Mechanisms of long-term presynaptic plasticity at Schaffer-collateral synapses

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DECLARATION

I, Zahid Padamsey, declare that the work presented in this thesis is my own unless where explicitly noted. Extracts of this thesis have been used, with and without modification, in two publications ((Padamsey and Emptage, 2011; Padamsey and Emptage, 2013)), both of which have been written and prepared by myself, with comments from my supervisor, Professor Nigel Emptage.

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ABSTRACT

Synaptic plasticity is thought to be integral to learning and memory. The two most common forms of plasticity are long-term potentiation (LTP) and long-term depression (LTD), both of which can be supported either by presynaptic changes in transmitter release probability (Pr), or by postsynaptic changes in AMPA receptor number. It is generally thought that the induction of LTP and LTD at Schaffer-collateral synapses in the hippocampus depends on the activation of NMDA receptors (GluN). Recent studies, however, have demonstrated that both increases and decreases in Pr can be induced under blockade of postsynaptic GluN receptors, suggesting that the activation of postsynaptic GluN receptors by glutamate is only a strict requirement for postsynaptic plasticity. In this thesis, I therefore re-examined the role of glutamate in presynaptic plasticity. I used single synapse imaging along with electrophysiological and pharmacological techniques to independently manipulate and monitor the levels of glutamatergic signalling during synaptic activity. I discovered that glutamate is inhibitory and unnecessary for the induction of LTP at the presynaptic locus. My findings support a novel model of presynaptic plasticity in which the net activity-dependent changes in Pr at an active presynaptic terminal is jointly determined by two opposing processes that can be simultaneously active: 1) postsynaptic depolarization, which, via the activation of L-type voltage-gated Ca^{2+} channels, increases Pr by driving the synthesis and release of nitric oxide from neuronal dendrites and 2) glutamate release, which through the activation of presynaptic GluN receptors, decreases Pr. Computationally, this model suggests that plasticity functions to reduce prediction-errors that arise during synaptic activity, and, thereby offers a biologically plausible mechanism by which neuronal networks may optimize learning at the level of single synapses.

TABLE OF CONTENTS

1. INTRODUCTION

The ability to learn and remember information is integral to survival. It enables organisms to generate useful predictions about their environment, and to modify these predictions as the environment changes. Learning and memory, therefore, are likely to play a central role in information processing in the brain, and understanding their biological basis remains a fundamental question in neuroscience.

1.1 Basic properties of the synapse

Synapses are nodes of communication between neurons. Within the central nervous system, excitatory synapses tend to be structurally comprised of: 1) a presynaptic swelling along the axon, known as a varicosity or a *bouton en passant*, and 2) a postsynaptic protrusion along the dendrite, known as a spine (Figure 1). The axonal bouton and dendritic spine contain the relevant machinery for neurotransmission, which enables the transfer of electrochemical signals from the pre- to the post- synaptic neuron.

1.1.1 Presynaptic bouton

Glutamate is the major neurotransmitter released at excitatory synapses in the mammalian brain. At the presynaptic bouton, glutamate can be synthesized either from glutamine or glucose, which are generally transported from the extracellular space (Palmada and Centelles, 1998). Free cytosolic glutamate is then packaged into vesicles via a glutamate transporter, which uses an electrochemical gradient generated by a vesicular proton pump (Edwards, 2007). Packaged vesicles are sorted into three functional release pools (Rizzoli and Betz, 2005): 1) the readily releasable pool (RRP), which comprises of vesicles that are

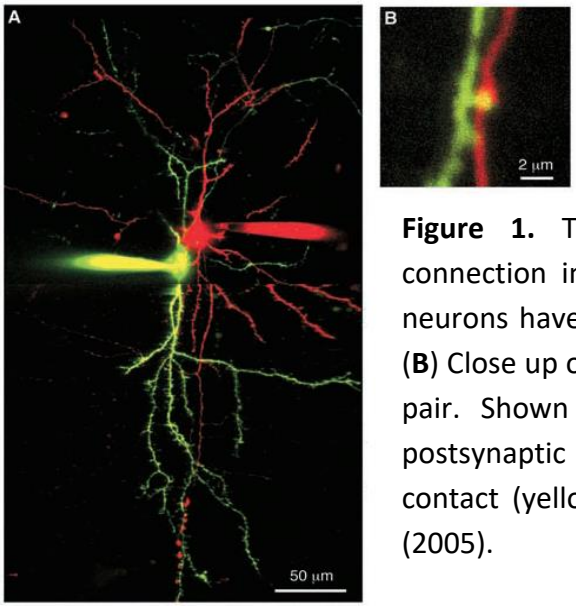


Figure 1. Two-photon image of an excitatory synaptic connection in a neocortical rat acute slice. (**A**) Two cortical neurons have been patched and loaded with fluorescent dye. (**B**) Close up of a putative synaptic connection between the cell pair. Shown is a presynaptic axonal bouton (red) and a postsynaptic dendritic spine (green) coming into close spatial contact (yellow). Image modified from Koester and Johnston (2005).

docked at the active zone, where the machinery for membrane fusion is concentrated. 2) The reserve pool, which serves to replenish the RRP, and 3) a resting pool of vesicles, which is refractory to activity-dependent release. Principally, only vesicles of the RRP fuse in response to electrical stimulation, and in a manner that depends on Ca^{2+} influx from voltage-gated channels (Sudhof, 2004).

Vesicle fusion at the active zone is mediated by the interactions between three SNARE proteins: syntaxin and SNAP-25, both of which are present on the plasma membrane at the active zone, and VAMP2, which is present on the vesicular membrane (Jahn and Scheller, 2006). When in contact, the alpha-helical domains of the SNARE proteins undergo spontaneous zippering to release the necessary energy for membrane fusion. Complete zippering of SNARE proteins, however, is generally subjected to Ca^{2+}-dependent regulation by two proteins: 1) a cytosolic protein known as complexin and 2) a Ca^{2+}-sensitive vesicular-bound protein known as synaptotagmin. Complexins bind and clamp the SNARE machinery, and so increase the required energy required for vesicle fusion. When Ca^{2+} enters the presynaptic terminal, synaptotagmin undergoes a confirmation change that then provides the additional energy necessary for completing SNARE zippering, which in turn enables the completion of vesicle fusion with the plasma membrane (Sudhof, 2004). Vesicular fusion is probabilistic (Del Castillo and Katz, 1954). At central synapses, a single action potential releases a single quantum of glutamate, corresponding to the transmitter contents of a single vesicle, with a probability of ≤ 1.0 (Walmsley et al., 1988; Raastad et al., 1992) (Figure 2). The release probability is heterogeneous across boutons and depends on 1) the number of docked vesicles, 2) the sensitivity of vesicular fusion proteins to Ca^{2+}, and 3) the effective Ca^{2+} concentration in the vicinity of vesicular fusion protiens

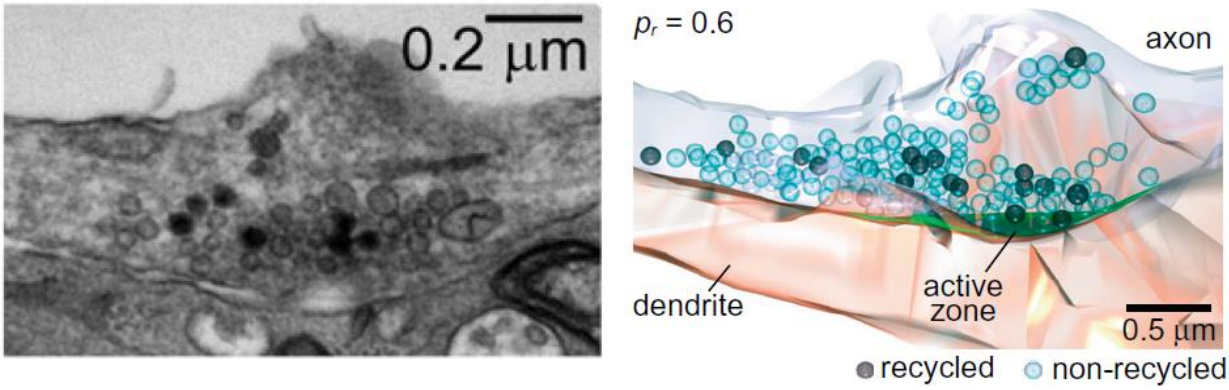


Figure 2. High-resolution electron micrographs of presynaptic bouton structure and function. (**Left**) Electron micrograph of a presynaptic bouton in hippocampal neuronal culture. Prior to fixation, the bouton was electrically stimulated 30 times in the presence of a fixable reagent (FM1-43FX), which is incorporated into the vesicle membrane following vesicle fusion. The dye can be photo-converted into a dense precipitate, causing stained vesicles to appear black in the image. (**Right**) 3D electron microscope reconstruction of the bouton. A total of 18 stained vesicles are present, suggesting that in response to 30 stimuli, the bouton had a 0.60 probability of releasing transmitter. Image modified from Branco et al. (2010).

(Walmsley et al., 1988; Bolshakov and Siegelbaum, 1995; Harris and Sultan, 1995; Dobrunz and Stevens, 1997; Murthy et al., 1997; Dobrunz, 2002). The latter of these depends not only on the average level of intraboutonal Ca^{2+}, but also on the proximity of the vesicle to voltage-gated Ca^{2+} channels; in general, vesicle release at central synapses varies with the third or fourth power of the intraboutonal Ca^{2+} concentration (Schneggenburger and Neher, 2005).

1.1.2 Postsynaptic spine

Glutamate released at the synaptic cleft affects postsynaptic activity by the activation of glutamate-sensitive receptors, which are expressed on dendritic spines. Activation of ionotropic receptors, which include the AMPA (GluA), kainate (GluK), and NMDA (GluN) receptors, produce direct depolarization of the membrane. These receptors are non-selective ion channels that are comprised of four subunits (Nakanishi and Masu, 1994). There are several genes in each family that encode receptor subunits, and each subunit can be modified by RNA editing and phosphorylation. Subunit composition and modifications greatly influence receptor properties, including channel trafficking, number, conductance, as well as activation, inactivation, and desensitization kinetics (Nakanishi, 1992; Nakanishi and Masu, 1994; Raymond et al., 1994). There are, however, general attributes associated with each class of receptor. GluA receptors are largely impermeable to Ca^{2+} and are a principle source of glutamate-mediated excitation for most neurons; these receptors have rapid opening times and rapid desensitization kinetics, making the duration of GluA receptor signalling is quite brief (Gouaux, 2004). GluK receptors have a similar channel conductance to GluA receptors, but have slower activation and inactivation kinetics, and show some Ca^{2+} permeability, which varies depending on receptor subtype (Frerking and

Nicoll, 2000). GluN receptors are Ca^{2+} permeable channels with relatively slow kinetics of activation (MacDermott et al., 1986; Jahr and Stevens, 1987; Ascher et al., 1988). However, owing to their high affinity to glutamate and slow rate of desensitization, these receptors remain active for relatively long periods of time (Lester et al., 1990; Lester and Jahr, 1992; Cull-Candy et al., 2001). GluN channel conductance and open channel probability are negatively regulated by Mg^{2+} in a voltage-dependent manner (Nowak et al., 1984): at resting membrane potential, conductance through the channel is generally 10-20% of maximum, but receptor conductance increases with depolarization, and is maximal at -20mV (Nowak et al., 1984; Jahr and Stevens, 1990). GluA, GluK, and GluN receptors have also all been reported to localize to the presynaptic terminal, where they can modulate transmitter release (Pinheiro and Mulle, 2008).

In addition to ionotropic receptors, metabotropic glutamate receptors (mGluR) are also present at the dendritic spine, though generally found perisynaptically (Lujan et al., 1996). These receptors are coupled to trimeric G-proteins, which can modulate neuronal activity over relatively long time scales (seconds to minutes) (Kimura et al., 1998). There are at least eight subtypes of mGluRs, which have been categorized into three groups based on their pharmacological and functional properties (Conn and Pin, 1997; Ferraguti and Shigemoto, 2006). Group I mGluRs are positively coupled to the production of inositol 1,4,5-trisphosphate (IP_3), which, by promoting Ca^{2+} release from endoplasmic reticular stores, influences local Ca^{2+}-sensitive processes like synaptic plasticity and protein synthesis (Raymond, 2007). Activation of Group 1 mGluRs can also augment neuronal activity via the inactivation of K^+ channels (Chemin et al., 2003). Group II and III receptors are negatively coupled to cyclic AMP signalling, and reduce neuronal activity by activating K^+ channels and

inactivating voltage-gated Ca^{2+} channels (Conn and Pin, 1997; Ferraguti and Shigemoto, 2006). These receptors are also found at the presynaptic terminal, where they generally inhibit transmitter release, namely by the inactivation of voltage-gated Ca^{2+} channels (Takahashi et al., 1996; Pinheiro and Mulle, 2008).

1.2 Early observations and theories of plasticity at the mammalian synapse

In his Croonian address to the Royal Society of London in 1894, Ramon y Cajal postulated that learning could be supported by changes in synaptic connectivity between neurons in the brain (Cajal, 1894; DeFelipe, 2006). The rules governing such changes were later theorized in the late 1940s, by Jerzy Konorski and Donald Hebb. Konorski was interested in understanding the neuronal substrates supporting the classical conditioned responses described by Ivan Pavlov in the 1890s. In 1948, he proposed that there existed potential synaptic connections between neuronal centers and that these connections would become actualized if activity in one center preceded the activation of the other (Konorski, 1948; Zielinski, 2006). In 1949, Hebb developed the idea further into a more generalized model of learning and memory (Kandel, 2009). He suggested that memory engrams were formed by enhancing connectivity between coactive neurons, resulting in an auto-associated cellular assembly. Activation of a subset of the neurons would result in reverberant activation of the entire assembly, enabling stable and holistic engrams to be generated from a subset of its components (Hebb, 1949; Sejnowski, 1999).

In the 1950s, several laboratories were exploring whether synapses were a plausible substrate for long-term memory storage in vertebrates. From work done in the 1940s,

transient synaptic enhancements were already known to occur at the frog neuromuscular junction following motoneuron stimulation (Feng, 1941; Magleby and Zengel, 1975b, a; Magleby and Zengel, 1976a; Magleby and Zengel, 1976b, c, 1982). Whereas a single stimulus could enhance responses to subsequent stimuli for a few hundred milliseconds, a brief train of stimuli could enhance responses for several seconds, and a high-frequency train of stimulation could enhance responses for several minutes; these processes were respectively referred to as facilitation, augmentation, and post-tetanic potentiation. All three phenomena were thought to reflect an increase in transmitter release, resulting from a sustained elevation in presynaptic Ca^{2+} levels following axonal stimulation (Katz and Miledi, 1968; Magleby and Zengel, 1975b, 1976c; Zengel and Magleby, 1982). Similar synaptic enhancements were also reported in the spinal cord, cortex, and hippocampus throughout the 1950s and 1960s (Lloyd, 1949b, a; Cragg and Hamlyn, 1955; Green and Adey, 1956; Cragg and Hamlyn, 1957; Andersen, 1960a, b; Andersen et al., 1961; Gloor et al., 1964; Beswick and Conroy, 1965; Spencer and Wigdor, 1965). However, the transient nature of these enhancements made them unlikely substrates for longer-term information storage.

Thus, despite an emerging theoretical framework, experimental support for a synaptic locus for long-term memory was lacking. In the 1950s a rival theory of learning emerged, championed by Karl Lashley, which suggested that information was not locally stored within synapses but distributed within the electromagnetic fields generated by dispersed neuronal activity (Lashley, 1950). The theory was based on Lashley's early failures to find specific memory deficits associated with targeted cortical lesions in rodents, which suggested against localized information storage (Lashley, 1920, 1924, 1926). However, in

the late 1950s, Scoville and Milner's work on patients receiving psychosurgery showed that deficits in declarative memory were tightly correlated with the bilateral removal of the hippocampus and surrounding regions (Scoville and Milner, 1957). Their observations provided compelling evidence for localized memory storage in the brain.

The hippocampus was a favorable model for plasticity research throughout the 1950s and 1960s. Not only was it implicated in learning and memory, but its distinct laminar structure and simple network architecture enabled synaptic responses to be easily identified and recorded for prolonged periods of time using extracellular electrodes; such long-duration recordings would be necessary for assessing the long-term stability of synaptic changes (Andersen et al., 1969) (Figure 3). Although at the time several groups were examining synaptic plasticity within the hippocampus, none were able to elicit any synaptic change with high frequency stimulation that would last longer than a few minutes (Green and Adey, 1956; Andersen, 1960a, b; Andersen et al., 1961; Gloor et al., 1964). In 1964, however, Terje Lømo observed that repeated trains of high frequency stimulation in the perforant pathway of the hippocampus resulted in an enhancement of synaptic strength that would outlast the decay of post-tetanic potentiation. Lømo later revisited these findings with Tim Bliss, and in 1973 the two published their seminal work demonstrating, for the first time, that electrical stimulation could produce stable and long-lasting enhancements in synaptic transmission *in vivo* (Bliss and Lømo, 1973). These enhancements came to be known as long-term potentiation (LTP) (Douglas and Goddard, 1975).

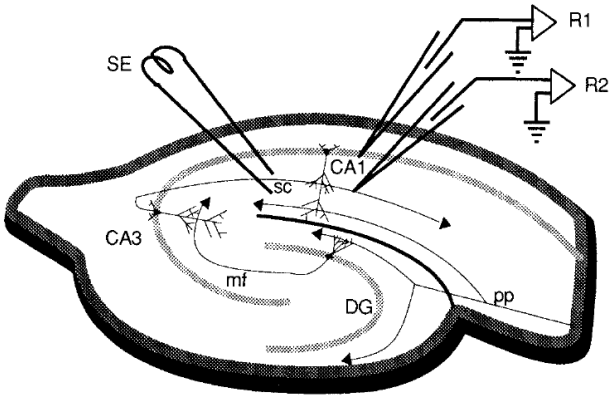


Figure 3. Schematic of a hippocampal slice preparation. The hippocampus has a simple tri-synaptic circuit: 1) Entorhinal cortical afferents synapse with granule cells of the dentate gyrus (DG) via the perforant pathway (pp). 2) Granule cells synapse with CA3 pyramidal neurons via the mossy fiber pathway (mf), and 3) CA3 neurons synapse with CA1 pyramidal neurons via the Schaffer-collateral pathway (sc). Stimulation and long-term recording of all three major synapses is possible. In the above figure, a stimulating electrode (SE) is used to stimulate the Schaffer-collaterals (sc), and the evoked synaptic responses are recorded by an extracellular field electrode (R2). Intracellular recordings (R1) are also possible. Image taken from Power et al. (1997).

1.3 An emerging role of postsynaptic GluN receptors in plasticity

The mechanism underlying LTP induction remained elusive for a decade following its discovery. Then in 1983, LTP induction at Schaffer-collateral synapses was shown to be blocked by the intracellular chelation of Ca^{2+} by Lynch et al. (1983) and with antagonists to the GluN receptor by Collingridge et al. (1983). The significance of these finding became clear in the following years, when it was demonstrated that the GluN receptor was 1) gated by Mg^{2+} in a voltage-dependent manner, (Nowak et al., 1984) and 2) permeable to Ca^{2+} (MacDermott et al., 1986; Jahr and Stevens, 1987; Ascher et al., 1988). These findings suggested that, GluN receptor activation could induce LTP via Ca^{2+} mediated signalling, and that this signalling would require presynaptic glutamate release to coincide with strong postsynaptic depolarization (Douglas et al., 1982; Gustafsson and Wigstrom, 1988; Bliss and Collingridge, 1993). This model of LTP was consistent with the coincidental pre- and post- synaptic activity that Hebb and Konorski had predicted to be required for synaptic strengthening decades earlier.

The GluN receptor-dependence of synaptic plasticity also explained the cooperative and site-specific nature of LTP. Cooperativity was an important facet of LTP induction. Early studies had demonstrated that a minimum stimulation intensity was needed for the induction of LTP, suggesting that LTP required the co-activation of a minimum number of synapses for its induction (Bliss and Gardner-Medwin, 1973; McNaughton et al., 1978). As such, low stimulation intensities could still induce LTP if paired with stronger, co-stimulation of a separate afferent pathway (McNaughton et al., 1978; Lee, 1983; Gustafsson and Wigstrom, 1986; Wigstrom and Gustafsson, 1986; Wigstrom et al., 1986). The

requirements for cooperativity were consistent with the properties of the GluN receptor, whose activation required a voltage-dependent relief of the Mg^{2+} block (Nowak et al., 1984). Receptor activation, therefore, was optimally achieved when glutamate release coincided with strong postsynaptic depolarization, as driven by the cooperative efforts across a larger number of co-active synapses. Certainly, the need for such cooperativity could be bypassed using lower frequency or lower intensity stimulation of an afferent pathway, provided that afferent activity was paired with postsynaptic depolarization artificially generated by current injection through the recording electrode (Wigstrom and Gustafsson, 1986; Wigstrom et al., 1986). Conversely, LTP induction by strong, high frequency tetanic stimulation could be prevented by postsynaptic hyperpolarization either by co-stimulation of inhibitory afferents (Douglas et al., 1982) or by the postsynaptic injection of hyperpolarizing current (Malinow and Miller, 1986). In addition to cooperativity, LTP induction had another striking property: when induced, it was always restricted to synapses that were active during postsynaptic depolarization (Andersen et al., 1977; Lynch et al., 1977). This site specificity was proposed to result from the spatially compartmentalized nature of GluN receptor-mediated Ca^{2+} signalling in dendritic spines, a notion that was later confirmed with optical techniques (Guthrie et al., 1991; Yuste and Denk, 1995).

In 1992 a second form of hippocampal plasticity was reported by Dudek and Bear, who demonstrated that prolonged, low-frequency stimulation resulted in a site-specific, long-term depression (LTD) of synaptic responses (Dudek and Bear, 1992). In line with Hebb's and Konorski's predictions, LTD appeared to result from the repeated failure of presynaptic activity to trigger postsynaptic spiking, and was later shown, like LTP, to depend on GluN

receptor activation and postsynaptic Ca^{2+} influx (Mulkey and Malenka, 1992). Based on these findings, it was hypothesized that the levels of Ca^{2+} influx through the GluN receptor determined the direction of plasticity (Artola et al., 1990; Nishiyama et al., 2000; Castellani et al., 2001; Lisman, 2001; Shouval et al., 2002). According to this model, brief but large elevations of intracellular Ca^{2+}, resulting from strong GluN receptor activation by glutamate and postsynaptic depolarization, would promote LTP induction. In contrast, more prolonged, but moderate increases in Ca^{2+}, resulting from weak GluN receptor activation by glutamate alone, would promote LTD induction. Consistent with this notion, Yang et al. (1999), demonstrated that emulating the patterns of Ca^{2+} signalling observed in cells during high- and low- frequency tetanic stimulation using caged-Ca^{2+} was sufficient to drive LTP and LTD, respectively (Yang et al., 1999; Zucker, 1999). Thus, by the late 1990s, a parsimonious model of synaptic plasticity had emerged, which obeyed Hebbian and Konorskian principles, and firmly linked both LTP and LTD to postsynaptic GluN receptor signalling (Castellani et al., 2001; Lisman, 2001; Shouval et al., 2002).

1.4 The locus of LTP expression

In the late 1980s, with some understanding of the mechanisms underlying LTP induction, focus turned to the mechanisms underlying LTP expression. In theory, synaptic plasticity can be supported either presynaptically, by changes in the amount of glutamate release, or postsynaptically, by changes in the sensitivity to glutamate release. Since at presynaptic terminals in the hippocampus, single action potentials trigger probabilistic glutamate release, changes in presynaptic function were generally thought to be mediated by changes in transmitter release probability rather than a change in the amount of glutamate loaded

into single vesicles, which was thought to be more or less constant across synapses (Del Castillo and Katz, 1954; Walmsley et al., 1988; Raastad et al., 1992; Harris and Sultan, 1995) [but see (Wilson et al., 2005; Schenck et al., 2009)]. Postsynaptic changes were mainly thought to involve changes in GluA receptor conductance and number, since these receptors were the main mediators of excitatory transmission (Collingridge et al., 1983). In the coming decades, a debate would emerge over the locus of LTP expression, particularly at Schaffer-collateral synapses, and would bring with it the development of novel experimental techniques to dissect out the pre- and post- synaptic components of transmission and plasticity.

1.4.1 Measurement of amino acid release

The earliest attempt at assessing the locus of LTP expression came from Skrede and Malthe-Sorenssen (1981) who used radioactive D-^{3}H-asparate to estimate the levels of L-glutamate released in hippocampal slices, taking advantage of the fact that exogenously applied aspartate was reliably loaded into glutamatergic vesicles (Malthe-Sorenssen et al., 1979). The authors found long-lasting elevations both in the rates of resting and evoked transmitter release at CA3-CA1 synapses following tetanic stimulation of the Schaffer-collaterals, as assessed by an increase in the radioactivity of the tissue perfusate. Their results suggested that tetanic stimulation can drive long-lasting presynaptic changes; however, a major limitation of the study was that no recording electrode was used to actually confirm the successful induction of LTP by tetanic stimulation. In the following year, Dolphin et al. (1982) assayed transmitter release in the dentate gyrus *in vivo.* They used a push-pull cannula both to locally deliver ^{3}H-glutamine and to measure the evoked release of newly synthesised ^{3}H-glutamate. Additionally, the cannula was attached to an

extracellular electrode, which was used to confirm and monitor the induction and time course of LTP. The authors found that LTP, as induced by tetanic stimulation of the perforant path, was accompanied by long-lasting increases in evoked glutamate release. Bliss et al. (1986) later used high-performance liquid chromatography to directly measure the evoked levels of endogenous glutamate and aspartate release in the dentate gyrus without the use of radioactive markers; they similarly reported long-term enhancement in presynaptic function following LTP induction. Errington et al. (1987) then demonstrated that these enhancements were blocked by bath application of the GluN receptor antagonist, AP5, during tetanic stimulation. Collectively, these studies demonstrated that LTP was supported, at least in part, by enhancements in presynaptic function. However, a subsequent study by Aniksztejn et al. (1989), which also used a push-pull cannula to measure evoked glutamate release *in vivo*, failed to find any evidence of enhanced transmitter release in CA1 or dentate gyrus following tetanic stimulation of the Schaffer-collaterals or perforant pathway, respectively (Aniksztejn et al., 1989). The study, however, lacked a positive control to confirm that the experimental preparation used was sensitive enough to detect the elevations in transmitter release that would be expected following LTP induction (Bliss and Collingridge, 2013).

