al*., 1994). Other studies demonstrate reduced retention of learning using discrimination avoidance tasks when either Group I mGluRs or IP_3Rs are blocked in chicks (Baker *et al*., 2008).

Relevant to LTP3, impaired L-type VGCC signalling selectively impairs long-term memory beyond a few days, without affecting acquisition or working memory (Bading *et al*., 1993; Davis *et al*., 2000; Moosmang *et al*., 2005). Similarly disruption of CREB-mediated transcription (Pittenger *et al*., 2002), CBP activity (Korzus *et al*., 2004) or Erk/MAPK signalling (Morozov *et al*., 2003) impairs spatial long-term memory with no effect on contextual memory. These findings are suggestive of a role for LTP1, 2 and 3 in different types of learning and memory under various physiological conditions.

If LTP1, 2 and 3 are able to coexist at a single synapse, what might be the consequences of this? It is conceivable that weak incoming afferent information may induce LTP1, which will decay rapidly (over the course of approximately 3 days in vivo; Abraham, 2003) after which time encoding of this afferent information will be lost. Delivery of subsequent stronger input might induce either LTP2 or LTP3 and this would result in new protein synthesis, reinforcing the posttranslational protein modifications initiated during LTP1. This would ultimately produce persistent changes in both the postsynaptic compartment and the presynaptic terminal. Alternatively such progression might not be necessary in all cases and stronger stimulation could induce *de novo* LTP3. In this manner the pattern of afferent input may dictate the probability of an event being encoded in the hippocampus.

In accordance with this reasoning, a study by Raymond (2008) demonstrated that LTP1 could be 'transformed' into LTP3 by following the 1TBS stimulation with delivery of 7 trains of TBS-patterned action potentials to the soma. This suggests that postsynaptic action potentials alone are sufficient to activate LTP3 maintenance, a finding that has substantial implications for late-associativity whereby a weaker form of LTP can be strengthened by more persistent forms of LTP at other inputs. Therefore, the gene products necessary for LTP3 maintenance could be trafficked along the dendritic arbor and utilised by synapses that have been 'tagged' by the induction of LTP1 (Frey & Morris, 1997, 1998a). Additionally, this process seems to be dependent upon the activation of D1/D5 dopamine receptors (Hunt & Raymond, unpublished), which appear to interact synergistically with L-type VGCCs to mediate the maintenance mechanisms of LTP3. In this manner weak LTP could be strengthened by subsequent strong input at other synaptic sites.

In this scenario it is logical to assume that if weak afferent signals are not reinforced by stronger forthcoming input, the information will be lost. As the hippocampus is thought to only store information in the short-term (Squire, 1986; Teyler & Rudy, 2007), the information from robustly potentiated circuits could be relayed to the neocortex where it would persist as an indexed trace of a learned task, event or episode.

5.6.4. Future directions

There are several avenues of further experimentation that may provide useful insight into the data presented here. It would be of particular interest to unravel the signalling pathways that lead from postsynaptic translation to enhanced presynaptic release. As previously mentioned, given the current results it is only possible to speculate about whether this translation is upstream or downstream of NO signalling. Varying the timing of NO signalling inhibition could help to elucidate this and would clarify whether NO-dependent processes are important beyond the induction period. For example, there may be a second wave of NO signalling that is required for the late onset of the enhanced exocytosis and this could be determined by applying cPTIO and L-NAME for the duration of the post-TBS period. Intermediates in the various kinase pathways known to be important for LTP2 and LTP3 could also be systematically inhibited to dissect which pathways are of particular importance for the increased rate of exocytosis. A particularly powerful technique that has recently been developed utilises a metabolic labelling approach to track the behaviour and movement of newly translated proteins in different cellular compartments (Dieterich *et al*., 2010). The application of such a technique to the present problem would allow for precise identification of newly synthesised proteins during LTP2 and LTP3 and may provide insight into the identity of the postsynaptic biochemical cascades that are required for the enhanced release.