1.4.2 GluN receptor-mediated synaptic transmission

A second technique established in 1988, independently by Kauer et al. (1988) and Muller and Lynch (1988), compared differential changes in the GluN receptor and GluA receptor component of synaptic transmission following LTP induction in order to dissociate the pre- and post- synaptic component of LTP. In theory, whereas presynaptic changes should equally affect both the GluN receptor and GluA receptor components of the synaptic

response, postsynaptic changes should selectively enhance only the GluA receptor component. Both Kauer et al. (1988) and Muller and Lynch (1988) reported that the GluN receptor-mediated transmission was minimally affected by LTP induction at CA3-CA1 synapses, suggesting that potentiation was predominantly expressed postsynaptically. Results, however, were inconsistent across subsequent studies employing similar techniques, with presynaptic enhancements reported to comprise anywhere between 0 - 100% of total LTP expression; the general consensus of the field, nonetheless, was that the presynaptic locus was unlikely to be a major contributor of LTP expression (Kauer et al., 1988; Muller et al., 1988a; Muller and Lynch, 1988, 1989; Bashir et al., 1991; Asztely et al., 1992; Perkel and Nicoll, 1993; Kullmann et al., 1996; Plant et al., 2006).

The underlying assumption of using the GluN receptor-component of synaptic transmission as a reporter of presynaptic efficacy is that GluN receptor number and function are not altered with LTP induction; although this is generally true for the experimental conditions under which LTP is normally assessed, GluN receptor transmission can in fact be modified in an activity-dependent manner (Slutsky et al., 2004; Watt et al., 2004; Sobczyk et al., 2005; Kwon and Castillo, 2008). At Schaffer-collateral synapses, in particular, GluN receptor-mediated synaptic transmission does appear to be selectively up-regulated within 1-2 hours of tetanic stimulation (Watt et al., 2004), and in older animals, LTP induction drives the synaptic insertion of GluN receptors (Grosshans et al., 2002). Thus, the use of the GluN receptor-mediated synaptic potentials to assess presynaptic function is not without confounds.

1.4.3 Exogenous application of glutamate and glutamate receptor agonists

Davies et al. (1989) took a more direct approach at assessing postsynaptic changes following LTP induction at Schaffer-collateral synapses by iontophoretically applying GluA receptor agonists to assess GluA receptor currents in CA1 neurons. They found that following LTP induction, depolarization induced by GluA receptor agonists increased slowly over the course of 1-2 hours. Since synaptic potentials were rapidly and stably elevated after induction, the authors concluded that LTP had an initial presynaptic component of expression, and then a later postsynaptic component of expression.

Rather than application of glutamate receptor agonists, the use of caged-glutamate is now commonly used both to induce LTP at single spines, and to assess postsynaptic changes that accompany LTP induction (ex. (Matsuzaki et al., 2001; Matsuzaki et al., 2004; Bagal et al., 2005; Harvey and Svoboda, 2007; Kasai et al., 2008)). These studies provide direct proof that LTP can be expressed by postsynaptic changes; however, their findings cannot rule out the possibility of presynaptic changes, especially under more physiological conditions, in which LTP is produced by afferent stimulation.

1.4.4 Quantal analysis using the binomial theorem

In the late 1980s, another method was developed to resolve the pre- and post- synaptic component of LTP that made use of co-efficient of variation (CV) analysis. This analysis applies the binomial theorem to synaptic transmission, whereby the mean (μ=Npq) and the standard deviation ($\sigma=[q^2Np(1-p)]^{1/2}$) of synaptic responses is expressed in terms of three quantal parameters: the probability of release (p), the number of active synapses (N), and the quantal amplitude (q), which is defined as the average postsynaptic response elicited by

the release of a single quantum of glutamate. The co-efficient of variation (CV), then, is equal to the standard deviation divided by the mean of the synaptic response ($CV=\sigma/\mu=[(1-p)/Np]^{1/2}$), and when expressed as CV^{-2}, co-varies with N and p ($CV^{-2}=Np/(1-p)$) (Faber and Korn, 1991). Under the assumption that LTP does not alter the number of active synapses (N), changes in CV^{-2} are attributable to changes in p alone.

Using CV^{-2} analysis, both Bekkers and Stevens (1990) and Malinow and Tsien (1990) demonstrated that LTP at Schaffer-collateral synapses was accompanied by an increase in CV^{-2}, which was interpreted to reflect an enhancement in p (Bekkers and Stevens, 1990; Malinow and Tsien, 1990). Similar findings were also reported in the majority of subsequent studies using similar or related statistical techniques (Kamiya et al., 1991; Malinow, 1991; Kullmann and Nicoll, 1992; Larkman et al., 1992; Manabe and Nicoll, 1994) [but see (Foster and McNaughton, 1991)]. However, the merits of CV^{-2} analysis were questioned following the experimental discovery of postsynaptic silent synapses. These synapses lack functional GluA receptors but can be converted into operational sites after LTP induction, resulting in an increase in N (Isaac et al., 1995; Liao et al., 1995). As such, reported increases in CV^{-2} following LTP induction may have resulted from an increase in N rather than an increase in p. However, because both μ and CV^{-2} linearly depend on N, increases in CV^{-2} that exceeds increases in μ (i.e. $CV^{-2} > \mu$) cannot be attributable to increase in N alone, and therefore, must additionally depend on increases in p. Therefore, by using the criterion of $CV^{-2} > \mu$, CV^{-2} analysis can still be used to detect changes in p; the criterion, however, is a conservative method for detecting increases in p, and is not generally satisfied following LTP induction (Kamiya et al., 1991; Malinow, 1991; Kullmann and Nicoll, 1992; Larkman et al., 1992; Manabe and Nicoll, 1994).

1.4.5 Single synapse recordings

Quantal parameters have also been assessed more directly, by recording electrical activity at single synapses. The first variant of this technique is minimal stimulation, in which the intensity of stimulation is adjusted until uniquantal, probabilistic responses are detected in the postsynaptic cell; under such conditions, it is assumed that stimulation is activating a small number of axonal fibres (ideally one) making synapse onto the recorded neuron (Raastad et al., 1992). A second variant involves recording from pairs of monosynaptically connected neurons. Using either technique, and assuming a bimodal distribution of synaptic responses (i.e. release is uniquantal and the number of stimulated synapses is one), the average amplitude of the evoked synaptic response reflects the quantal amplitude (q), whereas the proportion of stimulation trials that results in a synaptic response reflects the release probability (p) of the recorded synapse. Minimal stimulation was used first by Stevens and Wang (1994) to demonstrate that LTP at CA3-CA1 synapses was selectively associated with an increase in p (Stevens and Wang, 1994); though others later demonstrated that LTP was more commonly associated with an increase in q, especially at postsynaptically silent synapses in tissue from young animals (Isaac et al., 1995; Liao et al., 1995; Isaac et al., 1996; Isaac et al., 1998). Studies using paired recordings, in contrast, have revealed a predominantly presynaptic component of LTP expression (Malinow, 1991; Bolshakov and Siegelbaum, 1995).

Notably, both paired recordings and minimal stimulation techniques assume that a single synaptic site is being stimulated throughout the experiment (N=1), which is difficult to verify; changes in p or q after LTP induction can, therefore, be confounded by the unmasking of silent synapses, especially in tissue from young animals (Isaac et al., 1995;

Liao et al., 1995). Morevoer, owing to the low intensity of stimulation used with the minimal stimulation technique, axonal stimulation failures may constitute a realistic confound in measurements of p. In particular, McNaughton et al. (1994) demonstrated that in minimal stimulation experiments, increases in axonal excitability following repetitive axonal stimulation at room temperature, as often occurs in experiments during LTP induction, can be misinterpreted as artificial increases in p (McNaughton et al., 1994). It should be noted, however, that axonal failures are not a major confound with the higher intensity stimulation that is used to evoke synaptic responses in most LTP experiments (Bliss and Gardner-Medwin, 1973; Allen and Stevens, 1994).

1.4.6 Spontaneous and asynchronous release

Presynaptic terminals spontaneously release glutamate in the absence of action potentials. Spontaneous transmission is uniquantal and asynchronous across synapses, meaning that individual events recorded at the soma reflect glutamate release at single synapses. The frequency and amplitude of spontaneous release co-varies with pre- and post- synaptic efficacy, respectively, under various experimental manipulations, and have thus been used as proxy measures of p and q, respectively (Katz and Miledi, 1969; Eliot et al., 1994; Prange and Murphy, 1999); however, it is becoming increasingly clear that spontaneous and evoked glutamate release are distinct modes of synaptic transmission that can be independently regulated (Ramirez and Kavalali, 2011). Manabe et al. (1992) demonstrated that LTP induction in hippocampal slices by electrical stimulation of the Schaffer-collaterals resulted in an enhancement of the amplitude of spontaneous events, with no consistent change in the frequency of events; these findings were confirmed by a later study (Cormier and Kelly, 1996). These results would suggest that LTP is selectively expressed

postsynaptically. However, recordings of spontaneous release are derived from a large number of synapses, only a fraction of which will be potentiated by electrical stimulation. As such, recordings of spontaneous release may not provide a sufficiently sensitive assay to detect presynaptic changes at potentiated synapses following LTP induction. To circumvent this issue, Malgaroli and Tsien (1992) induced LTP at a large number of synapses in cultured neurons by using bath application of glutamate in Mg^{2+} free conditions in order to globally activate synaptic GluN receptors. Following LTP induction, the authors reported an increase in the frequency but not amplitude of spontaneous, release. Although their findings argue for a presynaptic component of LTP, chemically-induced LTP in dissociated cultures may be of little physiological relevance. Moreover, since LTP can be expressed by the unmasking of postsynaptically silent synapses, changes in the frequency of spontaneous release do not necessarily reflect changes in p (Isaac et al., 1995; Liao et al., 1995).

Glutamate release during evoked synaptic activity can be desynchronized by replacing a portion of extracellular Ca^{2+} with Sr^{2+}. As a consequence, uniquantal synaptic events can be resolved in the milliseconds following axonal stimulation; as with spontaneous release, the frequency and amplitude of these events are thought to reflect pre- and post- synaptic function, respectively (Zengel and Magleby, 1980; Bekkers and Clements, 1999). However, unlike spontaneous release, asynchronous release reflects activity at only stimulated synapses, and therefore, improves the ability to detect quantal changes at potentiated synapses after the induction of LTP under standard experimental conditions. Using this technique, Oliet et al. (1996) demonstrated increases in both the amplitude and frequency of uniquantal asynchronous events following LTP induction in hippocampal slices, suggesting that LTP was expressed at both the pre- and post- synaptic locus; however, as

with spontaneous release, an increased frequency of quantal events may result from unmasking of postsynaptically silent synapses (Oliet et al., 1996).

1.4.7 Use-dependent receptor blockade

Given the inconsistent findings from previous studies, several groups combined standard electrophysiological techniques with pharmacological tools in order to dissociate the pre- and post- synaptic components of LTP expression. One such technique utilized use-dependent glutamate receptor blockers.

In 1983, Rosenmund et al. (1983) and Hessler et al. (1983) used MK-801, a use-dependent, open-channel blocker of GluN receptors, to assess presynaptic function. Since MK-801 blockade requires GluN receptor activation, and therefore presynaptic glutamate release, the rate at which GluN receptor-mediated synaptic transmission decays in the presence of MK-801 can be used to qualitatively assess average transmitter release probability across a set of stimulated synapses (Hessler et al., 1993; Rosenmund et al., 1993). The technique was later used by Manabe and Nicoll (1994) to monitor presynaptic changes at CA3-CA1 synapses following the induction of LTP; though they reported no LTP-dependent changes in the rate of MK-801 blockade, their findings were not corroborated by a subsequent study using the same technique (Manabe and Nicoll, 1994; Kullmann et al., 1996).

A later study by Mainen et al. (1998) re-examined the locus of LTP using use-dependent blockers of GluA receptors. Two previous studies had reported that polyamine compounds acted as open-channel blockers of homomeric GluA1 receptors (Washburn and Dingledine, 1996; Bahring and Mayer, 1998). Using the polyamine-derivative HPP-SP, Mainen et al. (1998) reported that the rate of GluA receptor blockade was unchanged following LTP

induction at CA3-CA1 synapses. Though their finding supported a postsynaptic locus of LTP expression, their study required the use of GluA2 knockout mice, which, unlike wildtype animals, express a high proportion of GluA1 homomeric receptors at the synapse; such alterations of synaptic function may potentially affect the mechanisms of LTP expression.

1.4.8 Pharmacological manipulation of synaptic function

Pharmacological manipulations of either pre- or post- synaptic function have been used to probe the locus of LTP expression at CA3-CA1 synapses. The first example of this strategy was reported by Schulz (1997). In the study, Schulz demonstrated that LTP, which was previously saturated by delivering repeated trains of tetanic stimulation, could be induced by tetanus if transmitter release probability was first reduced by partial blockade of voltage-gated Ca^{2+} channels, using low concentrations of either ω-conotoxin or Cd^{2+}. LTP, in contrast, was not rescued when GluA receptor function was reduced using sub-saturating concentration of CNQX (Schulz, 1997). These findings provided evidence that LTP was supported by pre- rather than post- synaptic changes. A subsequent study, however, by Hjelmstad et al. (1997) demonstrated that saturation of transmitter release probability with the K^+ channel blocker 4-AP, failed to impair subsequent LTP induction. Moreover, the authors showed following saturation of LTP with repeated tetanic stimulation, enhancement of presynaptic function by 4-AP was no different than in control, untetanized conditions. Their findings suggest that LTP is mainly expressed postsynaptically (Hjelmstad et al., 1997).

1.4.9 Paired pulse ratio

Short-term plasticity, such as facilitation, augmentation, and post-tetanic potentiation, are thought to be mediated by transient increases in presynaptic function owing to residual intraboutonal Ca^{2+} following axonal stimulation (Katz and Miledi, 1968; Magleby and Zengel, 1975b, 1976c; Zengel and Magleby, 1982) [but see (Felmy et al., 2003; Vyleta and Jonas, 2014)]. These forms of plasticity are influenced by experimental manipulations of pre-, but not post-, synaptic function (Schulz et al., 1994), and as such, have been used to probe presynaptic changes following LTP induction. Presynaptic facilitation is commonly assayed for this purpose, and is assessed by delivering two presynaptic stimuli, spaced 50-200ms apart. Since intraboutonal Ca^{2+} levels after the second stimulus are higher than after the first, it is more likely to trigger glutamate release (Zengel et al., 1980). However, since release is generally uniquantal (Raastad et al., 1992; Isaacson and Walmsley, 1995), and because release probability cannot exceed 1, enhancement of presynaptic function should produce a greater fold change in the synaptic response triggered by the first of the two stimuli. The paired pulse ratio (PPR), as defined by ratio of the second postsynaptic response to the first postsynaptic response, should therefore be inversely affected by changes in release probability; in contrast, postsynaptic changes should influence both first and second response similarly and should, therefore, have no affect on PPR (Schulz et al., 1994).

McNaughton (1982) first measured PPR in order to examine changes in presynaptic function following LTP induction in dentate gyrus, and reported no change in the ratio. Results from subsequent studies, either examining LTP-induced changes in PPR in dentate gyrus or CA1 have been inconsistent (Anwyl et al., 1989; Muller and Lynch, 1989; Zalutsky

and Nicoll, 1990; Foster and McNaughton, 1991; Manabe et al., 1993; Schulz et al., 1994; Kleschevnikov et al., 1997; Schulz, 1997; Pananceau et al., 1998; Emptage et al., 2003; Volianskis and Jensen, 2003; Palmer et al., 2004; Johnstone and Raymond, 2013). Kleschevnikov et al. (1997) found that decreases in PPR accompanied LTP induced by strong, but not weak, tetanic stimulation, suggesting that inconsistencies across studies may result from differences in stimulation protocols; induction of presynaptic changes may simply require higher levels of synaptic activity than induction of postsynaptic changes.

1.4.10 Burst stimulation

Given inconsistent reports on PPR changes following LTP induction, several studies have used short bursts of stimulation (10-50Hz) instead of paired pulses to probe activity-dependent changes in presynaptic function. At synapses with high release probabilities, with each subsequent stimulus in the burst, glutamate release tends to decline due to depletion of the readily releasable pool of vesicles, whereas at synapses with low release probabilities, glutamate release tends to first increase due to an increase in intraboutonal Ca^{2+}, before then declining due to vesicle depletion (Pananceau et al., 1998; Dobrunz, 2002). As a consequence, the profile of synaptic responses can be used to gauge the average release probability of stimulated synapses. The technique was first employed by Markram and Tsodyks (1996) at neocortical synapses to demonstrate that LTP resulted in presynaptic enhancements, and that these enhancements resulted in redistribution, rather than a net increase, in glutamate released across a short train of stimulation. At CA3-CA1 synapses, however, LTP-induced changes in burst stimulation are not consistently reported across studies (Pananceau et al., 1998; Selig et al., 1999; Yasui et al., 2005; Volianskis et al., 2013).

1.4.11 Synaptic refractory period

Hjlemstad et al. (1997) developed an electrophysiological technique to probe presynaptic function based on an earlier observation by Stevens and Wang (1995) that synapses become refractory to stimulation for a few milliseconds following glutamate release (Stevens and Wang, 1995). As such, if two presynaptic stimuli are delivered at a sufficiently high frequency ($\geq$250Hz), a given synapse can only release glutamate at most once. Whereas synapses with high release probabilities will tend to release glutamate on the first pulse, synapses with low release probabilities will tend to release glutamate on the second pulse, owing to Ca^{2+}-dependent facilitation. The ratio of the synaptic response following the second stimulus to the response following the first stimulus will therefore be inversely related to the average release probability of the stimulated synapses. Following LTP induction, Hjlemstad et al. (1997) demonstrated no change in the ratio of responses at CA3-CA1 synapses, suggesting that LTP was supported exclusively by postsynaptic changes.

1.4.12 Glial transport currents

As an independent means of assessing presynaptic function, Luscher et al. (1998) and Diamond et al. (1998) used glial transport currents to assay evoked glutamate release in hippocampal slices. The technique took advantage of the fact that glial uptake of glutamate at the synaptic cleft is electrogenic, and can be detected via patch recordings of glial cells. These studies demonstrated that glial transport currents were unchanged following LTP induction at Schaffer-collateral synapses. Transport currents, however, were sensitive to enhancements in glutamate release following 1) paired pulse stimulation, 2) post-tetantic potentiation, and 3) chemical potentiation of mossy fibre synapses, all of which are thought

to be mediated by presynaptic changes. Kawamura et al. (2004) later used voltage-sensitive dyes to optically monitor glial transport currents and similarly reported that these currents were enhanced following the induction of LTP by tetanic stimulation at mossy fiber synapses, but not Schaffer-collateral synapses. Collectively, all three studies provide evidence against a presynaptic locus of LTP expression at CA3-CA1 synapses.

1.4.13 Antibodies

By the late 1990s, the locus of LTP expression, as assessed by electrophysiological techniques, remained inconclusive, and attention turned to the use of optical methods to more effectively assess pre- and post- synaptic function independently. The first of these methods, developed by Malgaroli et al. (1995), used fluorescently-tagged antibodies against the luminal domain of the vesicular protein synaptotagmin. Since the antibody only labels vesicles following their exocytosis, the fluorescence intensity at a presynaptic terminal should reflect the magnitude of transmitter release. Using this technique, Malgalori et al. (1995) reported that antibody uptake during periods of spontaneous glutamate release was enhanced following LTP induction. The study, however, had several limitations. 1) It relied on spontaneous glutamate release to indirectly probe presynaptic function and 2) was conducted in dissociated neuronal cultures, in which membrane bound proteins are readily accessible to antibody binding. 3) Moreover, LTP was induced by bath application of glutamate in Mg^{2+} free conditions. As such, it is difficult to extend the findings of this study to plasticity in evoked transmitter release that occurs under more physiologically-relevant conditions.

Although not specifically used in LTP studies, antibody labelling of presynaptic vesicles and postsynaptic receptors is still often used by several groups to assess both pre- and post-synaptic efficacy with single synapse resolution in dissociated cultures [e.g. (Park et al., 2004; Hou et al., 2008; Tokuoka and Goda, 2008)].

1.4.14 FM dyes

A more effective and more commonly used optical assay of presynaptic function was developed by Betz and Benwick (1992), and makes use of fluorescent styryl dyes, such as FM1-43, which readily intercalate with the plasma membrane. During bath application of FM1-43, vesicles generated by endocytosis become dye-labelled. Given that endocytosis follows activity-dependent vesicle fusion, electrical stimulation or elevation of extracellular [K^+] will load synaptic vesicles with dye; FM dye within the extracellular fluid or bound to the plasma membrane is then washed off leaving only intracellular vesicles labelled (Figure 4A). Either the fluorescence intensity of the bouton following FM loading, or the rate at which a subsequent round of electrical stimulation de-stains the bouton can be used as measure of presynaptic efficacy (Padamsey and Emptage, 2011).

In 1999, Ryan et al. used FM1-43 to examine the presynaptic component of LTP expression. They reported that dye loading was enhanced by tetanic stimulation, suggesting the probability of vesicle fusion was increased at imaged synapses. The experiment, however, owing to the levels of non-specific binding of FM dyes, was carried out in dissociated neuronal cultures, in which the low cellular density and the absence of a complex extracellular matrix facilitates washout of the dye. Other groups later successfully adapted the technique for use in acute slices by either 1) extracellular application of cyclodextrin

(ADAVSEP) to chelate unbound dye, or 2) extracellular application of sulforhodamine to quench fluorescence, or 3) the use of multi-photon microscopy to minimize background fluorescence (Kay and Laurent, 1999; Pyle et al., 1999; Stanton et al., 2001; Zakharenko et al., 2001, 2002; Stanton et al., 2003; Zakharenko et al., 2003; Stanton et al., 2005; Winterer et al., 2006; Ahmed and Siegelbaum, 2009). Given the inconsistencies in dye loading in hippocampal slices, however, the rate of FM de-staining in loaded boutons is used as a measure of presynaptic efficacy rather than the fluorescence intensity of boutons after dye loading (Figure 4B). In the study by Zakharneko et al. (2001,2003), the authors reported that 200Hz tetanic stimulation enhanced FM-destaining rates at Schaffer-collateral synapses, in contrast to 50Hz or 100Hz stimulation, suggesting that whether or not presynaptic changes accompanied LTP induction depended on the frequency of stimulation (Zakharenko et al., 2001; Zakharenko et al., 2003). Similar findings were confirmed by later studies (Stanton et al., 2005; Johnstone and Raymond, 2011, 2013). Stanton et al. (2005), in particular, used FM dyes in hippocampal slices to show that tetanus-induced enhancements in vesicle fusion were restricted to the readily releasable pool of vesicles. A more recent study in dissociated neuronal cultures, however, reported that LTP increased the total size of the recycling pool of vesicles (Ratnayaka et al., 2012). Although the reason for this discrepancy is not clear, FM dye studies provide compelling evidence that presynaptic changes can support LTP expression at Schaffer-collateral synapses, at least under some conditions. FM dyes have also been used to confirm presynaptic changes following LTD induction (Stanton et al., 2001; Zakharenko et al., 2002), and following LTP induction at perforant path-CA1 synapses (Ahmed and Siegelbaum, 2009).

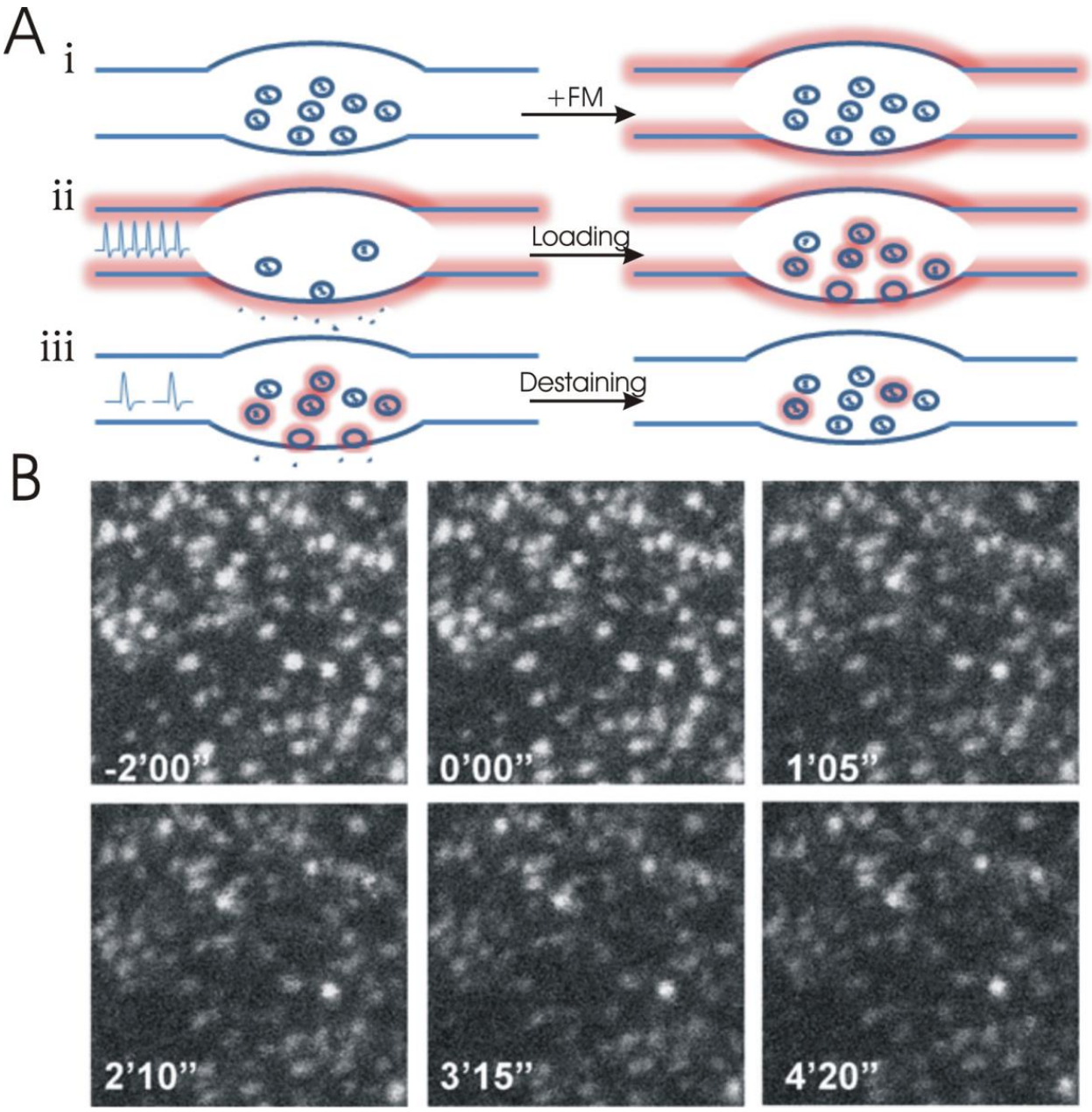


Figure 4. Imaging vesicle fusion with FM dyes. (**A**)(i) Bath applied FM dye intercalates with the plasma membrane. (ii) High frequency stimulation results in vesicular fusion followed by compensatory endocytosis, which generates FM-labelled synaptic vesicles. (iii) Following removal of bath applied FM-dye, a subsequent round of lower frequency stimulation results in vesicle fusion and FM de-staining. The rate or magnitude of fluorescence loss is used as a measure of presynaptic function at imaged boutons. (**B**) FM dye loaded boutons in the stratum radiatum of an acute hippocampal slice. At time 0, stimulation of the Schaffer-collaterals causes de-staining of labelled puncta. Image and caption taken from Padamsey and Emptage (2011). Figure 1B image originally reprinted from Neuron, 39 (6), Zakharenko, S.S., et al. (2003), with permission from Elsevier.

1.4.15 Ca^{2+} -sensitive dyes

Since the late 1990s, with improvements in the sensitivity of Ca^{2+}-indicator dyes and optical imaging, it has been possible to detect Ca^{2+} influx at individual spines following uniquantal glutamate release (Yuste and Denk, 1995; Denk et al., 1996). This Ca^{2+} influx results from the GluA receptor-mediated activation of local GluN receptors and voltage-gated Ca^{2+} channels (Emptage et al., 1999; Grunditz et al., 2008), and at a portion of synapses, Ca^{2+} influx is augmented by local Ca^{2+}-induced Ca^{2+} release from the endoplasmic reticulum (Emptage et al., 1999). Spine Ca^{2+} transients in response to axonal stimulation are stochastic, and are thought to reflect the probabilistic nature of glutamate release. As such, the proportion of Ca^{2+} events evoked by single presynaptic stimuli can be used as a measure of transmitter release probability (Pr) at the imaged synapse (Emptage et al., 2003) (Figure 5).

Using Ca^{2+} imaging, Emptage et al. (2003) and others have demonstrated that LTP induced by tetanic stimulation of the Schaffer-collaterals is accompanied by an increase in Pr at imaged synapses (Emptage et al., 2003; Ward et al., 2006; Enoki et al., 2009). Since Ca^{2+} transients depend on GluA receptor activation, Ward et al. (2006) additionally examined changes in Pr, specifically at postsynaptically silent synapses. At these sites, evoked Ca^{2+} transients are only observed in Mg^{2+}- free conditions, when GluN receptor-mediated Ca^{2+} influx can occur in the absence of GluA receptor activity. They reported that tetanic stimulation resulted in the insertion of GluA receptor at silent synapses without changing Pr; though a subsequent round of stimulation did reliably increase Pr (Ward et al., 2006). Their findings suggest that the locus of LTP expression, whether pre- or post- synaptic, depends on the state of the synapse, whether silent or active.

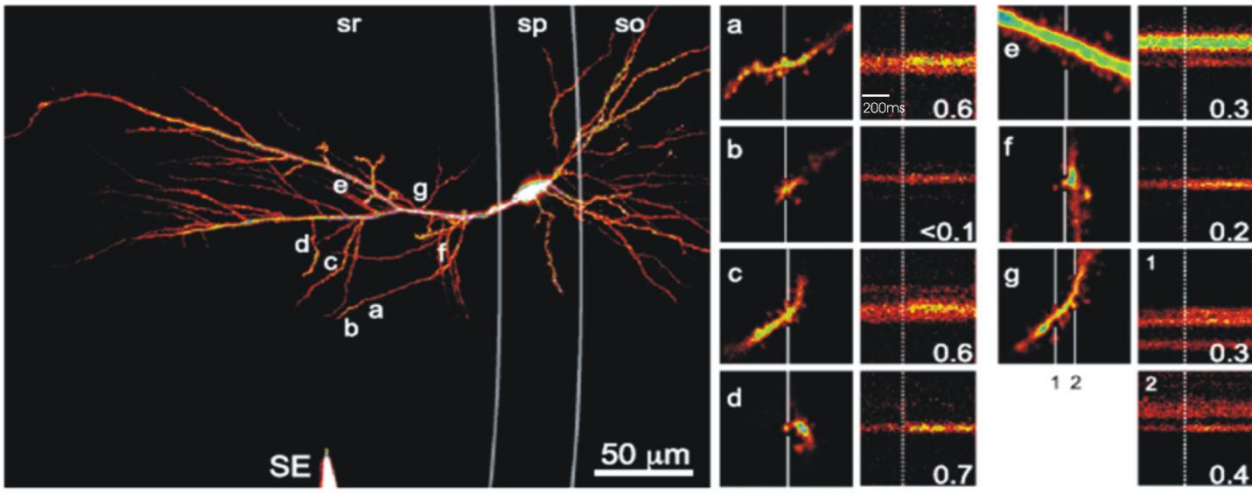


Figure 5. Optical quantal analysis using spine-Ca^{2+} transients. (**Left**) Activation of the axons within stratum radiatum (sr) with a stimulating electrode (SE) resulted in stochastic Ca^{2+} signalling in several spines (**a-g**) of a dye-filled CA1 neuron in an acute hippocampal slice. To enable rapid imaging of Ca^{2+} events over time, laser scanning was restricted to a line (white vertical line) through responsive spines. Line scans depicting successful transmitter release events are presented next to each imaged spine; the broken vertical line indicates the point of stimulation. The probability of detecting a calcium event is displayed under each line scan and is used as a measure of the probability of transmitter release at the associated bouton. Image and caption taken from Padamsey and Emptage (2011). Image originally taken from Enoki et al. (2009) with permission from Elsevier.

In addition to using the probabilistic nature of spine Ca^{2+} transients to measure Pr, Reid et al. (2001) demonstrated that the amplitude of spine Ca^{2+} transients at mossy fibre synapses could be used as a reporter for the GluA receptor activity in single spines. In a subsequent study, they then used spine Ca^{2+} transients to measure both pre- and post- synaptic efficacy, and demonstrated that LTP at mossy fibre synapses was expressed solely by presynaptic changes (Reid et al., 2001; Reid et al., 2004). At Schaffer-collateral synapses, however, owing to Ca^{2+}-induced Ca^{2+} release from internal stores, the amplitude of the evoked Ca^{2+} transient does not linearly correlate with GluA receptor activity and, therefore, cannot be used to assess postsynaptic function (Emptage et al., 1999; Emptage et al., 2003).

Recently, however, Enoki et al. (2009) simultaneously and independently assessed both pre- and post- synaptic components of LTP at Schaffer collateral synapses by combining Ca^{2+} imaging with electrophysiological recordings (Enoki et al., 2009). In one set of experiments the authors stimulated a single axon that made synaptic contact with the recorded neuron, and imaged the responsive spine; single-synapse stimulation was confirmed by obtaining a perfect correlation between the successes and failures of evoked Ca^{2+} transients imaged at the spine, and the successes and failures of synaptic potentials recorded at the soma. Under such conditions, the authors reported that LTP induction increased the probability of evoking a Ca^{2+} transient (and its associated synaptic potential) without altering the magnitude of the recorded synaptic potential. Their findings suggest that, at least at some synapses, LTP can be exclusively expressed presynaptically (Enoki et al., 2009). It is, however, important to recognise that Ca^{2+} imaging may incur a selection bias. The technique requires the experimenter to first search and identify a synapse that shows increases in Ca^{2+} fluorescence in response to afferent stimulation; because of this,

spines associated with larger Ca^{2+} transients are more likely to be selected for experimentation. It is therefore possible that imaged spines represent a specific subset of synapses that express LTP presynaptically.

Although, the detection of Ca^{2+} events within dendritic spines is generally used to calculate Pr at Schaffer-collateral synapses, McGuinness et al. (2010) recently demonstrated that a similar measure could be derived by examining the amplitude of evoked Ca^{2+} events within axonal boutons (McGuinness et al., 2010) (Figure 6). The study reported that single action potentials generate either low or high amplitude Ca^{2+} events within imaged boutons; whereas low amplitude events were shown to be dependent on Ca^{2+} influx from voltage-gated channels, high amplitude events additionally required the activation of presynaptic GluN receptors by glutamate. Given the dependency of high amplitude events on transmitter release, the authors used the probability of evoking such events as a measure of Pr, and demonstrated that Pr increased following LTP induction. Notably, the use of pre-, as opposed to post-, synaptic Ca^{2+} events for optical quantal analysis circumvents the observer bias involved in searching for stimulation-evoked Ca^{2+} responses in dendritic spines.

1.4.16 pHlourins

pHlourins provide an additional means of assessing presynaptic function. Originally developed by Meisenbock et al. (1998), pHlourins are a pH-sensitive variant of green fluorescent protein, that when fused to the luminal domain of a vesicular protein, act as genetically-encoded detectors for vesicular fusion (Miesenbock et al., 1998). Specifically, pHlourins are quenched when residing within the acidic lumen of the vesicle, but upon vesicle fusion, they fluoresce in the pH neutral environment of the extracellular fluid;

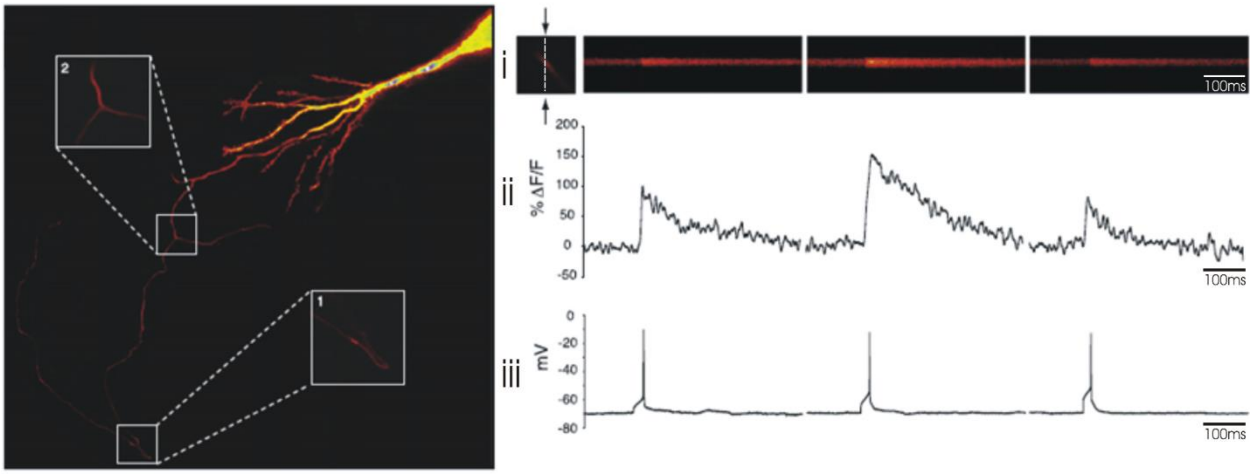


Figure 6. Optical quantal analysis using axonal Ca^{2+} transients. (**Left**) Axonal projection of CA3 hippocampal neuron loaded with a Ca^{2+-} sensitive dye. Laser scanning was restricted to a line through an axonal bouton in order to rapidly monitor fluorescence, in response to single action potentials, over time. (**Right**) (i) Three such line scans are presented, along with (ii) the quantified change in fluorescence (%ΔF/F) and (iii) the recorded membrane potential. Note that in the second linescan, the action potential generates a larger Ca^{2+} transient than in the first and third linescan. This high-amplitude Ca^{2+} transient reflects the additional activation of presynaptic GluN receptor following the release of glutamate, and can be used as a measure of the probability of transmitter release at the associated bouton. Image and caption taken from Padamsey and Emptage (2011). Image originally taken from McGuiness et al. (2010) with permission from Elsevier.

following vesicle endocytosis and re-acidification their fluorescence is then re-quenched (Figure 7). Though pHlourins are generally not used in plasticity studies, they were recently used by Bayazitov et al., (2007) to optically assess presynaptic function at multiple time points following the induction of LTP at CA3-CA1 synapses. The study reported that LTP induction was accompanied by an increase in presynaptic function, but that this increase gradually evolved over time.

pHlourins have also been used to assess postsynaptic function, particularly when fused to the extracellular domain of GluA receptor subunits (Padamsey and Emptage, 2011). Under such conditions, pHlourin fluorescence selectively reflects the fraction of cellular GluA receptors that are expressed on the pH-neutral surface of the cell, and not the fraction of GluA receptors residing within intracellular acidic stores. Using pHlourin-GluA receptor fusion proteins, several groups have demonstrated increases in surface GluA receptor expression at dendritic spines following LTP induction, either by chemical activation of GluN receptor, standard tetanic stimulation, glutamate photolysis at single spine, or following in vivo plasticity paradigms (Kopec et al., 2006; Kopec et al., 2007; Makino and Malinow, 2009; Petrini et al., 2009; Patterson et al., 2010; Makino and Malinow, 2011). These studies corroborate the seminal work by Shi et al., (1999) who first used GFP-tagged GluA receptors to demonstrate postsynaptic enhancements following tetanic stimulation.

1.4.17 The locus of LTP expression - summary

The locus of LTP expression at Schaffer-collateral synapses has been debated for decades. Quantal analyses at these synapses using standard electrophysiological techniques have produced inconsistent results, whereas studies using optical imaging consistently provide

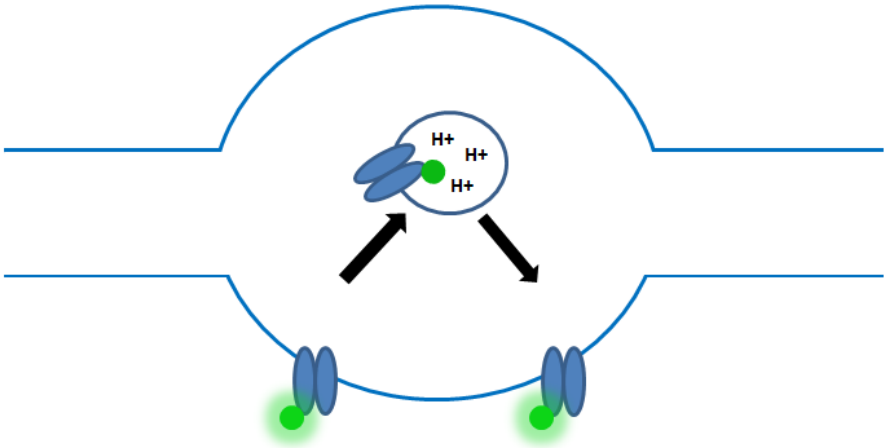


Figure 7. Imaging vesicular fusion with pHlourins. pHlourins are generally fused to the luminal domain of vesicular-bound proteins. pHlourin fluorescence, which is quenched in the acidic domain of the vesicle, is detected upon vesicular fusion following exposure of the luminal face of the vesicle to the pH-neutral extracellular fluid. Fluorescence is then re-quenched following vesicular endocytosis and re-acidification. Image taken from Padamsey and Emptage (2011).

evidence for both a pre- and post- synaptic locus of expression (Padamsey and Emptage, 2011). Nonetheless, presynaptic enhancements at Schaffer-collateral synapses are not reliably reported across laboratories (Luscher and Malenka, 2012; Bliss and Collingridge, 2013). In contrast, LTP at mossy fiber synapses is widely agreed to be expressed presynaptically. However, that the induction of LTP at mossy fiber synapses, in contrast to Schaffer-collateral synapses, is independent of GluN receptors casts further doubt as to the role of presynaptic changes in the expression of GluN receptor-dependent LTP (Luscher and Malenka, 2012; Bliss and Collingridge, 2013).

1.5 Linking GluN receptor activation with long-term postsynaptic changes

Although several lines of evidence suggested that Ca^{2+} influx from the GluN receptor was necessary and sufficient for LTP and LTD induction (Lynch et al., 1983; Malenka et al., 1988; Yang et al., 1999), how a transient increase in spine Ca^{2+} led to long-lasting changes in synaptic efficacy was unknown. In the late 1980s, the finding that LTP required both the activation of protein kinases and the Ca^{2+}-sensitive protein calmodulin, raised the possibility that the stable alteration in protein phosphorylation states could convert the transient nature of GluN receptor activation to longer-lasting changes in synaptic efficacy. In the years to follow, the role of several kinases, phosphatases, and related downstream targets were examined in the induction and expression of LTP and LTD. Then, as evidence accumulated through the late 1980s and 1990s supporting the postsynaptic expression of plasticity, an increasing number of studies began to study the role of kinases and phosphatases, specifically in the modulation of synaptic GluA receptor number and/or conductance.

1.5.1 Ca^{2+}/calmodulin dependent kinase II (CAMKII)

In 1985, Lisman proposed that kinases and phosphatases could interact to support a binary molecular switch of plasticity, in which long-lasting synaptic changes were generated and sustained by kinase autophosphorylation (Lisman, 1985). In the following year, Miller and Kennedy (1986) reported that, CAMKII, the most abundant protein kinase in the brain, exhibited the property of Ca^{2+}-dependent autophosphorylation in a manner dependent on calmodulin (Miller and Kennedy, 1986). Given the high levels of CAMKII expression at the synapse (Kelly et al., 1984), the enzyme became a candidate protein kinase for translating short-lived GluN receptor-mediated Ca^{2+} transients into long-lasting molecular changes (Lisman and Goldring, 1988a; Lisman and Goldring, 1988b). In the years following, CAMKII activation was shown to be both necessary and sufficient for the induction for LTP (Malinow et al., 1988; Malenka et al., 1989; Malinow et al., 1989; McGlade-McCulloh et al., 1993; Lledo et al., 1995; Otmakhov et al., 1997; Otmakhov et al., 2004; Zhang et al., 2008). Moreover, CAMKII phosphorylation accompanied LTP and learning *in vivo* (Fukunaga et al., 1993; Giese et al., 1998; Glazewski et al., 2000), and CAMKII mutations that prevented autophosphorylation impaired learning and memory in rodents (Silva et al., 1992a; Silva et al., 1992b; Cho et al., 1998).

Since the early 1990s, protein kinases were suspected to alter synaptic efficacy by interacting directly with GluA receptors, principally because several studies demonstrated that intracellular perfusion of active protein kinases in hippocampal pyramidal neurons could potentiate GluA receptor function (Greengard et al., 1991; McGlade-McCulloh et al., 1993; Wang et al., 1994). Soon after, specific kinase phosphorylation sites were identified on the C-terminal tail of the GluA1 receptor subunit (Roche et al., 1996). In 1997, Barria et

al. demonstrated that the CAMKII phosphorylated the GluA1 receptor subunit following LTP induction (Barria et al., 1997a; Barria et al., 1997b). This phosphorylation was shown to augment single channel conductances (Derkach et al., 1999) and to promote LTP-induced increases in GluA receptor number at the synapse by mediating interactions between the GluA1 receptor subunit and the PDZ domains of postsynaptic density proteins (Hayashi et al., 2000). CAMKII has more recently been shown to increase the capture of synaptic GluA receptors indirectly, by triggering the exocytosis of intracellular GluA receptors to extrasynaptic sites via activating Ras-ERK (Harvey et al., 2008; Patterson et al., 2010), and also by promoting the structural enlargement of the spine head via activation of RhoA and Cdc42, which remodel the actin cytoskeleton (Murakoshi et al., 2011).

1.5.2 Protein Kinase A (PKA)

The necessity of calmodulin in the induction of LTP also prompted an examination of the role of another of its downstream targets, adenylate cyclase, in synaptic plasticity. In particular, Chetkovich et al. (1991,1993) reported GluN receptor-dependent increases in cyclic adenosine monophosphate (cAMP) following tetanic stimulation, which required the activation of adenylate cyclase by calmodulin. This increase likely played a role in LTP induction as artificially elevating cAMP signalling via the bath application of a membrane-permeant cAMP analogue could alone induce LTP (Frey et al., 1993). Subsequent studies demonstrated: 1) that the downstream target kinase of cAMP, PKA, was upregulated following tetanic stimulation (Roberson and Sweatt, 1996), 2) that intracellular perfusion of PKA in hippocampal neurons was sufficient to potentiate synaptic responses (Greengard et al., 1991), and 3) that PKA inhibition reduced the initial magnitude of LTP and prevented the longer-term maintenance of LTP (Frey et al., 1993; Huang and Kandel, 1994;

Otmakhova et al., 2000). Collectively these findings implicated the cAMP-PKA pathway in the induction of LTP.

Several groups have since demonstrated that PKA-mediated phosphorylation of the GluA1 subunit is essential for synaptic GluA receptor insertion following LTP induction. In the case of naïve synapses, Esteban et al., (2003) demonstrated that phosphorylation of both the GluA1 serine 845 and 831 residues by PKA and CAMKII, respectively, is required for the full expression of LTP. In contrast, at previously depressed synapses, Lee et al. (2010) demonstrated that PKA phosphorylation alone was necessary for LTP induction. PKA activation has also been reported to prime LTP induction, by promoting surface trafficking of GluA1-containing receptors (Oh et al., 2006; Man et al., 2007), and by stabilizing the surface expression of perisynaptic GluA1-containing receptors (He et al., 2009).

1.5.3 Protein Kinase C (PKC)

In 1985, the Routtenberg laboratory examined alterations in the phosphorylation state of proteins after LTP induction. They identified protein F1, which showed long-term, GluN receptor-dependent enhancements in phosphorylation, the level of which correlated with the magnitude of synaptic potentiation (Akers and Routtenberg, 1985; Nelson and Routtenberg, 1985; Routtenberg, 1985b, a; Routtenberg and Lovinger, 1985; Linden et al., 1988). F1 phosphorylation required activation of PKC, which was dependent both on Ca^{2+} influx and the presence of phospholipid (Akers and Routtenberg, 1985; Lovinger et al., 1985). PKC activation and translocation were then shown to be associated with LTP induction *in vitro* (Akers et al., 1986) and learning *in vivo* (Sheu et al. 1993). Whereas exogenous activation of PKC by phorbol esters appeared to potentiate synaptic responses

(Malenka et al., 1986; Colley et al., 1989) inhibition of PKC prevented LTP induction (Lovinger et al., 1987; Malinow et al., 1988; Malinow et al., 1989; Wang and Feng, 1992). Although these findings suggest that PKC is necessary and sufficient for LTP induction, several studies have demonstrated that phorbol ester-induced potentiation does not actually occlude subsequent induction of LTP by tetanic stimulation (Muller et al., 1988b), and that PKC inhibitors appear to prevent LTP by reducing GluN receptor currents (Muller et al., 1990; Lopez-Molina et al., 1993). As such, whether PKC is involved in the early induction of LTP is not clear; however, there appears to be more compelling evidence in support for a role of PKC in the late-phase expression of LTP (Colley et al., 1990).

With regards to GluA receptor trafficking, PKC has been shown to promote synaptic incorporation of the GluA receptor1 subunit, potentially by phosphorylating the serine 818 residue (Boehm et al., 2006; Yang et al., 2010). Though its involvement in LTP has been controversial (Muller et al., 1988b), the importance of PKC-mediated phosphorylation of GluA receptors in plasticity may depend on the pattern of synaptic stimulation (Muller et al., 1990; Lopez-Molina et al., 1993; Yang et al., 2008b).

1.5.4 Other kinases

It should be mentioned that, although much research has focused on CAMKII, PKA, and PKC, a number of additional kinases have also been implicated in LTP induction, including: extracellular signal-regulated kinase (ERK) (Harvey et al., 2008; Patterson et al., 2010), mitogen-activated protein kinase (MAPK) (English and Sweatt, 1997), Rho-associated protein kinase (RhoK) (Murakoshi et al., 2011), tyrosine kinase Fyn (O'Dell et al., 1991a;

Grant et al., 1992; Prieto et al., 2007), and p21-activated kinase 1 (PAK1) (Asrar et al., 2009).