The role of NO signalling in the induction of LTP2 and LTP3 could be further investigated by determining whether LTP1 could be transformed into LTP2 or LTP3 upon application of a NO donor during induction. Such an experiment would help to resolve the mechanisms that promote rapid decay of LTP1. For example, if the presence of a NO donor during 1TBS induced LTP with a presynaptic expression component and similar decay characteristics to that of LTP2 or LTP3 this would indicate that enhanced presynaptic release plays a large role in the robustness of more persistent forms of LTP. However if this protocol induced LTP with a presynaptic expression component yet LTP1-like decay characteristics it would seem much more

likely that mechanisms in the postsynaptic cell (such as new protein synthesis) dictate persistence.

A particular limitation of using FM dyes is that they restrict the frequency with which presynaptic function can be monitored in a single experiment. The loading and unloading of dye within a slice takes approximately 30 minutes and excessive use of these protocols risk photodamage and excessive background staining. A recent technique that has overcome these issues utilises pH-sensitive GFP molecules linked to synaptic vesicle proteins in which the intensity of fluorescence is altered during exocytosis (Miesenböck *et al*., 1998). These synaptopHlourin mice have already been utilised in LTP experiments to measure the temporal onset of enhanced FM 1-43 destaining with various less well characterised forms of LTP (Bayazitov *et al*., 2007). This technique would also be interesting to apply to LTP2 and LTP3 experiments to ascertain the precise onset of enhanced release with these forms of LTP and determine the exact delay in onset for LTP2. Such results would provide further insight into the specific mechanisms underlying enhanced presynaptic function.

It would also be interesting to extend the recording time frame to determine how long LTP2 and LTP3 can persist for *in vitro*. Our results suggest that LTP3 is essentially non-decremental over the entire 160 min recording period and given that LTP can be recorded for up to 8 hours *in vitro* (Frey *et al*., 1988), it would be useful for our purposes to investigate whether LTP3 may represent a permanent form of potentiation. In these experiments presynaptic function could also be monitored over this extended period to determine whether enhanced release is crucial for the entire duration of LTP, or whether it diminishes over time allowing the postsynaptic component to dominate the potentiation. Indeed, an expression pattern such as this may be desirable to allow a 'resetting' of P_r in order to enable expression of further plasticity.

It would also be useful to selectively inhibit presynaptic translation to determine the effects on the enhanced release. The current data indicate that postsynaptic translation is required, but do not rule out a role for presynaptic protein synthesis. To discern this, Gelonin could be applied to the CA3 cut end of the hippocampal slice, allowing for its axonal diffusion to the terminals and release could be measured during LTP2 and LTP3 as per the experiments described here in which postsynaptic translation was blocked. It would be necessary to trace the diffusion of Gelonin along the axon (perhaps with a fluorescent marker) to ensure it reached the axon terminal. It would be similarly useful to repeat the protein synthesis inhibition experiments with CA3 intact. As discussed

previously, the CA3 cell bodies containing the presynaptic transcriptional machinery are removed during our hippocampal slicing protocol; therefore it remains possible that presynaptic transcription may contribute to LTP persistence.

Given the supposition discussed here that BDNF may work in concert with NO (yet at a later stage) to establish the presynaptic LTP component it would be intriguing to perform experiments in which BDNF was blocked (either via genetic deletion or pharmacological inhibition of TrkB receptors). Presynaptic function could then be assessed during LTP2 and LTP3 over the same time frames used here to determine whether BDNF signalling is required for these forms of LTP, as has been reported for other TBS-induced LTP (Zakharenko *et al*., 2003).

Finally, and perhaps most importantly, it would be useful to examine the role that LTP1, 2 and 3 may play *in vivo*. It would be interesting to ascertain whether LTP1, 2 and 3 exist in synaptic pathways outside the hippocampus. If learning and memory events encoded by the hippocampus are indeed relayed to the cortex where they are indexed, perhaps these cortical circuits may exhibit forms of LTP that utilise similar mechanisms and exhibit similar characteristics to those of hippocampal LTP1, 2 and 3. Perhaps more interesting, however, is the possibility that LTP1, 2 and 3 might be necessary only for the unique processing performed in the hippocampus. If this were so, further understanding of the function of LTP1, 2 and 3 in the hippocampus *in vivo* could help unravel its specific role in learning and memory.

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