1.5.5 Protein phosphatase 1 and 2A (PP1/PP2A)

The necessity of kinases in LTP induction would suggest a necessary role for their counterpart phosphatases in synaptic plasticity. In 1994, Mulkey et al. demonstrated that LTD induction by prolonged low-frequency stimulation required the activation of either serine protein phosphatase 1 (PP1) or 2A (PP2A), in a manner that depended on GluN receptor activation and Ca^{2+} influx. Activation of PP1 was mediated by calcineurin, a calmodulin-dependent phosphatase, which exerted its effects by inactivating inhibitor-1, a negative regulator of PP1 activity (Mulkey et al., 1994). Phosphatase activity was also shown to be necessary for the reversal of LTP induction by low frequency stimulation (O'Dell and Kandel, 1994). However, whereas the reversal of LTP required PP1/2A-mediated deposphorylation of the GluA1 serine 831, the target residue for CAMKII phosphorylation, LTD additionally required dephosphorylation of the GluA1 serine 845 residue, the target residue for PKA phosphorylation (Lee et al., 1998; Lee et al., 2000). PP1/2A-mediated dephosphorylation of GluA receptor subunits is currently thought to mediate LTD by promoting the lateral diffusion and subsequent endocytosis of synaptic GluA receptors (Blitzer et al., 1998; Beattie et al., 2000; Hayashi et al., 2000; Lin et al., 2000; Esteban et al., 2003; He et al., 2009; Yang et al., 2010)

In addition to their actions on GluA receptor subunits, protein phosphatases and protein kinases appear to negatively interact. PKA phosphorylation activates inhibitor-1, which negatively regulates PP1 activity (Blitzer et al., 1998). PP1 and PP2a can also inactivate

CAMKII by de-phosphorylation (Shields et al., 1985; Allen et al., 1997; Strack et al., 1997; Chen et al., 2001b). Such negative interactions likely impose a bistable switch of plasticity, in agreement with theoretical and computational models of synaptic plasticity (Lisman, 1985; Lisman and Goldring, 1988b; Castellani et al., 2001; Shouval et al., 2002).

1.6 Current model of GluN receptor-dependent postsynaptic plasticity

The current model of postsynaptic plasticity depends on both kinases and phosphatases to bidirectionally control synaptic GluA receptor trafficking, with each class of protein being differentially sensitive to GluN receptor-mediated Ca^{2+} influx (Lisman, 2001; Shouval et al., 2002) (Figure 8). In general, brief and large levels of GluN receptor-mediated Ca^{2+} influx, which occurs when glutamate release coincides with strong postsynaptic depolarization, is thought to preferentially induce LTP by activating Ca^{2+}- and/or calmodulin-sensitive kinases (Lisman, 2001; Shouval et al., 2002). These kinases then, through protein phosphorylation, promote the exocytosis of GluA1-containing receptors to the extrasynaptic membrane as well as the subsequent capture of these receptors at the synapse (Ehlers et al., 2007; Heine et al., 2008; Yang et al., 2008b; He et al., 2009; Makino and Malinow, 2009; Opazo et al., 2010; Yang et al., 2010). In contrast, moderate and prolonged levels of GluN receptor-mediated Ca^{2+} influx, which results when glutamate release occurs in the absence of strong depolarization, is thought to preferentially induce LTD by activating Ca^{2+}- and/or calmodulin-sensitive phosphatases (Lisman, 2001; Shouval et al., 2002). These phosphatases, through protein de-phosphorylation, promote the loss of GluA receptors from the synapse into the extrasynaptic membrane as well as their subsequent endocytosis (Tardin et al., 2003; Yang et al., 2008a; Yang et al., 2010). The

inhibitory interactions between kinases and phosphatases ensure that synaptic strength transitions between discrete states of potentiation and depression (Lisman, 1985; Lisman and Goldring, 1988b; Castellani et al., 2001; Shouval et al., 2002).

1.7 Linking GluN receptor activation with long-term presynaptic changes: a role for retrograde signalling

In contrast to the strong mechanistic understanding of GluN receptor signalling in postsynaptic plasticity, the link between GluN receptor activation and presynaptic plasticity is not well-established and is far more controversial. Early studies on plasticity suggested that presynaptic changes might accompany LTP induction. However, since LTP was generally accepted to require postsynaptic GluN receptor signalling, Bliss et al. (1986) first suggested that the presynaptic expression of LTP would require a retrograde signal, which would be released postsynaptically in response to GluN receptor-dependent Ca^{2+} influx.

Candidate retrograde messengers are thought to require several properties to mediate retrograde signalling during LTP induction. The messenger must be: 1) postsynaptic in origin 2) synthesized/released in response to GluN receptor-mediated Ca^{2+} influx, 3) capable of enhancing transmitter release at the presynaptic terminal, 4) selective, only influencing active presynaptic terminals, and 5) short-lived. Experimentally, inhibition of retrograde signalling should impair LTP, particularly the component of LTP that is expressed presynaptically, whereas exogenous application of the retrograde signal should induce LTP with a presynaptic component of expression, in a manner independent of GluN receptors or postsynaptic activity.

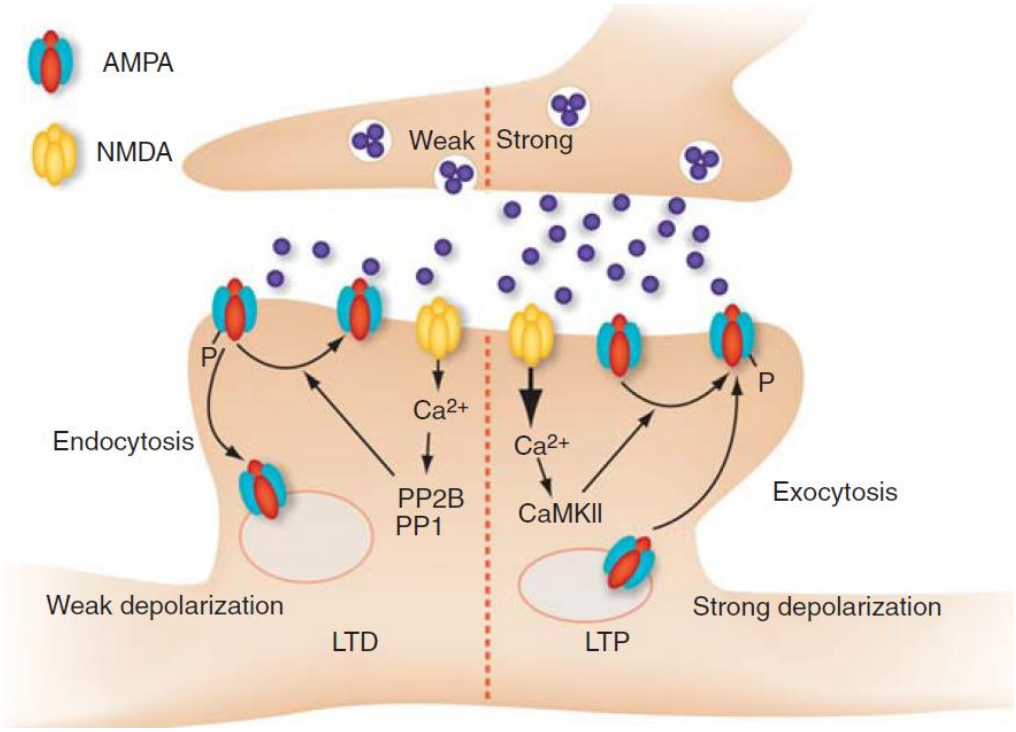


Figure 8. Current model of postsynaptic plasticity. LTP is induced when glutamate release coincides with strong postsynaptic depolarization, resulting in high levels of Ca^{2+} influx from GluN receptors, which preferentially activate Ca^{2+}-sensitive kinases, such as CAMKII. These kinases trigger the exocytosis and subsequent synaptic capture of AMPARs. In contrast, LTD is induced when glutamate release coincides with only modest levels of postsynaptic depolarization, resulting in low levels of Ca^{2+} influx from GluN receptors, which preferentially activate Ca^{2+}-sensitive phosphatases, such as PP1 and PP2B. These phosphatases trigger the lateral dispersion and subsequent endocytosis of AMPARs. Image taken from Luscher and Malenka (2012).

1.7.1 Arachidonic acid

In 1987, Piomelli et al. identified arachidonic acid and its eicasanoid metabolites as the mediators of presynaptic inhibition of Aplysia sensory neurons induced by the neuropeptide FMRFamide. Owing to its diffusible nature, the authors suggested that arachidonic acid, if released at CA3-CA1 synapses of the mammalian hippocampus, could act as a retrograde messenger to influence presynaptic function following LTP induction (Piomelli et al., 1987). Arachidonic acid was later shown to be released from striatal and cerebellar neurons following the activation of Ca^{2+}-dependent phospholipase A2 by GluN receptor-mediated Ca^{2+} influx (Dumuis et al., 1988; Lazarewicz et al., 1988); a similar mechanism was also found in hippocampal neurons (Sanfeliu et al., 1990). In 1989, Williams et al. first proposed that arachidonic acid or its lipoxygenase metabolites as a possible retrograde messenger necessary for the presynaptic expression of LTP (Williams et al., 1989). They demonstrated that arachidonic acid alone failed to potentiate synaptic responses in the dentate gyrus, but when coupled with weak tetanic stimulation, generated a slow onset potentiation that was accompanied by an increase in glutamate release and occluded subsequent LTP induction by tetanic stimulation. Bath application of arachidonic acid was later shown to enhance glutamate release at Schaffer-collateral synapses (Drapeau et al., 1990) and in synaptosome preparations (Lynch and Voss, 1990). Other studies confirmed that LTP induction resulted in the activation of phospholipase A2 (Clements et al., 1991), and induced a measurable release of arachidonic acid in hippocampal slices (Lynch et al., 1989); inhibition of arachidonic acid production by the phospholipase A2/ lipoxygenase inhibitor, nordihydroguaiaretic acid, prevented both the induction of LTP at perforant path (Lynch et al., 1989) and Schaffer-collateral synapses (Williams and Bliss,

1989). Thus, arachidonic acid appeared to have the desired properties of a retrograde signal: its synthesis was postsynaptic and dependent on GluN receptor-Ca^{2+} influx, and it appeared to act at the presynaptic terminal to increase glutamate release.

Nonetheless, later studies questioned the role of arachidonic acid as a retrograde signal, owing to the supraphysiological concentrations (50-200μM) required to exert synaptic potentiation, and the slow onset of its effects. Moreover, arachidonic acid was found to inhibit glial glutamate uptake (Barbour et al., 1989) and to augment postsynaptic GluN receptor currents (Miller et al., 1992); both effects would augment GluN receptor activation during tetanic stimulation, and increase the likelihood of inducing LTP. O'Dell and Kandel (1991) and Kato et al. (1991) later demonstrated that facilitatory effects of arachidonic on LTP could be blocked by the GluN receptor-inhibitor AP5 (Kato et al., 1991; O'Dell et al., 1991b), thereby providing compelling evidence that arachidonic acid was exerting its effects through post-, rather than pre-, synaptic mechanisms of action.

1.7.2 Platelet-activating factor

Activation of phospholipase A2 was also known to drive the synthesis of platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), receptors for which were shown to be expressed at the synapse (Marcheselli et al., 1990). In the early 1990s, PAF receptor (PAF-R) antagonists were reported to inhibit the induction of LTP in CA1 and dentate gyrus (del Cerro et al., 1990; Arai and Lynch, 1992; Wieraszko et al., 1993; Kato et al., 1994; Kato and Zorumski, 1996). In addition, exogenous application of a nonhydrolyzable PAF analogue induced long-lasting potentiation of synaptic responses, which occluded LTP generated by electrical stimulation (Clark et al., 1992; Kato and

Zorumski, 1996). A subsequent study reported similar synaptic enhancements with bath application of PAF; though these enhancements were mediated by GluN receptor activation (Wieraszko et al., 1993), Kato et al. (2004) showed that postsynaptic intracellular application of a nonhydrolyzable PAF analogue (methylcarbamyl-PAF), when paired with weak tetanic stimulation, generated long-lasting synaptic enhancements that were independent of GluN receptor activation. This enhancement was accompanied by increases in the frequency but not amplitude of spontaneous events, suggesting that PAF acted at the presynaptic locus to generate LTP (Clark et al., 1992; Kato et al., 1994; Kato and Zorumski, 1996). Consistent with the potentiating effects of PAF, Teather et al. (1998) reported that spatial memory in rats was augmented by intrahippocampal infusions of methylcarbamyl-PAF, but impaired by infusions of PAF-R antagonists. Koboyashi et al. (1999), however, failed to show an effect of PAF on synaptic responses or of PAF-R antagonists on LTP induction in CA1, and further demonstrated that Schaffer-collateral LTP in PAF-receptor knockout mice did not differ from wild types (Kobayashi et al., 1999); PAF-R knockout mice did, however, show deficits in LTP induction in the lateral perforant pathway (Chen et al., 2001a). Although differences in experimental conditions might account for these discrepancies, results from Kobayashi et al. (1999) casts doubt over the role of PAF as a necessary retrograde signal at hippocampal synapses.

1.7.3 Nitric oxide

In the 1980s, it was shown that endothelial cells, in response to vasodilators such as acetylcholine, released a diffusible factor that generated smooth muscle relaxation via a cyclic guanosine monophosphate (cGMP)-dependent mechanism (Furchgott and Zawadzki, 1980; Fiscus et al., 1983; Rapoport et al., 1983; Rapoport and Murad, 1983). The diffusible

factor was initially termed endothelial-derived relaxing factor, and was later identified as nitric oxide (NO) (Ignarro et al., 1987; Palmer et al., 1987). In 1988, Garthwaite et al. explored whether NO-signalling similarly mediated the glutamate-induced increase in cGMP levels previously reported in the cerebellum (Drummond, 1983). They reported that, in response to glutamate, cerebellar neurons did in fact release NO in a GluN receptor-dependent manner, and that the released NO increased cGMP via activation of guanylate cyclase. Moreover, extracellular chelation of NO by haemoglobin blocked cGMP accumulation, suggesting that NO was acting as a transsynaptic signal (Garthwaite et al., 1988). As such, in the 1990s when there was a strong interest in identifying a GluN receptor-dependent retrograde signal in LTP, nitric oxide appeared to be a promising candidate.

In 1991, several groups demonstrated that that inhibition of NO signalling impaired the induction of LTP (Bohme et al., 1991; O'Dell et al., 1991b; Schuman and Madison, 1991). Schuman and Madison (1991) additionally reported that LTP impairments could be achieved by bath application of the NO scavenger, haemoglobin, suggesting that NO-dependent synaptic potentiation required trans-synaptic signalling. Moreover, Bohme et al. (1991) demonstrated that bath application of the NO donor generated long-lasting potentiation of synaptic responses; other groups similarly reported similar results following exogenous application of NO, either by dissolving NO gas in aqueous form, using an NO donor, or photoreleasing caged NO (O'Dell et al., 1991b; Bon et al., 1992; Zhuo et al., 1993; Zhuo et al., 1994; Arancio et al., 1996; Malen and Chapman, 1997; Zhuo et al., 1998; Nikonenko et al., 2003). Notably, Zhuo et al. (1993,1994) demonstrated that exogenous NO had no effect on synaptic responses unless paired with a weak tetanus, which alone failed to

generate LTP; this NO-induced potentiation was not inhibited by the GluN receptor-antagonist AP5. Their findings suggested that the potentiating effects of NO were restricted to active synapses; such a mechanism would prevent the inherently diffuse nature of NO signalling from violating the site-specificity of LTP(Zhuo et al., 1993; Zhuo et al., 1994)(Zhuo et al., 1993; Zhuo et al., 1994).

In subsequent years, NO synthesis was shown to be activity-dependent (von Bohlen und Halbach et al., 2002), and both neuronal and endothelial variants of nitric oxide synthase (NOS) were found to be expressed postsynaptically in CA1 pyramidal neurons (Dinerman et al., 1994; Wendland et al., 1994). NOS was found to be co-localized with postsynaptic density protein 95 (PSD-95) (Brenman et al., 1996b; Brenman et al., 1996a; Burette et al., 2002), and functionally coupled with GluN receptor activation (Sattler et al., 1999). LTP induction was impaired at Schaffer-collateral synapses following pharmacological inhibition of NOS *in vivo* (Doyle et al., 1996), and also following genetic deletion of NOS, with maximal impairments requiring deletion of both endothelial and neuronal variants (O'Dell et al., 1994; Son et al., 1996; Wilson et al., 1999),

Then, in 1996, Arancio et al. provided compelling evidence that retrograde NO signalling had a presynaptic locus of effect (Arancio et al., 1996). They first demonstrated that LTP was impaired by: 1) extracellular NO scavengers, 2) pre- or post- synaptic injection of NO scavengers or 3) intracellular application of NO synthase inhibitors in the postsynaptic, but not presynaptic, neuron. Moreover, they demonstrated that photolytic release of caged NO in the postsynaptic neuron elicited LTP, which could be blocked by extracellular NO scavengers. Photorelease of NO in the presynaptic neuron similarly elicited LTP, even in

the presence of AP5, suggesting that the actions of NO, unlike that of arachidonic acid, was independent of postsynaptic signalling. Collectively, their findings suggested that the trans-synaptic diffusion of NO from the postsynaptic neuron to the active terminals of the presynaptic neuron was necessary and sufficient for LTP induction.

The findings of Arancio et al. (1996) suggest that NO acts at the presynaptic terminal. In more recent years, studies have extended these findings by showing that NO is necessary and sufficient for the enhancement of presynaptic strength. The first of these studies, by Nikonenko et al. (2003), demonstrated that tetanic stimulation could induce structural changes within the axon, including the restructuring of presynaptic terminals and the emergence of new axonal boutons. These changes were prevented by NO inhibitors and elicited by NO donors. In 2005, Stanton et al. (2005) then demonstrated that the presynaptic expression of LTP, as assessed with FM dyes, depended on NO signalling; similar findings were reported by Johnstone and Raymond (2011), and Ratnayaka et al. (2012) who used FM dyes and paired pulse ratio to monitor presynaptic enhancements. NO has also been shown to be necessary for some forms of presynaptic LTD (Stanton et al., 2003).

Despite this evidence, NO remains a controversial retrograde signal, owing to inconsistences in laboratory results. In particular, not all studies report LTP impairments following the inhibition of NO signalling. Several reasons have been proposed for these inconsistencies: Williams et al. (1993) reported that the effects of NO on LTP were only apparent at room temperature and in young animals, and Gribkoff & Lum-Ragan (1992) suggested that only LTP produced by strong, but not weak, tetanic stimulation is sensitive

to NO inhibition; these findings, however, are not consistent as some studies, which report the induction of NO-sensitive LTP, do so at near-physiological temperatures and using both weak and strong tetanic stimulation (Williams et al., 1993; Padamsey and Emptage, 2013). Moreover, not all labs report synaptic potentiation following exogenous application of NO. Some studies report transient presynaptic depression during donor application (Boulton et al., 1994; Murphy et al., 1994), while others report that NO photorelease generates long-term depression in GluN receptor-mediated synaptic currents (Murphy and Bliss, 1999), which serves to inhibit subsequent induction of LTP. The reason for these inconsistencies is unclear, though likely results from differences in experimental conditions across studies (Garthwaite and Boulton, 1995).

1.7.4 Carbon monoxide

Carbon monoxide (CO) has similar signalling actions as nitric oxide and, as such, has been examined as a candidate retrograde messenger in the hippocampus. Stevens and Wang (1993) and Zhuo et al. (1993) demonstrated that inhibition of heme oxygenase-2, which catalyzes the synthesis of CO, using either zinc protophyrin IX (ZPP) or zinc deuteroporphyrin IX 2,-4 bisglycol, prevented the induction of LTP, despite having no effect on basal synaptic responses or on the induction of LTD. Zhuo et al. (1993) additionally demonstrated that CO application, which alone had no effect on synaptic transmission, produced LTP when paired with presynaptic stimulation, suggesting CO-induced potentiation was restricted to active synapses. Poss et al. (1995), however, later demonstrated that LTP induction was unaltered in mice lacking a major brain isoform of heme oxygenase; nonetheless, the heme oxygenase inhibitor ZPP still suppressed LTP in both mutant and wild type mice, suggesting that inhibition of LTP may have resulted from a

non-specific effect of the drug (Poss et al., 1995). Consistent with this notion, Meffert and Schumann demonstrated that ZPP additionally inhibited NO synthase, and therefore was likely inhibiting LTP by interfering with NO, rather than with CO, signalling (Meffert et al., 1994).

1.7.5 Brain-derived neurotrophic factor (BDNF)

Since their discovery at the neuromuscular junction, neurotrophic factors have been recognized as potent transsynaptic signals, capable of influencing synaptic development, function, and plasticity. Within the forebrain, the effects of brain-derived neurotrophic factor (BDNF) have been particularly well characterized. At hippocampal synapses in particular, exogenous BDNF application was shown to increase transmitter release probability, likely by kinase-dependent increases in the number of docked vesicles, though also influenced postsynaptic excitability and GluN receptor function (Kang and Schuman, 1995; Gottschalk et al., 1998; Pozzo-Miller et al., 1999; Schinder et al., 2000; Xu et al., 2000; Tyler and Pozzo-Miller, 2001; Lu, 2003). Although several studies reported that BDNF-induced enhancements were transient (Figurov et al., 1996; Jakawich et al., 2010), Kang et al. (1995) demonstrated that longer-term application of higher concentrations of BDNF could generate long-lasting synaptic potentiation (Kang and Schuman, 1995). This potentiation occurred even in the presence of GluN receptor antagonists, and was likely presynaptic, as it was associated with a decrease in paired pulse ratio. Subsequent studies demonstrated that the release of BDNF was activity-dependent (Goodman et al., 1996), and that the inhibition of BDNF signalling could reduce the magnitude of LTP (Korte et al., 1995; Patterson et al., 1996; Kang et al., 1997; Korte et al., 1998; Chen et al., 1999). Several lines of evidence, however, have suggested against BDNF as a retrograde messenger for LTP.

Firstly, BNDF can be released by neurons in an GluN receptor-independent manner (Goodman et al., 1996; Hartmann et al., 2001; Balkowiec and Katz, 2002). Secondly, inhibition of BDNF reduces basal synaptic transmission, suggesting that impaired LTP may simply result from decreased synaptic activity during tetanic stimulation (Figurov et al., 1996; Patterson et al., 1996). Thirdly, BDNF-induced potentiation neither occludes nor is occluded by the induction of LTP, suggesting that it mechanistically differs from standard Hebbian plasticity (Kang and Schuman, 1995). Finally, both the axon and dendrite are capable of releasing BDNF (Goodman et al., 1996; Hartmann et al., 2001; Balkowiec and Katz, 2002), and it appears that axonal, rather than dendritic, release of BDNF is necessary for presynaptic potentiation. Consistent with this notion, Zakharenko et al. (2003) demonstrated that presynaptic enhancements accompanying LTP were absent in BDNF knockout mice, but could be rescued by viral-mediated expression of BDNF in presynaptic CA3 neurons, but not in postsynaptic CA1 neurons (Zakharenko et al., 2003). Dendritic release of BDNF, in contrast, is more likely to be involved in enhancing presynaptic function in the context of homeostatic plasticity, which functions to maintain levels of neuronal activity within a range optimal for network function (Jakawich et al., 2010; Turrigiano, 2011).

1.7.6 Contact-dependent proteins

Retrograde-signalling in LTP is generally thought to be mediated by diffusible, short-lived signals whose postsynaptic synthesis is dependent on GluN receptor-mediated Ca^{2+} influx. However, membrane-bound proteins such as neuronal cell adhesion molecules (N-CAMs) (Luthl et al., 1994; Ronn et al., 1995; Muller et al., 1996) , N-cadherins (Bozdagi et al., 2000; Mendez et al., 2010)., GluA receptors (Ripley et al.), as well as ephrin and ephrin receptors

(ephs) (Grunwald et al., 2001; Contractor et al., 2002) have all been implicated in transsynaptic retrograde signalling. The function of contact-dependent transsynaptic signalling, however, appears to be restricted to co-regulation of pre- and post- synaptic efficacy over long time scales. Although they may be permissive for LTP induction, evidence has yet to emerge that these membrane-bound proteins can signal postsynaptic activity to the presynaptic terminal during LTP induction.

1.7.7 Structural changes

Structural enlargements of dendritic spines have been reported to accompany LTP induction. Blundon et al. (2013) have postulated that such changes may provide a synapse-specific means of influencing presynaptic function. Although the persistence of spine expansion varies across studies and induction protocols (Kasai et al., 2004; Lang et al., 2004; Bagal et al., 2005; Govindarajan et al., 2011), transient spine expansion is reliably observed at active synapses, and is dependent on GluN receptor-dependent Ca^{2+} influx, suggesting that structural change may be a useful transsynaptic signal for postsynaptic GluN receptor activity (Lang et al., 2004). Such a mechanism would require the presynaptic terminal to detect changes in spine size, for example, by conformation changes in yet to be identified presynaptic mechanoreceptors. However, it is not clear how these mechanisms would distinguish structural changes in the axon terminal, which also occur with activity (Nikonenko et al., 2003; Becker et al., 2008), from changes in dendritic spine, as both would mechanically distort the synapse. Moreover, weak tetanic stimulation, which fails to induce LTP, also can elicit transient spine expansion (Lang et al., 2004), suggesting that structural changes do not always reflect long-lasting functional changes.

1.8 Downstream targets of retrograde signalling

1.8.1 NO-cGMP-PKG pathway

Despite controversies in the field, nitric oxide (NO) remains the most studied and arguably, the most promising candidate for retrograde signalling (Padamsey and Emptage, 2013); though the precise mechanism by which NO potentially affects presynaptic transmission remains unknown. In the early 1980s, it was discovered that retrograde signalling from endothelial cells resulted in smooth muscle relaxation by a cyclic guanosine monophosphate (cGMP) dependent mechanism. NO, in particular, was found to activate guanylate cyclase, resulting in cGMP production and the activation of downstream kinases, namely protein kinase G (PKG) (Fiscus et al., 1983; Rapoport et al., 1983; Rapoport and Murad, 1983); NO was later shown to similarly elevate cGMP in cerebellar neurons (Garthwaite et al., 1988), and so, a similar cGMP-dependent mechanism was thought to mediate the presynaptic effects of NO at hippocampal synapses. Consistent with this notion, several groups demonstrated that tetanic stimulation induced GluN receptor-dependent elevations in cGMP in hippocampal slices (East and Garthwaite, 1991; Chetkovich et al., 1993). These elevations were necessary for potentiation since inhibition of guanylate cyclase or PKG, the downstream target of cGMP activation, blocked the induction of LTP induction (Zhuo et al., 1994; Boulton et al., 1995; Son et al., 1998; Arancio et al., 2001). LTP impairments induced by guanylate cyclase blockade occluded and were occluded by inhibition of NO synthesis, suggesting that cGMP and NO shared a common signalling pathway (Boulton et al. (1995)). Moreover, bath application of membrane-permeable cGMP analogues could also rescue LTP induction blocked by NO synthase inhibition, suggesting that cGMP signalling lay downstream from the actions of NO (Haley et al. (1993). Moreover,

bath application of membrane-permeable cGMP, when paired with tetanic stimulation, also induced LTP in a GluN receptor-independent manner (Zhuo et al., 1994; Boulton et al., 1995; Son et al., 1998; Arancio et al., 2001), and pharmacological activation of guanylate cyclase during LTP induction augmented the levels of synaptic potentiation (Chien et al., 2003). The potentiating effects of cGMP-signalling were localized to the presynaptic terminal since LTP induction was only inhibited when antagonists of either guanylate cyclase or PKG were specifically applied to the pre-, but not post-, synaptic neuron. Moreover, injection of cGMP into the presynaptic neuron was sufficient to induce LTP in a GluN receptor-independent manner, and pre-, rather than post-, synaptic injections of active PKG directly potentiated synaptic transmission (Arancio et al., 2001).

Despite the growing body of evidence supporting the role of presynaptic cGMP signalling in mediating NO-dependent LTP, some studies have failed to find a role of cGMP in LTP induction. Some groups have instead found a role of cGMP signalling in presynaptic depression rather than potentiation (Boulton et al., 1994; Stanton et al., 2001; Stanton et al., 2003). Other groups have reported that application of cGMP, when paired with weak tetanic stimulation, fails to elicit GluN receptor-independent LTP (Schuman et al., 1994; Selig et al., 1996; Kleppisch et al., 1999), or that inhibiting guanylate cyclase or cGMP-sensitive kinases does not impair LTP induction (Schuman and Madison, 1994; Kleppisch et al., 1999). Kleppisch et al. (1999) has additionally shown that LTP in mice lacking cGMP-sensitive kinases is of comparable magnitude to that in wild types, and can still be abolished with inhibitors of NO signalling, suggesting that NO might act on mechanisms other than cGMP signalling to potentiate synaptic responses. The reasons for these inconsistencies, are not clear, though differences in experimental conditions and the pattern of tetanic stimulation

appear to influence likelihood of observing a cGMP-dependent form of LTP (Kleppisch et al., 1999).

1.8.2 NO-ADP-ribosyltransferase pathway

In 1994, Schuman et al., after failing to find an involvement of either cGMP or its downstream kinase in LTP induction, examined the role of a second downstream target of NO, adenosine diphosphate ribosyltrasferase (ADPR) (Schuman and Madison, 1994). ADPR covalently modifies proteins with an ADP-ribose moiety, trasnferred from either nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$). Earlier works suggested that NO could stimulate the activity of ADPRs and generate ADPR-dependent ribosylation of neuronal proteins (Brune and Lapetina, 1989, 1990; Williams et al., 1992; Brune et al., 1994), and that ribosylation induced by exogenous NO application occluded ribosylation induced by tetanic stimulation (Duman et al., 1993). Consistent with past studies, Schuman et al. (1994) found that NO stimulated ADPR activity in the CA1 region, and that the ADPR inhibitor nicotinamide prevented LTP induction when bath applied, but not when injected into the postsynaptic neurons, suggesting that activation of presynaptic ADPR was important for synaptic potentiation. Pavlidis et al. 1998, however, later demonstrated that presynaptic injection of nicotinamide did not impair LTP induction. Taken together with the results from Schuman et al. (1994), these findings suggest that nicotinamide likely inhibits LTP through its effects on targets that are neither pre- nor post- synaptic. As such, the NO-cGMP pathway remains the most promising retrograde signalling mechanism triggered by LTP induction.

1.8.3 Kinases and Phosphatases

Although PKA, PKC, and CAMKII have been implicated in plasticity, their effects have been predominantly explored postsynaptically, and the link between retrograde signalling and the activation of these kinases, presynaptically, has not been well-established. Pavildis et al. (2000) did, however, demonstrate that the injection of a non-specific serine-threonine kinase inhibitor into the presynaptic neuron reduced the magnitude of LTP, whereas injection of the inhibitor into the postsynaptic neuron completely abolished LTP. These findings suggest that presynaptic kinases do contribute to LTP induction, likely by enhancing presynaptic function, but that LTP, whether pre- or post- synaptically expressed, also depends on the activation of postsynaptic kinases.

The link between kinase activity and presynaptic machinery is not well established at Schaffer-collateral synapses. However, at mossy fibre synapses, in which synapses express an GluN receptor-independent form of LTP, PKA has been shown to be necessary for activity-dependent increases in transmitter release probability and works by increasing the interaction between Rab3a, a vesicular GTPase, and the active zone protein, RIMα (Castillo et al., 1997; Castillo et al., 2002). Whether a similar mechanism mediates plasticity at Schaffer-collateral synapses remains to be elucidated.

More recently, the phosphatase calcineurin has been shown to be necessary for GluN receptor-dependent increases in recycling vesicle pool size at hippocampal synapses, suggesting that it may also be important for presynaptic LTP (Ratnayaka et al., 2012).

1.9 Presynaptic expression of LTP

Changes in transmitter release probability can be mediated by three general mechanisms: 1) changes in the number of docked vesicles or 2) changes in the levels of action potential-evoked increases in Ca^{2+} concentration in the vicinity of docked vesicles or 3) changes in the sensitivity of the fusion machinery to Ca^{2+} (Sudhof, 2004). The mechanism(s) involved in the expression of presynaptic LTP at Schaffer-collaterals is not known. At perforant path synapses, LTP increases the contribution of N-type Ca^{2+} channels to transmitter release (Ahmed and Siegelbaum, 2009). However, at Schaffer-collateral synapses, the rate of FM dye destaining following LTP is selectively enhanced for the readily releasable pool (RRP), but not reserve pool, of vesicles (Stanton et al., 2005). This finding suggests that presynaptic enhancements at these synapses are likely to be supported by an increase in docked pool size rather than an increase in Ca^{2+} influx or an increase in the sensitivity of the vesicle release machinery to Ca^{2+}, as these latter two changes would be expected to enhance exocytosis of both the RRP and reserve pool of vesicles. Such a notion is consistent with findings that the intraboutonal Ca^{2+} concentration at Schaffer-collateral synapses is not affected after LTP induction (Wu and Saggau, 1994). FM dye studies in hippocampal culture similarly show LTP-induced enhancements that are selective to the RRP (Ryan et al., 1996); though one study reports additional enhancements in the total releasable pool as well (Ratnayaka et al., 2012). Despite this discrepancy, the results from these studies suggest that an increase in the number of docked vesicles is likely to be a major contributor to the expression of presynapitc LTP. The exact signalling mechanism linking postsynaptic GluN

receptor activation to long-term increases in the number of docked vesicles, however, is not well understood.

1.10 Current model of GluN receptor-dependent presynaptic plasticity

Whether the presynaptic terminal is a major locus of LTP expression at Schaffer-collateral synapses continues to be questioned. Moreover, the mechanism by which activity regulates presynaptic function, and how it is linked to GluN receptor-mediated Ca^{2+} influx, remains controversial. Perhaps the most promising mechanism involves retrograde signalling by NO, which is thought to be synthesized in response to high levels of Ca^{2+} influx from postsynaptic GluN receptors (Garthwaite et al., 1988; East and Garthwaite, 1991; Garthwaite and Boulton, 1995); it is unclear, however, how exactly NO affects presynaptic release, though it might involve cGMP and/or downstream kinases, and an increase in size of the RRP. Notably, the NO-cGMP pathway has also been implicated in the induction of a GluN receptor-dependent form of LTD with a presynaptic component of expression; though little is known about this form of plasticity (Stanton et al., 2003) (Figure 9).

1.11 Postsynaptic GluN receptor-independent forms of plasticity

1.11.1 GluN receptor-independent LTP

The link between GluN receptor activation and presynaptic plasticity is tentative, though in the 1990s, evidence emerged to suggest that postsynaptic GluN receptors were not required for all forms of plasticity. Grover and Teyler were first to report that LTP could be induced in the presence of GluN receptor antagonists (50μM AP5), provided that tetanic stimulation was delivered at 200Hz, rather than the 100Hz traditionally used to induce LTP (Grover and Teyler, 1990). LTP under these conditions was not simply a result of residual

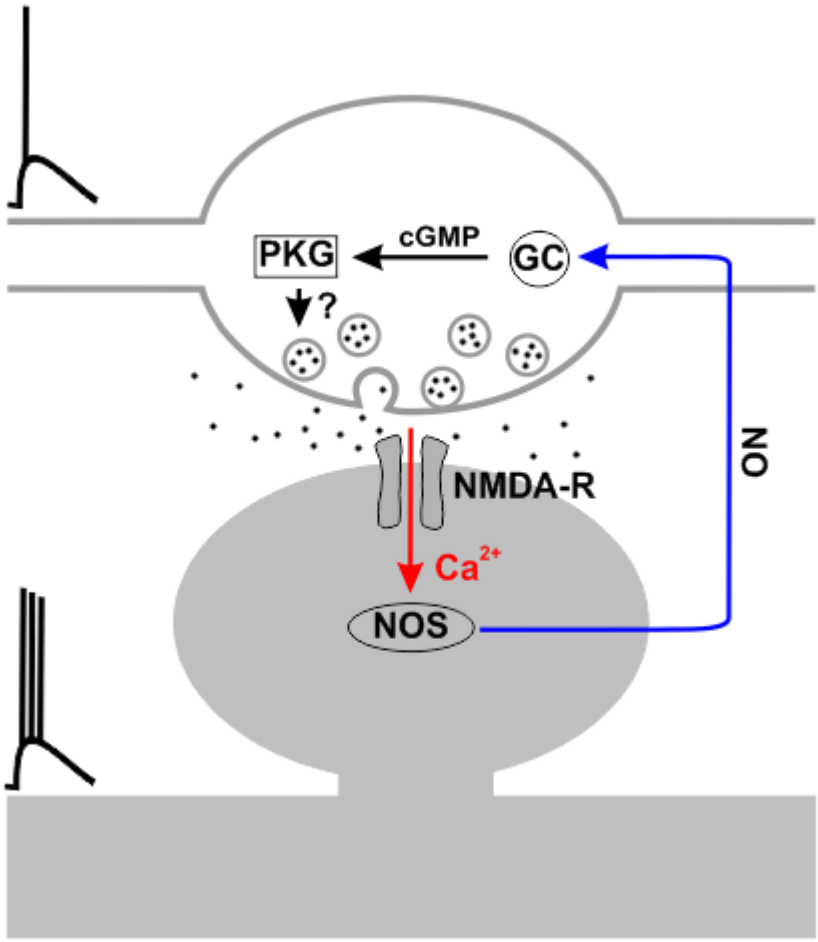


Figure 9. Potential model of GluN receptor-dependent presynaptic plasticity. LTP is induced at the presynaptic terminal when glutamate release coincides with strong postsynaptic depolarization, which results in high levels of Ca^{2+} influx through the postsynapitc GluN receptor. GluN receptor-dependent Ca^{2+} influx promotes the synthesis of gaseous nitric oxide (NO) via activation of NO synthase (NOS). The NO diffuses to the presynaptic terminal, where it promotes the synthesis of cyclic GMP (cGMP) via activation of guanylate cyclase (GC). cGMP then activates downstream kinases, such as protein kinase G (PKG), which increase transmitter release probability (Pr), possibly by increasing the number of docked vesicles . The NO-cGMP pathway may also be involved with the induction of presynaptic LTD, which results when glutamate release is not accompanied by strong postsynaptic depolarization. In this case, modest Ca^{2+} influx through the postsynaptic GluN receptors may still trigger NO synthesis, but with different spatiotemporal dynamics possibly resulting in the activation of a different set of downstream effectors in the presynaptic terminal (Stanton et al. 2003).

GluN receptor activity during high frequency stimulation, since it was induced with similar magnitude under a more potent receptor blockade (100μM AP5 + 20μM MK-801) (Grover, 1998) [but see (Pananceau and Gustafsson, 1997)]. GluN receptor-independent LTP depended on the activation of high-threshold L-type voltage gated calcium channels (L-VGCC), and some, but not all, studies also suggested a requirement for the activation of metabotropic glutamate receptors (mGluRs) (Petrozzino and Connor, 1994; Little et al., 1995; Platt et al., 1995; Grover, 1998; Grover and Yan, 1999a). In the following years, others reported that a similar form of LTP could be obtained by lower frequency stimulation, provided that some experimental manipulation was made to augment levels of postsynaptic depolarization experienced during LTP induction, presumably to activate L-VGCCs. Such protocols included delivering: 1) low-frequency axonal stimulation (≤0.1Hz) in the presence of voltage-gated potassium channel blockers (Aniksztejn and Ben-Ari, 1991; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Petrozzino and Connor, 1994; Huber et al., 1995; Platt et al., 1995), 2) delivering tetanic stimulation (25-100Hz) in slices treated with $GABA_A$ receptor antagonists (Grover and Yan, 1999b; Hsu et al., 1999) or 3) by pairing presynaptic stimuli (1-2Hz) with strong postsynaptic depolarization (Kullmann et al., 1992; Stricker et al., 1999). Notably, neither presynaptic stimulation in the absence of postsynaptic depolarization nor postsynaptic depolarization in the absence of presynaptic stimulation induced GluN receptor-independent LTP (Grover, 1998; Grover and Yan, 1999a), suggesting that, despite not requiring GluN receptors, this form of plasticity still obeyed Hebbian and Konorskian rules in requiring coincidental pre- and post- synaptic activity for its induction. Although the vast majority of protocols used to obtain GluN receptor-independent LTP may represent artificial experimental conditions that would be

unlikely to have physiological relevance, several groups have more recently demonstrated that a similar form of LTP can be produced by theta-burst stimulation (Morgan and Teyler, 2001; Zakharenko et al., 2001; Zakharenko et al., 2003; Bayazitov et al., 2007; Grover et al., 2009), which emulates *in vivo* patterns of hippocampal activity. Moreover, the finding that inhibition of L-VGCCs augments the impairment to spatial memory caused by GluN receptor blockade, suggests that L-VGCCs support some aspects of learning and memory *in vivo,* independent of GluN receptors (Borroni et al., 2000; Woodside et al., 2004; Moosmang et al., 2005).

The locus of expression of GluN receptor-independent LTP appears to be presynaptic (Stricker et al., 1999; Bayazitov et al., 2007; Blundon and Zakharenko, 2008) (but see (Grover, 1998)). The most compelling evidence comes from Bayazitov et al. (2007), who used synaptopHlourins to optically monitor activity-driven changes in presynaptic function. SynaptopHlourin is a pH-sensitive variant of GFP that has been fused to the luminal domain of the vesicular protein, VAMP2. The fluorophore is quenched within the acidic lumen of the vesicle and fluoresces upon vesicular exocytosis, when it is exposed to the pH-neutral extracellular environment. Using this construct, the authors demonstrated that presynaptic function was enhanced following either theta-burst or 200Hz stimulation, and that such increases could only be abolished with the L-VGCC antagonist, nitrendipine, but not with the GluN receptor antagonist, AP5; the resilience of presynaptic enhancement to AP5 was also evident in several studies using FM dyes to monitor presynaptic function (Ryan et al., 1996; Zakharenko et al., 2001; Ratnayaka et al., 2012). Moreover, in AP5, a similar fold potentiation was observed both for the presynaptic pHlourin response and the recorded field potential, suggesting that LTP was exclusively expressed presynaptically under GluN

receptor blockade. Conversely, tetanus in nitrendipine resulted in an enhancement of the recorded field potential but not in the presynaptic pHlourin response, suggesting that under L-VGCC blockade, LTP was exclusively expressed postsynaptically. Such findings strongly suggest that the induction of pre- and post- synaptic forms of LTP are mechanistically distinct, with the former requiring Ca^{2+} influx from L-VGCCs and the latter requiring Ca^{2+} influx from GluN receptors.

1.11.2 Presynaptic GluN receptor-dependent LTD

As with LTP, not all forms of LTD seem to rely on postsynaptic GluN receptors. In 1983, Levy and Steward demonstrated that LTP induced at a weak perforant path input depended on the temporal order in which it was paired with a strong input: when the weak input preceded the strong input, LTP was induced; when the order was reversed, LTD was induced instead (Levy and Steward, 1983). This form of LTD was later referred to as spike-timing dependent LTD (STDP-LTD), and is elicited when synaptic input follows postsynaptic spiking, within a few tens of milliseconds (Magee and Johnston, 1997; Markram et al., 1997; Bi and Poo, 1998; Debanne et al., 1998; Zhang et al., 1998). Although both LTP and LTD induced by STDP protocols were initially thought to depend on GluN receptors, several studies have now demonstrated that LTD at neocortical synapses specifically depends on the activation of pre-, but not post-, synaptic GluN receptors, and is expressed presynaptically (Nevian and Sakmann, 2006; Rodriguez-Moreno and Paulsen, 2008; Rodriguez-Moreno et al., 2011; Rodriguez-Moreno et al., 2013). The activation of presynaptic GluN receptors during the induction of STDP-LTD is mediated by glia, which release glutamate in response to mGluR-dependent synthesis of endocannabinoids from the postsynaptic cell (Min and Nevian, 2012). Recently, however, Rodriguez-Moreno et al.

(2013) demonstrated that patterned stimulation of the presynaptic neuron alone can induce a presynaptic form of LTD that is independent of endocannabinoid signalling, but still requires presynaptic GluN receptors, which in this case are directly activated by neuronal glutamate release from the presynaptic terminals. Whether similar presynaptic GluN receptor-dependent forms of LTD exist in the hippocampus is not known, although the possibility does exist since presynaptic GluN receptors were recently confirmed to be present at Schaffer-collateral synapses (McGuinness et al., 2010).

1.11.3 mGluR-dependent LTD

The activation of mGluRs have also been shown to mediate LTD independently of GluN receptor activation. mGluR-dependent LTD was initially reported at Schaffer-collateral synapses by Stanton et al. (1989,1990), who demonstrated that low frequency stimulation of one afferent pathway induced LTD when stimulation was delivered 50ms following high frequency stimulation of a second pathway. Similar mGluR-dependent forms of LTD were subsequently reported using a variety of induction protocols including: 1) low frequency stimulation, in which each stimulus preceded a postsynaptically generated action potential (STDP-LTD) (Normann et al., 2000), 2) prolonged low frequency stimulation with single or paired pulses (Oliet et al., 1997; Kemp and Bashir, 1999; Huber et al., 2000; Nosyreva and Huber, 2005), 3) bath application of a group I mGluR agonist (DHPG) (Fitzjohn et al., 2001; Xiao et al., 2001; Nosyreva and Huber, 2005; Qian and Noebels, 2006), and 4) low frequency afferent stimulation paired with either membrane hyperpolarization (Stanton and Sejnowski, 1989; Stanton et al., 1991) or 5) bath application of $GABA_A$ or $GABA_B$ agonists (Yang et al., 1994). Using either electrophysiological or optical means, several groups have now demonstrated that mGluR-dependent LTD is expressed, at least in part, by a decrease

in transmitter release probability (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997; Fitzjohn et al., 2001; Zakharenko et al., 2002; Nosyreva and Huber, 2005; Qian and Noebels, 2006), likely as a result of a decrease in the number of docked vesicles and a change in the mode of vesicle exocytosis, from full fusion to kiss-and-run (Zakharenko et al., 2002). Some studies, however, find evidence for a predominant postsynaptic locus of expression. For example, DHPG-application has been shown to trigger GluA receptor internalization (Xiao et al., 2001) and low-frequency activation of mGluRs on single spines using glutamate uncaging at single spines decreases GluA receptor activity (Holbro et al., 2009). Nosyreva and Huber (2005) have argued that such experimental discrepancies likely result from the differences in the age of the tissue used across studies; specifically, they have reported that DHPG-induced LTD is predominantly expressed presynaptically in young (2 week-old) tissue but postsynaptically in older tissue.

1.12 Aims and hypotheses

In contrast to the well-established link between GluN receptor-dependent Ca^{2+} influx and postsynaptic plasticity, the link between GluN receptor function and presynaptic plasticity is far less defined (Padamsey and Emptage, 2013). The finding that Hebbian/Konorskian-like LTP and LTD at the presynaptic locus can occur independently of postsynaptic GluN receptor function suggests that the mechanism by which glutamate and postsynaptic depolarization regulate synaptic function at the pre- and post- synaptic locus may fundamentally differ (Blundon and Zakharenko, 2008; Padamsey and Emptage, 2013).

Perhaps this point is best made when plasticity at presynaptically silent synapses is considered, in which only pre- but not post- synaptic changes would serve to enhance synaptic strength. These synapses are thought to exist, particularly early in development, and yet the mechanism by which they become activated is not well understood (Maggi et al., 2003; Slutsky et al., 2004; Yao et al., 2006; Crawford and Mennerick, 2011). However, recent studies have used electron microscopy to identify a significant portion (~35-50%) of synapses in adult rodent hippocampus that have presynaptic zones lacking synaptic vesicles in their near proximity (<170nm); these so-called "nascent zones" have been hypothesized to be functionally silent (Spacek and Harris, 1998; Bell et al., 2014) (Figure 10). If, at such sites, presynaptic activity coincides with strong postsynaptic depolarization, by Hebb's and Konorski's postulate, one might expect LTP to occur. And yet, according to the current GluN receptor-dependent view of plasticity, no change should occur since no glutamate was released and so no GluN receptors could have been activated. Under such circumstances, the current model of plasticity would appear to violate Hebb's and Konorski's postulate. If, however, presynaptic potentiation does not follow the conventional

rules of GluN receptor-dependent plasticity, and only requires presynaptic activity to coincide with postsynaptic depolarization, without the strict requirement for glutamate release, then terminals releasing little or no glutamate could be potentiated, provided that their activity coincided with postsynaptic depolarization, driven by glutamate release at other co-active presynaptic terminals. Although the existence of presynaptically silent synapses is controversial (Voronin and Cherubini, 2004), a similar argument could be made for synapses with very low release probabilities (<0.2), which comprise a substantial portion of hippocampal synapses (Holderith et al., 2012). At such synapses, since transmitter is released with a low probability, it is still conceivable that presynaptic activity could coincide with postsynaptic depolarization in the absence of glutamate release. The aim of this thesis, therefore, was to elucidate the precise role of glutamate and its receptors in presynaptic plasticity.

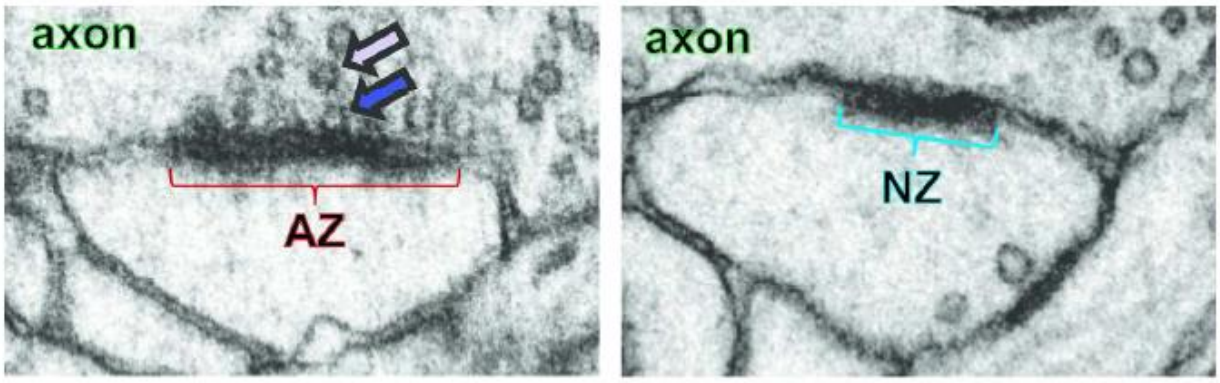


Figure 10. Putative presynaptic silent synapses. Electron micrographs of axonal boutons in hippocampal slices of adult rodents containing either (left) active zones (AZ) and (right) nascent zones (NZ), the latter being defined as a presynaptic zone lacking synaptic vesicles in the close proximity (<170nm). Synapses containing nascent zones are potentially presynaptically silent and comprise 35-50% of synapses in the slice preparation. Image modified from Bell et al. (2014).

2. METHODS

2.1 Slice preparation

2.1.1 Acute hippocampal slices

Acute hippocampal slices were prepared from 2-4 week old male Wistar rats. Hippocampi were dissected out of the whole brain in a sucrose-based ACSF solution (in mM: 85 NaCl, 65 sucrose, 26 $NaHCO_3$, 10 glucose, 7 $MgCl_2$, 2.5 KCl, 1.2 NaH_2PO_4, and 0.5 $CaCl_2$) and then sliced into transverse sections (350-400µm thick) using a Microm HM 650V vibratome (Thermo Scientific). Alternatively, whole brain sections (350-400µm thick) were cut along the coronal plane and individual hippocampi were subsequently dissected out of the dorsal sections. Isolated hippocampal slices were allowed to recover at 36^oC for 30-60 minutes in normal ACSF (in mM: 120 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 26 $NaHCO_3$, and 11 glucose), bubbled with carbogen (95% O_2 and 5% CO_2), after which they were stored at room temperature for subsequent use. During experimentation, slices were perfused with normal ACSF (2mL/min) containing picrotoxin (100µM). The ACSF was constantly bubbled with carbogen (95% O_2 and 5% CO_2) and heated to achieve near-physiological temperatures in the bath (33-36^oC).

2.1.2 Organotypic hippocampal slices

Organotypic hippocampal slices were prepared from 7-8 day old male Wistar rats (Harlan Laboratories, UK). Pups were killed by cervical dislocation and brains were extracted and placed in ice-cold dissection media. Dissection media comprised of Earl's Balanced Salt Solution (Invitrogen) with added glucose (+35mM) and HEPES (+20mM), and pH corrected to 7.2-7.4 using 5M NaOH. Hippocampi were dissected out and cut into 350µm thick slices

along the transverse plane with a McIlwain tissue chopper (Mickle, UK). Slices were observed under a dissection media and those that showed signs of tissue damage or that did not exhibit normal hippocampal morphology with a contiguous band of pyramidal cells, and an intact dentate gyrus were discarded. The remaining slices were transferred on to culture inserts (Merck-Millipore, UK), and placed in a 6-well plate containing 1mL/well of growth media. Growth media consisted of 50% Minimum Essential Media (Invitrogen), 25% heat-inactivated horse serum (Invitrogen), 23% Earl Balanced Salt Solution (Invitrogen), and 2% B-27 (Invitrogen) with added glucose (+35mM), and was replaced every 2-3 days. Slices were incubated at 36.0^0C and 5% CO_2 for 7-14 days before use.

For experiments, organotypic slices were transferred to a recording chamber and perfused (1.2mL/minute) with artificial cerebrospinal fluid (ACSF), which constantly bubbled with carbogen (95% O_2 and 5% CO_2) and heated to achieve near-physiological temperatures in the bath (31-33^{o}C). ACSF contained (in mM) 145 NaCl, 2.5 KCl, 2-3 $CaCl_2$, 1-2 $MgCl_2$, 1.2 NaH_2PO_4, 16 $NaHCO_3$, 11 glucose and, to minimize photodynamic damage in imaging experiments, 0.2 ascorbic acid and 1 Trolox.

2.2 Electrophysiology

2.2.1 High-resistance patch recordings

CA1 pyramidal neurons were patched using high resistance glass electrodes (18-25MΩ) to minimize intracellular dialysis. Recordings were made using an Axoclamp 2A amplifier (Axon Insturments). Patch electrodes were filled with internal solution containing (in mM): 135 KGluconate, 10 KCl, 10 HEPES, 1 $MgCl_2$, 4 Na_2ATP and 0.4 Na_3GTP. Access resistance was monitored using hyperpolarizing current pulses (-100pA) and varied from 50-120MΩ.

Experiments were abandoned if, during the experiment, access resistance increased beyond 120MΩ, if there was a sudden and considerable decrease in membrane resistance, or if the resting membrane potential depolarized beyond -50mV.

2.2.2 Sharp microelectrode recordings

Sharp microelectrodes (70-100MΩ) filled with 400mM $KMeSO_4$ and 100mM KCl were used to record from either CA3 or CA1 hippocampal pyramidal neurons. Recordings were made using an Axoclamp 2A amplifier (Axon Insturments). Microelectrodes were tip-filled with Ca^{2+} indicator dye (200-500μM Oregon Green BAPTA-1 dissolved in 200mM $KMeSO_4$) and backfilled with a solution containing 400mM $KMeSO_4$ and 100mM KCl. Since plasticity at CA3-CA3 and CA3-CA1 synapses is indistinguishable, data from both population of synapses were pooled. Experiments were abandoned if, during the experiment, there was a sudden and considerable drop in membrane resistance, or if resting membrane potential depolarized beyond -50mV.

2.2.3 Stimulation protocols

An ACSF-filled glass patch electrode (4-8MΩ) was used to stimulate Schaffer and association collateral synapses. The electrode was placed in stratum radiatum within 50-200μm of the postsynaptic cell. Stimulation pulses were 100μs in duration and delivered with an intensity (10-30μA) sufficient to evoke a 5-10mV EPSP. Continuous basal stimulation (0.05-0.10Hz) was present for all experiments, and was only interrupted to deliver paired-pulse or tetanic stimulation. Paired-pulse stimulation, when present, consisted of two presynaptic stimuli delivered 70ms apart, every 30 seconds.

Long-term potentiation (LTP) was induced by tetanic stimulation or paired stimulation. Tetanic stimulation consisted of three trains delivered 1.5 seconds apart, each consisting of 20 pulses delivered at 100Hz; pulse intensity was increased by 50% during stimulation. In some experiments, postsynaptic current was injected to depolarize the cell by 10-15mV for 200ms during each of the three trains of stimuli in the tetanus. Paired stimulation consisted of 30-60 pulses delivered at 5Hz. For induction of LTP by paired stimulation under standard conditions, each pulse was paired with a brief (50-70ms) postsynaptic current injection of sufficient magnitude to elicit 3-5 spikes; the first of these spikes followed the presynaptic stimulus by 7-15ms. For induction of LTP by paired stimulation in glutamate receptor blockade or in GluN receptor blockade postsynaptic current injection (1-2nA) emulated a complex spike that was approximately 60ms in duration, and consisted of a 7-10ms long rising phase, a 20ms long plateau phase, and a 30-33ms long falling phase. Current injection was of sufficient amplitude to trigger 3-6 spikes at a frequency of 50-100Hz, with the first spike commencing 7-15ms after the presynaptic stimulus. In these experiments, stimulating electrodes were placed within 50-70μm of the soma to ensure that postsynaptic depolarization reached stimulated synapses without significant attenuation.

Long-term depression (LTD) induction consisted of 60-120 presynaptic stimuli, delivered at 5Hz and in the absence of postsynaptic depolarization. In some cases, stimulation was delivered while the postsynaptic neuron was hyperpolarized to prevent spiking. In other cases, stimulation proceeded without any postsynaptic current injection. In such instances, the neuron rarely generated postsynaptic spikes.

2.2.4 EPSP slope analysis

Electrophysiological data were recorded using WinWCP software (Strathclyde Electrophysiology Software), and were analyzed using Clampfit (Axon Instruments) and Excel (Microsoft). The initial EPSP slope, calculated during the first 3ms of the response, was used to analyze changes in the EPSP throughout the recording. All data were normalized to the average EPSP slope recorded during baseline to yield ΔEPSP slope.

2.2.5 Paired pulse ratio analysis

Paired pulse ratios (PPRs) were used to monitor changes in presynaptic function (Schulz et al., 1994). Paired-pulse stimulation consisted of two presynaptic stimuli delivered 70ms apart, every 30 seconds. PPR was calculated as the average EPSP slope to the second pulse divided by the average EPSP slope to the first pulse, as previously described (Kim and Alger, 2001); averages were calculated from 10-20 paired-pulse trials. Decreases in PPR were assumed to reflect an increase in presynaptic function (Schulz et al., 1994).

2.2.6 Co-efficient of variation analysis

Co-efficient of variation analysis was used to monitor changes in presynaptic function (Faber and Korn, 1991). The coefficient of variation parameter CV^{-2} was calculated as $mean^2$/variance using the EPSP slopes collected over 25-30 trials. The CV^{-2} calculated for the last 25-30 trials of the recording were normalized to that calculated during the first 25-30 trials of the recording to yield Δ CV^{-2}. For some experiments, ΔCV^{-2} was plotted against ΔEPSP slope. Increases in presynaptic function were assumed to have occurred when ΔCV^{-2} > ΔEPSP slope (Faber and Korn, 1991) (see p24 for further details).

2.3 Imaging

Confocal imaging was achieved using a BioRad MRC-1000 scanhead attached to a Zeiss Axioskop upright microscope equipped with a 60X NA 0.90 water-immersion lens (Olympus) (Figure 1). A 488nm solid state laser provided the excitation source and galvanometer mirrors within the scanhead enabled rapid xy scanning of tissue. Emitted light passed through a dichroic mirror and a confocal pinhole, before being detected by a photomultiplier tube. LaserSharp software (Bio Rad) was used to acquire images, to control the area and speed of scanning, and to adjust the laser power, confocal pinhole size, and photomultiplier tube sensitivity.

2.3.1 Intracellular Ca^{2+} loading

CA1 pyramidal neurons were filled with Ca^{2+} sensitive dye using a low resistance patch electrode (4-8 MΩ) or sharp microelectrodes. Patch electrodes contained 1mM Oregon Green Bapta-1 (Invitrogen) dissolved in standard internal solution. Cells were loaded for 45-60s, after which the patch electrode was slowly withdrawn over the course of 1-2 minutes using a piezoelectric drive. Withdrawal of the electrode was very rarely associated with a rise in intracellular Ca^{2+}, suggesting that the procedure caused minimal damage to the cell. For sharp microelectrode loading, CA1 or CA3 neurons were impaled using microlectrodes (70-100MΩ) tip-filled with 200-500μM Oregon Green BAPTA-1 dissolved in 200mM $KMeSO_4$ and backfilled with a solution containing 400mM $KMeSO_4$ and 100mM KCl. Experiments were abandoned if action potentials failed to elict large increases in fluorescence (>50% ΔF/F) as a result of overloading the cell with dye.

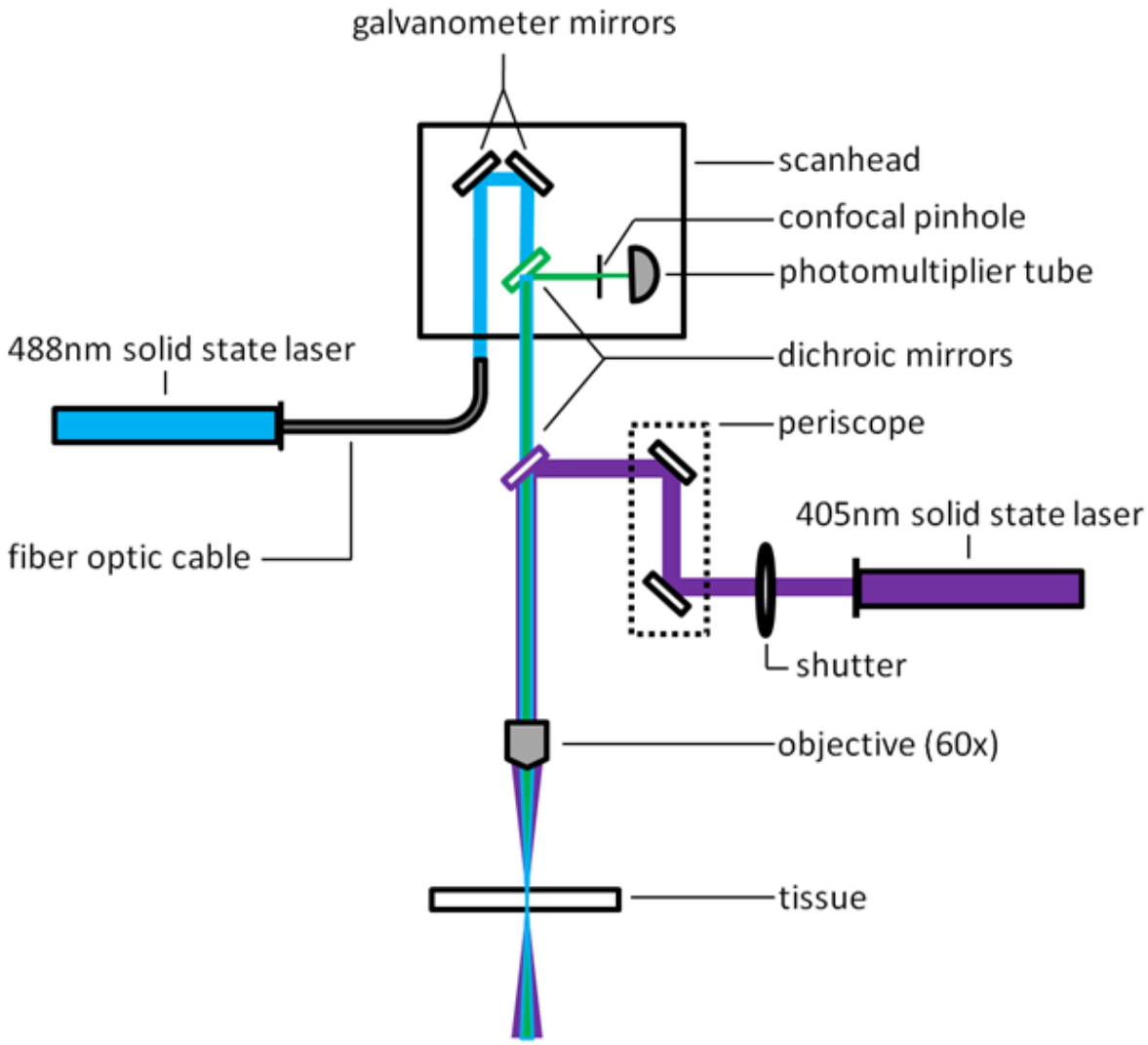


Figure 1. Confocal imaging and spot photolysis setup. Confocal scanning was achieved by using galvanometer mirrors to move a 488nm excitation beam along a xy plane of tissue. Emission light was re-directed via a dichroic mirror, through a confocal pinhole and into a photomultiplier tube for detection. A 405nm laser beam, used for chemical photolysis, was directed onto the tissue via a periscope and dichroic mirror. The beam was focussed to a ~1.2µm spot on the tissue using a 60X NA 0.90 objective. The duration of 405nm exposure was controlled using a shutter.

2.3.2 Single spine Ca^{2+} imaging

Spine Ca^{2+} imaging was used to calculate transmitter release probability at individual synapses. After hippocampal pyramidal cells were loaded with Ca^{2+} indicator dye, a stimulating glass electrode (4-8MΩ) was then brought near (5-20μm) to a branch of imaged dendrite within stratum radiatum in order to maximize the chances of stimulating a synapse in the vicinity (Yasuda et al., 2004). For visualization purposes, electrode tips were coated with bovine serum albumin Alexa 488 conjugate (Invitrogen), as previously described (Ishikawa et al., 2010). Briefly, a 0.05% BSA-Alexa 488 solution was made with 0.1M phosphate-buffered saline containing 3mM NaN_3. Pipette tips were placed in the solution for 2-5 minutes. A region of dendrite was monitored at 2-5Hz using an xy scan while axonal fibres were stimulated with brief trains of presynaptic stimuli; a train consisted of 2-4 pulses delivered 70ms apart. Given that uniquantal glutamate release can elicit detectable increases in spine Ca^{2+}, it was often possible to detect spines that fluorescently responded to stimulation. Once a responsive spine was found, laser scanning was restricted to a line through the spine head and underlying dendrite to enable rapid imaging (500Hz) during presynaptic stimulation. Stimulation-evoked fluorescence increases were often restricted to the spine, or at least preceded fluorescence increases in the dendrite. Line scans lasted a total of 300-400ms, and included 100ms of baseline imaging axonal stimulation, which consisted of two pulses delivered 70ms apart. Spine fluorescence was recorded for 20-40 stimulation trials at baseline and 25-30 minutes following LTP or LTD induction. Linescans were analyzed in ImageJ and in Microsoft EXCEL using custom written macros. Changes in fluorescence were quantified as $\%\Delta F/F = 100 \times (F - F_{baseline}/ F_{baseline} - F_{background})$, where $F_{baseline}$ reflected the average fluorescence intensity

during the 100ms of baseline recording. Pr was calculated as the proportion of trials in which presynaptic stimulation successfully resulted in an increase in fluorescence intensity from baseline; in the event of a dendritic spike, these increases had to precede or exceed the fluorescence increases imaged in the dendrite for the trial to be considered a success (Emptage et al., 1999; Emptage et al., 2003; Nevian and Helmchen, 2007).

2.3.3 Nitric oxide (NO) imaging

NO was imaged using the NO-sensitive fluorescent dye DAF-FM (Invitrogen). Experiments were carried out in Tyrodes buffer (in mM: 120 NaCl, 2.5 KCl, 30 glucose, 4 $CaCl_2$, 0 $MgCl_2$, and 25 HEPES) containing 50μM AP5, 10μM NBQX, 500μM MCPG, and 100μM LY341495 to block glutamate receptors, as well as 1μM Bay K-8644 to prevent L-type voltage gated Ca^{2+} channel desensitization during K^+ application (Sattler et al., 1999; Stanika et al., 2012). CA1 pyramidal neurons were transiently patched with glass electrodes (4-8MΩ) containing standard internal solution with 250μM of DAF-FM and loaded for 60s before withdrawing the electrode. Apical dendrites, often secondary or tertiary branches, within 100μm of the soma were imaged at one focal plane, once prior to, and once 5-10s following, the addition of a high K+ Tyrodes solution (in mM: 32.5 NaCl, 90 KCl, 30 glucose, 4 $CaCl_2$, 0 $MgCl_2$, 25 HEPES, 50μM AP5, 10μM NBQX, 500μM MCPG, and 100μM LY341495). Laser power and exposure were kept to a minimum to avoid photobleaching. DAF-FM basal fluorescence was not quenched by intracellular addition of cPTIO.

2.4 Photolysis

A 405nm laser (Photonics) was used for photolysis (Figure 1). The laser beam was directed via a periscope and dichroic mirror, and focussed to a small spot (~1.2μm diameter) by

overfilling the back aperture of the objective (60X NA 0.90; Olympus). Electrode manipulators and recording chambers were mounted on a movable stage, which enabled a region above the spine head to be positioned beneath the photolysis spot. Laser exposure was controlled using a fast shutter (LS6; Uniblitz).

2.4.1 Glutamate photolysis

For glutamate photolysis, MNI glutamate (Tocris) was focally delivered through a glass pipette (4-8MΩ; 10mM MNI glutamate) using a picospritzer (Science Products). Laser exposure was limited to 1-2ms and the laser intensity (0.5-2mW) was adjusted to generate a Ca^{2+} response in the underlying spine that was comparable to the response generated by electrical stimulation.

2.4.2 Nitric oxide (NO) photolysis

For NO photolysis, 0.5-1mM $RuNOCl_3$ (Sigma) was bath applied and uncaged using 30-60 laser pulses (25ms; 2mW) delivered at 5Hz; laser pulses were timed to precede or follow presynaptic stimulation by 10ms. Using the NO-indicator, DAF-FM (Invitrogen), a given laser pulse was estimated to liberate approximately 4-7nM of NO.

2.5 Pharmacology

Glutamate receptor blockade was achieved using AP5 (50-100μM; Abcam), NBQX (10μM; Abcam), MCPG (500μM; Abcam) and LY341495 (100μM; Abcam). In experiments requiring only NMDA receptors (GluN) to be blocked, AP5 (50-100μM) was added to the bath for the duration of the experiment. Postsynaptic GluN receptors were blocked by including MK-801 (0.5-2mM loaded for 60s via a 4-8MΩ patch electrode; Abcam) in the patch solution during

Ca^{2+} dye loading. L-type voltage gated Ca^{2+} channels (L-VGCCs) were blocked with nitrendipine (20μM; Abcam). NO synthase was inhibited by incubation with L-NAME (100μM; Sigma), 20 minutes prior to experimentation. Extracellular NO was scavenged by bath application of cPTIO (50μM; Sigma). Intracellular NO was scavenged by including cPTIO (5mM; loaded for 60s via a 4-8MΩ patch electrode) in the patch solution during Ca^{2+} dye loading.

2.6 Statistical analysis

The statistical significance of comparisons was assessed using two-tailed Mann-Whitney or Wilcoxon matched-pairs tests, respectively for paired and unpaired data. Chi-squared tests were used when assessing whether differences between the actual and expected distribution of data across defined categories were statistically significant. Pearson correlation coefficients were calculated to determine the significance of linear trends. Significance for all tests was assessed at the $p<0.05$ value. Averages and standard error of the mean (S.E.M.) are represented in the text as average±S.E.M.

3. THE NECESSITY OF GLUTAMATE IN THE LONG-TERM POTENTIATION OF PRESYNAPTIC FUNCTION

3.1 Introduction

One of the most widely studied forms of synaptic plasticity is long-term potentiation (LTP). Despite controversy, there is sufficient evidence to suggest that LTP can be supported either presynaptically, by an increase in transmitter release probability (Pr), or postsynaptically, by an increase in AMPA receptor (GluA) number or conductance. These changes are currently thought to require the activation of NMDA receptors (GluN) (Bliss and Collingridge, 2013; Padamsey and Emptage, 2013).

This GluN receptor-dependent view of plasticity suggests that glutamate release and postsynaptic GluN receptor activation are necessary for the induction of LTP (Luscher and Malenka, 2012). As a consequence, augmenting glutamate release at a synapse, provided that it is paired with strong postsynaptic depolarization, should increase the magnitude of LTP. This is certainly true at the postsynaptic locus, whereby augmenting the amount of glutamate released at dendritic spines, for example by glutamate photolysis, increases the likelihood of synaptic potentiation (Harvey and Svoboda, 2007; Lee et al., 2010). However, at the presynaptic locus, studies consistently demonstrate an inverse relationship between initial Pr and the magnitude of potentiation at both hippocampal and neocortical synapses, whereby presynaptic terminals that release little or no glutamate are most likely to show potentiation (Larkman et al., 1992; Ryan et al., 1996; Slutsky et al., 2004; Hardingham et al., 2007; Saez and Friedlander, 2009). Although such findings may arise as a result of a ceiling effect imposed by Pr, which cannot exceed 1, several studies have demonstrated that, whereas pairing pre- and post- synaptic activity at low Pr synapses generates presynaptic

potentiation, the same pairing protocol actually produces presynaptic depression at high Pr synapses (Hardingham et al., 2007; Saez and Friedlander, 2009). Such findings are not easily reconciled under the current view of plasticity, and suggest that glutamate release, if anything, impedes presynaptic potentiation whilst promoting presynaptic depression.

Moreover, under the GluN receptor-dependent model of plasticity, the supposed necessity of glutamate release for the potentiation of presynaptic function poses a significant theoretical problem, especially if one considers LTP induction at synapses that release no glutamate. The existence of presynaptically silent synapses is controversial, and definitive evidence of the existence of a population of axonal boutons that truly releases no glutamate, even in response to high frequency stimulation, has yet to emerge (Voronin and Cherubini, 2004). Nonetheless, data from studies using either optical imaging or electrophysiology to examine synaptic vesicle fusion do at least suggest the existence of presynaptically silent synapses in developing neuronal cultures and immature hippocampal slices (Kimura et al., 1997; Maggi et al., 2003; Slutsky et al., 2004; Yao et al., 2006; Kim and Ryan, 2010). Moreover, a recent study looking at adult hippocampal tissue in the rodent identified that approximately 35-50% of all presynaptic zones lack synaptic vesicles and are hypothesized to be functionally silent (Bell et al., 2014). There is, therefore, some justification to consider, at least in theory, plasticity at a presynaptically silent terminal. At such synapses then, if presynaptic activity were to coincide with strong postsynaptic depolarization, no LTP would occur according to the current model of plasticity, as no GluN receptors were activated on the synapse. This lack of potentiation would appear to violate Konorski's and Hebb's postulate, and would suggest that, at least on a theoretical level, the application of

the GluN receptor-dependent model of plasticity at the presynaptic locus is potentially flawed.

The GluN receptor, however, is not necessary for all forms of LTP. Since the 1990s, a GluN receptor-independent form of LTP has been known to exist, one which requires the activation of postsynaptic L-type voltage-gated Ca^{2+} channels (L-VGCCs) (Grover, 1998; Grover and Yan, 1999a). Induction of this form of plasticity generally requires strong postsynaptic depolarization, which is achieved by a variety of means, most commonly by increasing the frequency of tetanic stimulation from 50-100Hz to 200Hz (Grover and Yan, 1999b; Hsu et al., 1999; Zakharenko et al., 2001; Zakharenko et al., 2003; Bayazitov et al., 2007), or by delivering low-frequency stimulation (1Hz) in the presence of a voltage-gated K^+ channel blocker tetraethylammonium chloride (TEA) (Aniksztejn and Ben-Ari, 1991; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Petrozzino and Connor, 1994; Huber et al., 1995; Platt et al., 1995). Notably, despite not requiring GluN receptor activation, this form of LTP still exhibits Hebbian and Konorskian properties, as its induction requires that presynaptic activity coincide with strong postsynaptic depolarization. As such, neither presynaptic stimulation in the absence of postsynaptic depolarization nor postsynaptic depolarization in the absence of presynaptic stimulation induces GluN receptor-independent LTP (Grover, 1998; Grover and Yan, 1999a).

The locus of expression of GluN receptor-independent LTP appears to be presynaptic (Stricker et al., 1999; Bayazitov et al., 2007; Blundon and Zakharenko, 2008) (but see (Grover, 1998)). The most compelling evidence for this comes from Bayazitov et al. (2007) who, using synaptopHlourins to image vesicle fusion, recently demonstrated that the

induction of GluN receptor-independent LTP resulted in presynaptic increases in transmitter release, whereas the induction of GluN receptor-dependent LTP resulted only in enhancements of postsynaptic strength. These finding strongly suggest that LTP at the pre- and post- synaptic terminal are mechanistically distinct, and, as a consequence, will likely follow different rules of induction.

The results of Bayazitov et al. (2007) suggest that GluN receptor activation is not a strict requirement for increases in Pr. Notably, others have shown that LTP induction is possible, not only under GluN receptor blockade (Blundon and Zakharenko, 2008), but also under metabotropic glutamate receptor (mGluR) blockade (Wilsch et al., 1998) and GluA receptor blockade (Fuenzalida et al., 2007; Holbro et al., 2010). Such findings hint at the possibility that activation of any glutamate receptor may not be required for presynaptic potentiation. This notion is made all the more plausible by the observation that L-VGCC channels, which are necessary for presynaptic LTP induction (Bayazitov et al., 2007), are activated by postsynaptic depolarization rather than directly by glutamate release. As a consequence, it might be possible for synapses to be potentiated even if they do not release sufficient levels of glutamate to activate postsynaptic receptors, provided that their activity coincides with the necessary level of postsynaptic depolarization required to trigger the activation of L-VGCCs, which, in a physiological context, would be driven by glutamate release at other co-active synapses.

In this chapter, I examine the necessity of glutamatergic signalling in the induction of LTP at the presynaptic terminal. I used standard electrophysiological techniques to show that LTP, with a presynaptic component of expression, can be induced, first in GluN receptor

blockade and then in full glutamate receptor blockade, by pairing presynaptic activity with sufficiently strong postsynaptic depolarization. My findings suggest that the activation of glutamate receptors is not a strict requirement for the induction of presynaptic LTP.

3.2 Results

3.2.1 Induction of presynaptic LTP under blockade of GluN receptors

In order to determine whether the induction of presynaptic LTP requires glutamate, I first attempted to induce LTP in GluN receptor blockade. I used sharp microelectrodes to record from either CA3 or CA1 pyramidal neurons in organotypic hippocampal slices and evoked excitatory postsynaptic potentials (EPSP) by stimulating CA3 axons in stratum radiatum; since the properties of synaptic plasticity at CA3-CA3 synapses and CA3-CA1 synapses are indistinguishable, the results from both groups were pooled (Debanne et al., 1998, 1999; Emptage et al., 2003; Ward et al., 2006). Consistent with findings from other laboratories, standard tetanic stimulation, consisting of three 100Hz trains of 20 stimuli delivered at 1.5 seconds apart, led to a robust increase in the EPSP (196±17%; n=5) (Figure 1A,D); no such enhancements, however, were observed when stimulation was delivered in the presence of the GluN receptor blocker AP5 (93.7±4.6%) (control vs. AP5; n=5; p=0.012) (Figure 1B,D). Given that the induction of GluN receptor-independent LTP is often induced under conditions of strong postsynaptic depolarization, presumably to drive activation of L-VGCCs (Blundon and Zakharenko, 2008), I next examined whether LTP induction in AP5 could be rescued by augmenting postsynaptic activity during tetanus. To do so, I paired 100Hz stimulation with postsynaptic injection of sufficient magnitude to depolarize the neuron by 10-20mV. Under these conditions, standard 100Hz stimulation generated robust

LTP (197±22%; n=7) (Figure 1C) in the presence of AP5, which was of comparable magnitude to that generated in control conditions (LTP magnitude: AP5 + depolarization vs. control; 197±22% vs. 196±17%; n=7 and 5; p=0.87) (Figure 1D).

To examine whether postsynaptic depolarization rescues the presynaptic component of LTP expression in GluN receptor blockade, I used CV^{-2} analysis to assay presynaptic efficacy prior to and following tetanic stimulation. CV^{-2} analysis assumes that synaptic responses can be modelled using the binomial theorem, implying that the mean ($\mu=Npq$) and variance ($\sigma^2=Nq^2p(1-p)$) of synaptic responses can be defined by three quantal parameters: the number of synapses active (N), the average release probability (p), and the average postsynaptic potential in response to a single quanta of glutamate (q). As a consequence, the co-efficient of variation (CV), which is defined by the standard deviation of the synaptic response divided by the standard mean, becomes independent of q ($CV=(1-p/Np)^{1/2}$), and the square of its inverse ($CV^{-2}=Np/1-p$) increases with p. Assuming that LTP is not accompanied by changes in N, changes in CV^{-2} provide a purely presynaptic measure of changes in synaptic efficacy.

In control conditions, tetanic stimulation resulted in an increase in the CV^{-2}, suggesting that LTP had a presynaptic component of expression; no such increases were observed when tetanus was delivered in AP5 (fold ΔCV^{-2} control vs. AP5: 2.11±0.26 vs. 1.04±0.14; n=5; p=0.02) suggesting that, in agreement with other laboratories, presynaptic potentiation induced by standard 100Hz stimulation was dependent on GluN receptor activity (Figure 2A). However, in the presence of AP5, when postsynaptic depolarization was augmented during tetanic stimulation the induced LTP was accompanied by an increase in CV^{-2} (fold

ΔCV^{-2} AP5 + depolarization vs. AP5; 2.87±0.56 vs. 1.04±0.14; n=7 and 5; p=0.01) that was comparable to control conditions (fold ΔCV^{-2}: AP5 + depolarization vs. control; 2.87±0.56 vs. 2.14±0.28; n=7 and 5; p=0.42), suggesting that postsynaptic depolarization did in fact rescue a presynaptic component of LTP expression in GluN receptor blockade (Figure 2A).

Increases in CV^{-2} can be generated by the unmasking of postsynaptically silent synapses (Isaac et al., 1995; Voronin et al., 1996; Isaac et al., 1997); however, given that GluA receptor insertion requires GluN receptor activation (Luscher and Malenka, 2012), changes in CV^{-2} are likely to more accurately reflect presynaptic changes induced under my experimental conditions, in which GluN receptors are blocked. Nonethless, I sought to confirm presynaptic changes using a second, independent method: paired pulse ratio (PPR) analysis. PPR is calculated from the postsynaptic responses elicited by a pair of presynaptic stimuli, delivered 10-200ms apart, and is defined as the ratio of the postsynaptic response elicited by the second stimulus to the postsynaptic response elicited by the first stimulus. PPR is generally thought to only be influenced by presynaptic changes, and decreases as Pr increases (McNaughton, 1982; Schulz et al., 1994). I examined PPR in three experiments, by alternating basal synaptic stimulation with paired pulse stimulation, consisting of two stimuli delivered 70ms apart (Figure 2B). In all three experiments, the induction of GluN receptor-independent LTP resulted in an immediate, and long lasting decreases in PPR, which was stable for the duration of the recording (40 minutes) (PPR: baseline vs. post-tetanus: 2.19±0.15 vs. 1.25±0.10; n=3) (Figure 2B). These data suggest that LTP induction was accompanied by stable and long-lasting increases in transmitter release. Collectively, my findings strongly suggest that, provided postsynaptic depolarization is applied during

tetanus, the induction of stable, long-lasting presynaptic changes is not hindered by GluN receptor blockade.

3.2.2 Induction of presynaptic LTP under blockade of GluN receptors, GluA receptors, and Group I/II mGluRs

Given that the induction of presynaptic LTP does not strictly require GluN receptor activation, I asked whether it required the activation of other glutamate receptors. As with the GluN receptors, several studies have demonstrated that the need for GluA receptors (Fuenzalida et al., 2007; Holbro et al., 2010) or group I/II mGluRs (Wilsch et al., 1998) in LTP can be circumvented by augmenting postsynaptic depolarization either by current injection or by stronger presynaptic stimulation, suggesting that there is no strict requirement for any one of these receptors in the induction of LTP. Though, whether presynaptic LTP can be induced in the presence of a simultaneous blockade of GluN receptors, GluA receptors, and mGluRs has never been tested.

I, therefore, next attempted to induce LTP in a bath application of receptor antagonists against GluN receptors, GluA receptors, and group I/II mGluRs. I used sharp microelectrodes to record from either CA3 or CA1 neurons in organotypic slices, and evoked EPSPs by stimulating CA3 axons in stratum radiatum. Once a stable baseline was obtained, I applied 50-100μM AP5, 1μM NBQX and 200μM MCPG to block GluN receptors, GluA receptors, and group I/II mGluRs respectively. The antagonist cocktail abolished the EPSP within 5 minutes, and after 10 minutes I induced LTP by pairing 100Hz tetanic stimulation with postsynaptic depolarization. The drugs were than washed out in order to recover the EPSP. Compared to untetanized control experiments, tetanic stimulation induced a significant increase in EPSP amplitude following drug washout (LTP magnitude:

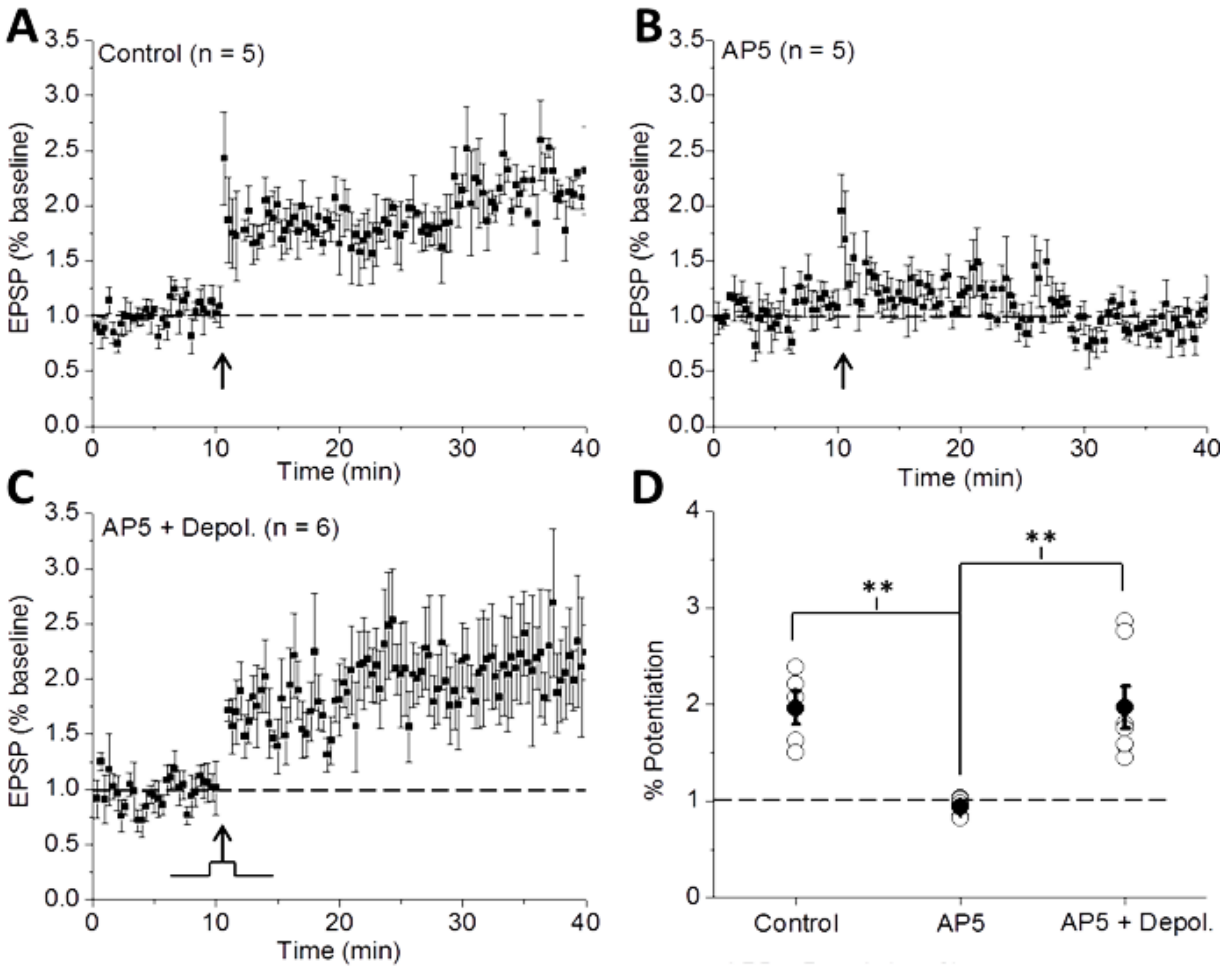


Figure 1. Postsynaptic depolarization during tetanic stimulation enables the induction of a GluN receptor-independent form of LTP. LTP was induced using 100Hz tetanus at CA3-CA3 or CA3-CA1 synapses in organotypic hippocampal slices (**A**) Tetanus stimulation (arrow) induced robust LTP under control conditions. (**B**) Tetanic stimulation failed to induce LTP in AP5. (**C**) Postsynaptic depolarization during tetanic stimulation resulted in robust LTP in AP5. (**D**) Group data representing the net potentiation in EPSP slope averaged 20-30 minutes after tetanization. Asterisks denote statistically significant differences between groups (Mann-Whitney test; $p<0.01$; n=5-7/condition). Error bars represent S.E.M.

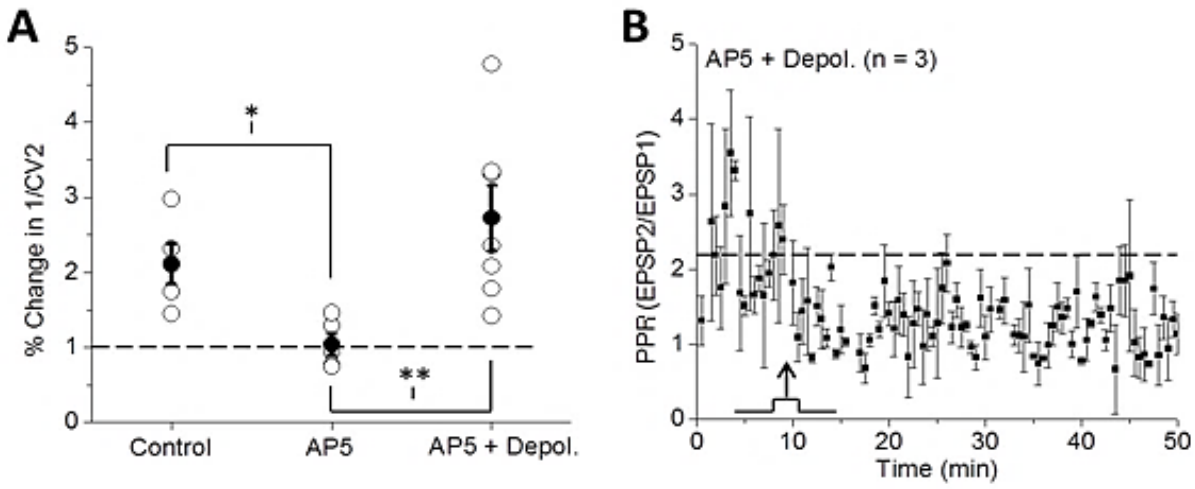


Figure 2. GluN receptor-independent long-term potentiation has a presynaptic component of expression. Tetanus-induced changes in presynaptic efficacy were assayed using CV^{-2} or PPR analysis. (**A**) Changes in CV^{-2} assessed 20-30 minutes post-tetanic stimulation. Tetanus-induced increases in presynaptic efficacy, as measured by an increase in CV^{-2}, were abolished in AP5, but rescued when tetanus was delivered during postsynaptic depolarization. (**B**) PPR was measured from a pair of stimulation pulses delivered 70ms apart. The induction of GluN receptor-independent LTP (arrow) resulted in an immediate increase in presynaptic efficacy, as measured by a decrease in the PPR, which remained stable for the duration of the recording. Asterisks denote statistically significant differences between groups (Mann-Whitney test; $p < 0.05$ (*) or $p<0.01$ (**); n=5-7/condition). Error bars represent S.E.M.

tetanized vs. control; 126±4.2% vs. 98.8±6.7%; n=6 and 5; p=0.008) (Figure 3A,C). The potentiation was modest, and accompanied by an equally modest increase of CV^{-2}, which, although suggestive of an increase in Pr, was not significantly different from control, untetanized recordings (CV^{-2}: tetanized vs. control;: 1.40±0.22 vs. 1.09±0.27; n=6 and 5; p =0.12)(Figure 3D).

The modest amount of potentiation achieved in glutamate receptor blockade may have resulted from a diminished ability to generate sufficient levels of postsynaptic depolarization at recorded synapses using somatically-evoked action potentials. Action potentials are known to attenuate as they travel away from the soma, primarily due to the presence of voltage-gated K^+ channels (I_A), the density of which increases along the somatodendritic axis (Hoffman et al., 1997; Migliore et al., 1999; Frick et al., 2003). Depolarizing the dendrite by GluA receptor-mediated synaptic activity or local current injection is known to inactivate these K^+ channels, thereby enhancing backpropagation of subsequently evoked action potentials into the dendrite (Spruston et al., 1995; Hoffman et al., 1997; Magee and Johnston, 1997; Migliore et al., 1999). Thus, in my experiments, dendritic depolarization evoked by synaptic activity during tetanic stimulation would normally facilitate the backpropagation of the action potential into the spine and dendrites under control conditions. However, this facilitation would presumably be absent under glutamate receptor blockade.

In an attempt to augment LTP induction in glutamate receptor blockade, I included 200mM tetraethylammonium chloride (TEA) in the sharp microelectrode, so as to improve action potential backpropagation by blocking voltage-gated K^+ currents within recorded cells.

Intracellular TEA led to a significant broadening of action potentials (TEA vs control AP width at half-height; 4.92ms±0.49ms vs. 1.74ms±0.20ms; n=4 and 5; $p < 0.01$), and, as predicted, intracellular application of TEA greatly improved the potentiation generated by pairing tetanic stimulation with postsynaptic depolarization during glutamate receptor blockade. (TEA vs. no TEA control LTP; 174±20%; vs 98.8±6.7%; n=4 and 5; $p<0.01$) (Figure 3B,C). Moreover, potentiation was associated with a large and significant change in CV^{-2} (TEA vs untetanized control ΔCV^{-2}: 5.49±0.90 vs 1.10±0.27; n=4 and 5; $p<0.01$) (Figure 3D), suggesting that this glutamate-independent form of potentiation had a strong presynaptic component of expression. Collectively, these findings suggest that, neither the activation of GluN receptor, GluA receptor nor group I/II mGluRs are strictly required for presynaptic potentiation, provided that presynaptic stimulation is accompanied by strong postsynaptic depolarization.

3.2.3 Induction of presynaptic LTP under full glutamate receptor blockade using complex spikes

In the previous set of experiments, a form of presynaptic LTP, independent of GluN receptors, GluA receptors, and group I/II mGluRs, was induced by high frequency stimulation in the presence of TEA. I next examined whether LTP could be elicited 1) in a full glutamate receptor blockade, including blockers against kainate receptors (GluK) and all of the mGluRs and 2) using a more physiologically-relevant paradigm, without the need for TEA. Studies recording from hippocampal pyramidal cells *in vivo* have shown that CA1 neurons generate either 1) single spikes that are phase-locked to the theta (~5Hz) oscillation or 2) complex spikes, that include 3-5 spikes at a high frequency 50-200Hz;

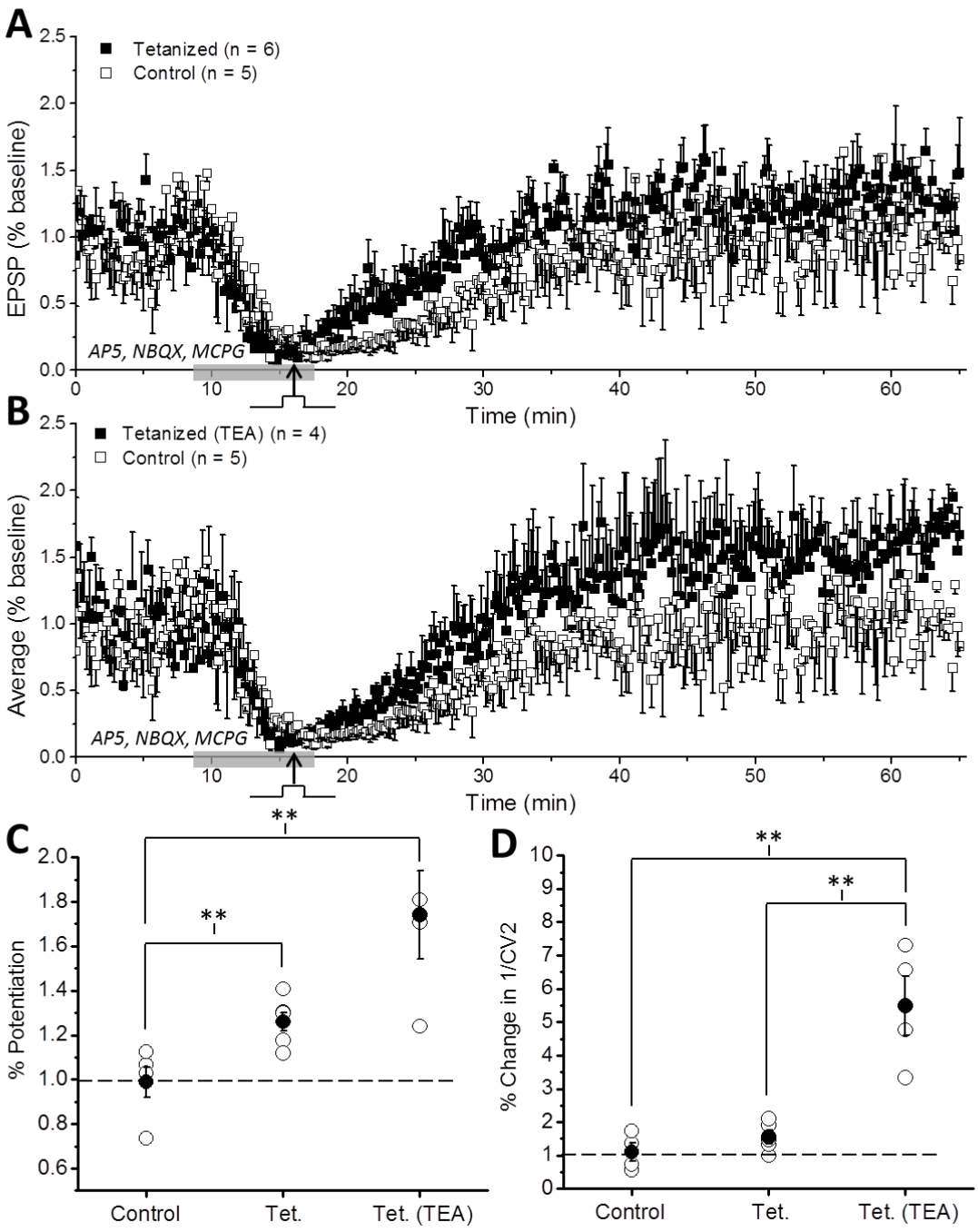

A
Tetanized (n = 6)
Control (n = 5)
EPSP (% baseline)
AP5, NBQX, MCPG
Time (min)
B
Tetanized (TEA) (n = 4)
Control (n = 5)
Average (% baseline)
AP5, NBQX, MCPG
Time (min)
C
% Potentiation
**
**
Control
Tet.
Tet. (TEA)
D
% Change in 1/CV2
**
**
Control
Tet.
Tet. (TEA)

Figure 3. Pairing presynaptic stimulation with strong postsynaptic depolarization in the presence of TEA induces LTP with a presynaptic component of expression in a GluN receptor, AMPAR and group I/II mGluR blockade (**A**) EPSP changes in tetanized and control experiments. Glutamate receptor antagonist cocktail (grey bar; 50uM AP5, 1uM NBQX, 500um MCPG) was applied for 10 minutes, during which one group of cells received 100Hz tetanic stimulation paired with postsynaptic depolarization and one group received no stimulation (control). (**B**) LTP experiments in which TEA was intracellularly applied to one group of cells to facilitate dendritic depolarization during tetanus. Control data are duplicated from (A). (**C**) Group data representing net potentiation, averaged 45-55 minutes after tetanization. Tetanic stimulation resulted in significant potentiation, which was augmented with the use of intracellular TEA. (**D**) Changes in CV^{-2} measured 45-55 minutes after tetanization. LTP induced in the presence of intracellular TEA was accompanied by a significant increase in CV^{-2}, and therefore likely had a presynaptic component of expression. Asterisks denote statistically significant differences between groups (Mann-Whitney test; $p < 0.05$ (*) or $p<0.01$ (**); n=4-6/condition). Error bars represent S.E.M.

postsynaptic patterns of firing have been shown to be efficient at inducing LTP (Ranck, 1973; Grienberger et al., 2013). I therefore examined whether pairing presynaptic stimuli, delivered at a theta frequency, could elicit glutamate-receptor independent LTP when each stimuli was paired with a postsynaptic complex spike. These complex spikes were simulated with postsynaptic current injection of sufficient magnitude to generate 3-5 postsynaptic spikes over a 50ms time course, with the first spike starting 7-10ms following the start of current injection (Figure 4).

For this experiment, I recorded from CA1 pyramidal neurons using high-resistance patch electrodes (18-25MΩ); these electrodes provide better electrical access to the postsynaptic cell than sharp microelectrodes, and compared to normal patch electrodes, are less likely to perturb the intracellular milieu during recording. In contrast to earlier experiments, I positioned stimulating electrodes close to the soma (50-70μm), rather than at more distal sites (100-200μm away from the soma) in order to ensure that postsynaptic current injections would reach recorded synapses without significant attenuation (Gasparini et al., 2007). Following a baseline recording, I applied a cocktail of antagonists to block all known glutamate receptors (50-100μM AP5, 10μM NBQX, 500μM MCPG, 100μM LY341495), which included a higher concentration of NBQX (10μM) to block GluK receptors and high concentration of LY341495 (100μM) to block all mGluRs. 7 minutes following drug application, LTP was induced by pairing each of 60 single presynaptic stimuli, delivered at 5Hz, with a simulated complex spike. The antagonists were then washed out. Because of the higher concentration of NBQX used, EPSP recovery was never complete and varied considerably between experiments (20-80%), likely reflecting differences in the depth of recording (Figure 5). To circumvent this problem, changes in the EPSP recorded from the

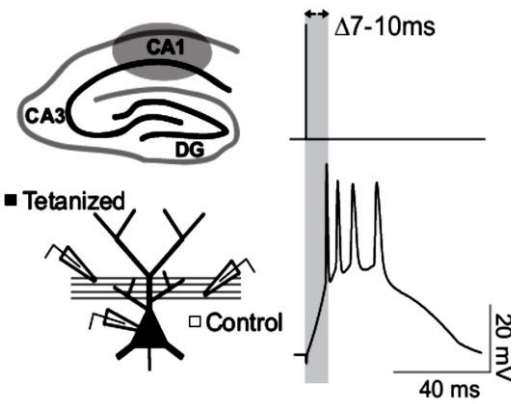
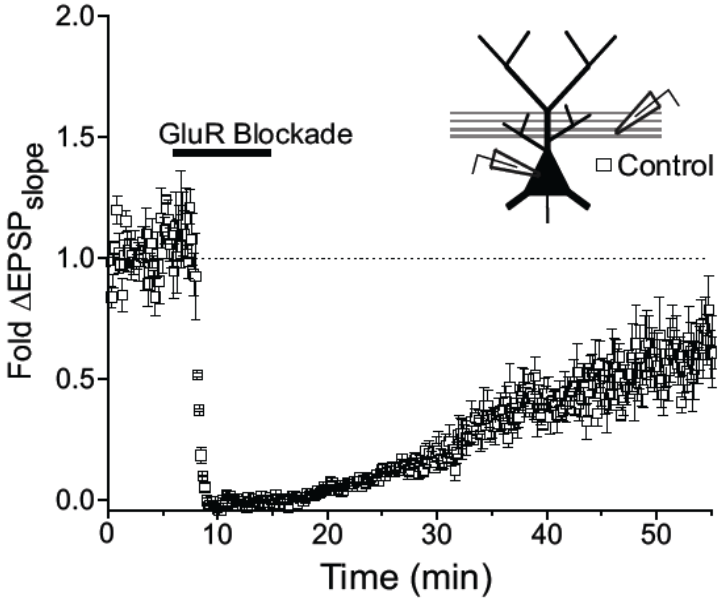


Figure 4. Experimental setup to examine the necessity of glutamate in LTP induction. (Left) CA1 neurons were recorded from using high resistance patch electrodes (18-25MΩ) to minimize dialysis. EPSPs were evoked by stimulation of two independent Schaffer-collateral pathways. (Right) Following glutamate receptor blockade, the tetanized pathway received paired stimulation, consisting of a single presynaptic stimulus paired with a postsynaptic complex spike, with the first postsynaptic action potential commencing 7-10ms after the presynaptic stimulus. The pairing was repeated 60 times at 5Hz, after which the glutamate receptor antagonists were washed out. Due to incomplete drug washout, EPSP amplitude in the tetanized and control pathway were compared to assess the magnitude of LTP.

Figure 5. Incomplete washout of glutamate receptor antagonists in hippocampal slices. EPSPs were recorded from CA1 neurons in organotypic slices. Application of glutamate receptor antagonists (black bar) (50µM AP5, 10µM NBQX, 500µM MCPG, 10µM LY341495) abolished the EPSP. EPSP recovery commenced after washout, but was incomplete 40 minutes post-washout (n=5). Error bars represent S.E.M.



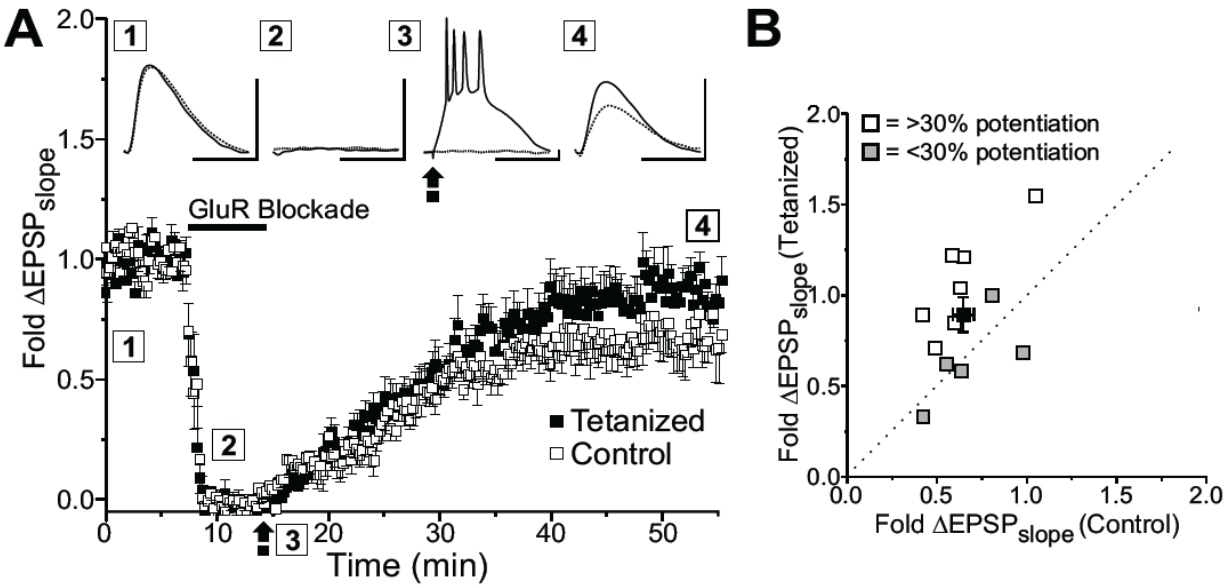


Figure 6. Glutamatergic signalling is not required for LTP induction. (**A**) Fold change in the EPSP slope is plotted against time in control and tetanized pathways (n=12). Sample EPSP traces shown are averages of 10 traces from the tetanized (solid line) and control (broken line) pathway for a single experiment at 4 different time points (scale bar: 4.0mV by 40ms for tetanized EPSP, 4.9mV by 40ms for control EPSP). Stimulation artifacts have been reduced for clarity. Paired pre- and post- synaptic stimulation was applied to the tetanized pathway following glutamate receptor blockade (arrow). (**B**) For each experiment, the fold change in EPSP slope in the tetanized pathway is plotted against the fold change in EPSP slope in the control pathway. The broken diagonal line represents the expected trend if EPSP recovery in both the tetanized and control pathway were equal. Robust potentiation (>30%) was observed in 7 of 12 experiments (white boxes). On average (including failures), tetanized values were significantly greater than control values (black box) (Wilcoxon matched-pairs test; $p<0.05$; n=12). Error bars represent S.E.M.

tetanized pathway were normalized to that recorded from a second, control pathway, which stimulated at a similar depth (Figure 4). Remarkably, in 7 of 12 cells, tetanization induced a robust enhancement of the EPSP slope (>30%; normalized fold $\Delta EPSP_{slope}$ tetanized/control: 1.73±0.11; fold $\Delta EPSP_{slope}$ tetanized vs. control: 1.07±0.11vs. 0.63±0.07; n=7; p=0.017), which lasted for the duration of the recording (40-90 minutes post-tetanus) (Figure 6). Failure to induce LTP in the remaining 5 experiments (normalized fold $\Delta EPSP_{slope}$ tetanized/control: 0.95±0.10; fold $\Delta EPSP_{slope}$ tetanized vs. control: 0.64±0.11 vs. 0.68±0.10; n=5; p=0.69) may have resulted from intracellular dialysis, since I found no tetanus-induced enhancements of the EPSP (>30%) when a low-resistance patch electrode was used (4-6MΩ; n=4; data not shown). However, when an ATP-regenerating internal solution (Kullmann et al., 1992) was used instead of the standard patch solution, I was able to consistently obtain LTP using low-resistance patch electrodes (data not shown). When data from all 12 experiments were pooled, the enhancement of the EPSP slope in tetanized pathway was still significant ($\Delta EPSP_{slope}$ in tetanized vs. control pathway: 0.89±0.10 vs. 0.65±0.06; n=12; p=0.027) (Figure 6). To examine whether the LTP induced had a presynaptic component of expression, I used CV^{-2} and PPR analysis. In experiments showing LTP (>30% potentiation), the tetanized pathway showed robust increases in CV^{-2} and decreases in PPR that were significantly greater than that of the control pathway, suggesting that LTP had a presynaptic component of expression (ΔCV^{-2}: tetanized pathway vs. control pathway: 1.55±0.19 vs. 0.61±0.09; n=7; p=0.01) (ΔPPR: tetanized pathway vs. control pathway: -0.27±0.05 vs. -0.07±0.05; n=7; p=0.02) (Figure 7B,C). The control pathway did show considerable decreases in CV^{-2} 30-40 minutes following paired stimulation of the tetanized pathway (0.61±0.09; n=7), though such decreases likely

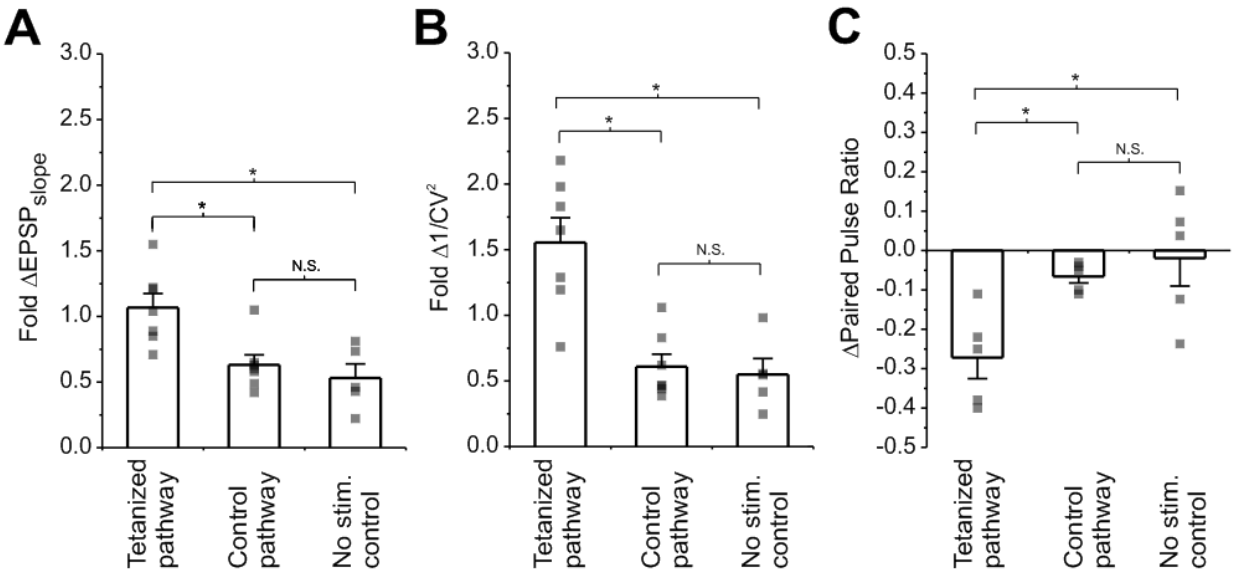


Figure 7. LTP induced in glutamate receptor blockade has a presynaptic component of expression and is site specific. Group data and averages plotted for (**A**) fold ΔEPSP slope (**B**) fold ΔCV^{-2} and (**C**) ΔPPR for 3 pathways: 1) The tetanized pathway, which received paired pre- and post- synaptic stimulation during glutamate receptor blockade and which showed >30% potentiation (Figure 6 data; n=7), 2) the corresponding control pathway, which was simultaneously recorded alongside the tetanized pathway but received no paired stimulation (Figure 6 data; n=7), and 3) an unstimulated control pathway in a separate set of experiments in which glutamate receptor antagonists were applied but no paired stimulation was delivered in any pathway (Figure 4 data; n=5). ΔEPSP slope, ΔCV^{-2}, and ΔPPR, in the control pathway were not different from unstimulated control experiments, but differed from the tetanized pathway, suggesting that LTP had a presynaptic component of expression and was selectively induced in the tetanized pathway. Asterisks denote statistically significant differences between groups (Mann-Whitney-Wilcoxon test; $p < 0.05$ level; n=5-7/condition). N.S. denotes no significant differences between groups. Error bars represent S.E.M.

resulted from an incomplete washout of the drugs and were also apparent in unstimulated control experiments, which received a 7 minute bath application of the drug, but in the absence of paired stimulation (fold ΔCV^{-2}: control pathway vs. unstimulated control; n= 7 and 5; p=0.75) (Figure 7B). The drug-induced decrease in CV^{-2} was likely not due to presynaptic depression, as PPR was unchanged in both the control pathway (PPR: baseline vs. post-drug washout; 1.36±0.08 vs. 1.29±0.08 n =5; p=0.53) and in unstimulated control experiments (PPR: baseline vs. post-drug washout; 1.54±0.19 vs. 1.52±0.19 n =5; p=1.00). The decrease in CV^{-2} likely resulted from an apparent decrease in the number of active synapses; it is possible that incomplete drug washout may have rendered a portion of synapses, particularly those with weak postsynaptic responses in the baseline period, effectively silent. Nonetheless, that changes in the EPSP slope, CV^{-2}, and PPR were not significantly different in the control pathway and in the unstimulated control experiments (ΔEPSP slope: control pathway vs. unstimulated control: 0.63±0.08 vs. 0.53±0.11; n=7 and 5; p=0.62) (ΔCV^{-2}: control pathway vs. unstimulated control: 0.61±0.09 vs. 0.55±1.20; n=7 and 5; p=0.75) (ΔPPR: control pathway vs. unstimulated control: -0.07±0.05 vs. -0.02±0.07; n=7 and 5; p=0.67) strongly suggested that the induction of presynaptic LTP was site specific, and was therefore restricted to the tetanized pathway (Figure 7A-C).

Since not all experiments showed successful LTP induction, as assessed by a >30% increase in the EPSP slope, I examined whether changes in EPSP slope across all experiments were correlated with changes in CV^{-2} and PPR. To estimate the magnitude of LTP induction, ΔEPSP slope in the tetanized pathway was normalized to values recorded in the control pathway. ΔCV^{-2} was similarly normalized. When ΔCV^{-2} were plotted against ΔEPSP slope for all 12 experiments, including the 7 which showed >30% potentiation and the 5 experiments

which did not, a significant positive correlation emerged (r=0.61; n=12; p=0.04) (Figure 8A). I also found a significant correlation between changes in PPR and the magnitude of LTP (r=-0.73 n=10; p=0.02; PPR was only measured in 10 of the 12 experiments) (Figure 8B). In both instances, only experiments demonstrating robust potentiation (>30%) showed substantial changes in CV^{-2} and PPR. These findings support the notion that LTP, when successfully induced, was predominantly supported by presynaptic changes.

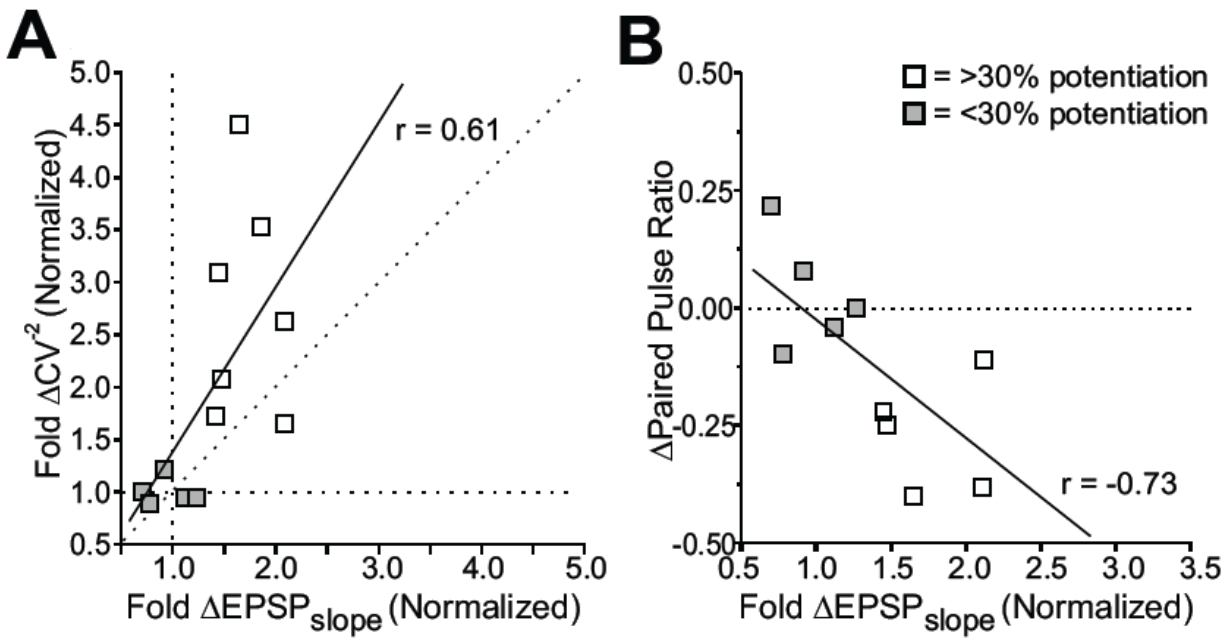


Figure 8. LTP induced in glutamate receptor blockade has a presynaptic component of expression. (**A**) Fold ΔCV^{-2} is plotted against the fold ΔEPSP slope for the 12 experiments in Figure 6; tetanized values are normalized to control values. White boxes represent experiment showing >30% potentiation (n=7). Grey boxes represent experiments showing <30% potentiation (n=5). The broken diagonal represents the expected trend if ΔCV^{-2} =ΔEPSP. (**B**) Changes in PPR are plotted against fold change in EPSP slope for 10 of the 12 experiments in Figure 6, in which PPR was recorded. Both fold ΔCV^{-2} and ΔPPR were significantly correlated with the magnitude of LTP ($p<0.05$; n=10-12/condition), suggesting that LTP, when it was induced, had a presynaptic component of expression.

3.3 Discussion

Here, I have demonstrated that LTP can be rescued both in GluN receptor blockade and in full glutamate receptor blockade by augmenting the levels of postsynaptic depolarization that accompany presynaptic stimulation. Remarkably, even physiologically relevant levels of postsynaptic depolarization, as driven by complex spikes, could induce glutamate-independent LTP in this way.

My findings suggest that the induction of presynaptic LTP, in contrast to postsynaptic LTP, requires no specific Ca^{2+} or metabotropic signalling by any glutamate receptor; rather, I would argue that the role of glutamate release in the induction of presynaptic LTP is to generate strong postsynaptic depolarization, likely to drive the activation of L-VGCCs, which have been previously implicated in presynaptic potentiation (Zakharenko et al., 2002; Zakharenko et al., 2003; Bayazitov et al., 2007). In a physiological context then, it may be possible for a presynaptic terminal that releases little or no glutamate to become potentiated provided that its activity coincides with strong postsynaptic activity, driven by release of glutamate at co-active synapses. In fact, given that plasticity appears to be preferentially driven by glutamate release at clusters of co-active synapses (Schiller and Schiller, 2001; Govindarajan et al., 2006; Losonczy et al., 2008; Govindarajan et al., 2011; Makino and Malinow, 2011; Fu et al., 2012; Takahashi et al., 2012), glutamate release at neighbouring synapses within a dendrite would be a far more efficient means of driving local dendritic depolarization than the somatic current injections used in this experiment.

The finding that presynaptic change can occur independently of GluN receptor activation appears to be at odds with findings from other laboratories, including our own, that

demonstrate that GluN receptor blockade abolishes, or at least reduces, presynaptic enhancement (Ryan et al., 1996; Zakharenko et al., 2001; Emptage et al., 2003; Bayazitov et al., 2007; Ratnayaka et al.). It is however important to recognize that the GluN receptor, in addition to acting as a Ca^{2+} source for the spine, is also a potent source of depolarization for the cell and dendrite. The GluN receptor is far more permeable to Na^{+} than it is to Ca^{2+}, and the activation of the receptor facilitates somatic and dendritic spiking (Herron et al., 1986; Mayer and Westbrook, 1987; Grover and Yan, 1999b; Schiller et al., 2000; Schiller and Schiller, 2001). Although postsynaptic enhancement depends on GluN receptors as a source of Ca^{2+}, presynaptic enhancement, may only rely on GluN receptors as a source of postsynaptic depolarization. This would explain why GluN receptor antagonists abolish presynaptic potentiation during standard 100Hz, but not during 200Hz or theta burst stimulation protocols, which are more effective at producing postsynaptic depolarization via GluA receptor activation (Zakharenko et al., 2001; Zakharenko et al., 2003; Bayazitov et al., 2007). It is important to note that presynaptic potentiation can also be obtained when single presynaptic stimuli are paired with postsynaptic depolarization, which rules out any specific requirement of high frequency presynaptic activity for the enhancement of presynaptic strength (Stricker et al., 1999; Enoki et al., 2009).

In addition to GluN receptors, GluA receptors and mGluRs can also be important for the full-expression of LTP induced by standard stimulation protocols (Bashir et al., 1993; Luscher et al., 1998; Fuenzalida et al., 2007; Holbro et al., 2010). GluA receptor-mediated depolarization is thought to facilitate LTP by augmenting Ca^{2+} influx from GluN receptors or VGCCs (Blundon and Zakharenko, 2008; Holbro et al., 2010; Luscher and Malenka, 2012), and mGluRs are thought to facilitate LTP by increasing synaptic Ca^{2+} levels via IP_3-mediated

release of Ca^{2+} from the endoplasmic reticulum (Petrozzino and Connor, 1994; Wilsch et al., 1998; Raymond and Redman, 2006)., and also by contributing to dendritic depolarization via G-protein-dependent mechanisms (Gee et al., 2003; Fan et al., 2010). Nonetheless, consistent with findings from my study, several groups have demonstrated the need for GluN receptors, GluA receptors or mGluRs can be circumvented by using stronger stimulation protocols, either by depolarizing the postsynaptic cell during presynaptic stimulation, increasing the stimulation intensity or frequency of tetanic stimulation, or using theta-burst stimulation to more effectively drive postsynaptic spiking (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Grover and Teyler, 1992; Kullmann et al., 1992; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Petrozzino and Connor, 1994; Hanse and Gustafsson, 1995; Huber et al., 1995; Little et al., 1995; Platt et al., 1995; Pananceau and Gustafsson, 1997; Grover, 1998; Luscher et al., 1998; Manahan-Vaughan et al., 1998; Thomas et al., 1998; Wilsch et al., 1998; Grover and Yan, 1999b, a; Hsu et al., 1999; Stricker et al., 1999; Borroni et al., 2000; Ramakers et al., 2000; Morgan and Teyler, 2001; Zakharenko et al., 2001; Zakharenko et al., 2003; Wang et al., 2004; Woodside et al., 2004; Moosmang et al., 2005; Raymond and Redman, 2006; Bayazitov et al., 2007; Fuenzalida et al., 2007; Galvan et al., 2008; Phillips et al., 2008; Pinheiro and Mulle, 2008; Grover et al., 2009; Holbro et al., 2010; Bonansco et al., 2011).

Contrary to my findings, however, there have been several studies that have demonstrated that LTP induced in AP5, either by stimulating at 200Hz or by delivering low frequency stimulation in the presence of TEA, is dependent on the activation of mGluRs (Grover and Teyler, 1990, 1992; Petrozzino and Connor, 1994; Little et al., 1995; Platt et al., 1995; Grover, 1998; Grover and Yan, 1999a). The reason for this discrepancy is not clear, but may

be due to the differential induction protocols used to induce LTP. It should be noted that there are also discrepancies across, and even within, studies concerning the ability of various mGluR inhibitors in blocking the induction of GluN receptor-independent LTP (Petrozzino and Connor, 1994; Grover and Yan, 1999a). Perhaps the most in-depth study of the role of mGluRs in the induction of GluN receptor-independent LTP was conducted by Grover et al. (1999). They found that LTP induced by 200Hz tetanic stimulation in AP5 was blocked by the Group II antagonist EGlu and the Group III antagonist CPPG, but not with the Group II antagonist MSOPPE, nor the Group III antagonist MSOP; the reason for these inconsistences is not known, but may relate to off-target drug effects. The study also reported no effect of Group I antagonists. It is also important to mention that, because of solubility issues, Grover et al. (1999) dissolved their mGluR antagonists in sodium hydroxide (NaOH), which, at the concentration used in their study (up to 1mM), could have caused long-lasting decreases in the EPSP, in part, by reducing vesicular glutamate concentration through vesicular de-acidification (Moriyama et al., 1990; Grover and Yan, 1999a). Such NaOH-induced decreases therefore, make it less likely that 200Hz stimulation will generate sufficient postsynaptic depolarization to circumvent the requirement of mGluRs in LTP induction. To prevent any non-specific effects of NaOH, the mGluR antagonists used in my study were water soluble sodium salts, which have recently been made available, and were applied at concentrations that are known to block mGluR-dependent responses (Little et al., 1995; Fitzjohn et al., 1998; Grover and Yan, 1999a; Wu et al., 2004).

In this study, I found that pairing presynaptic stimuli with complex spikes was an effective method for producing glutamate-independent LTP. This paradigm, however, resembles

spike-timing dependent plasticity (STDP) protocols, which have been shown to be dependent on GluA receptor and GluN receptor activation (Gustafsson et al., 1987; Dan and Poo, 2004; Nevian and Sakmann, 2006; Fuenzalida et al., 2007; Rodriguez-Moreno and Paulsen, 2008; Fuenzalida et al., 2010; Holbro et al., 2010). Although STDP protocols similarly pair presynaptic stimuli with bursts of postsynaptic spiking, there are two main differences between this protocol and the one used in my study. Firstly, the period of subthreshold depolarization that precedes the first postsynaptic spike in STDP protocols is generated by evoked glutamate release, rather than by postsynaptic current injection, and is therefore sensitive to glutamate receptor blockade. This period of depolarization is thought to facilitate the induction of LTP by inactivating dendritic voltage-gated K^+ channels within the dendrite, which would otherwise attenuate action potential backpropagation (Hoffman et al., 1997; Johnston et al., 1999; Gasparini et al., 2007). Secondly, the current injection I used elicited broadened action potentials in the neuron, reminiscent of the complex spikes recorded in vivo (Ranck, 1973); these broadened action potentials are likely to generate stronger dendrite depolarization than the simple spikes used in STDP protocols (Hoffman et al., 1997; Migliore et al., 1999). Consequently, the postsynaptic waveforms used in my study were more likely to generate stronger levels of postsynaptic depolarization, and in a manner independent of glutamate release, than those used in STDP studies.

3.4 Conclusion

The application of the GluN receptor-dependent model of plasticity to the induction of presynaptic LTP poses a significant theoretical problem, in that it fails to provide a

mechanism by which the presence of correlated pre- and post- synaptic activity can generate Hebbian/Konorskian LTP at a synapse releasing no glutamate. In this chapter, I have examined the necessity of glutamatergic signalling in the induction of LTP at the presynaptic terminal using standard electrophysiological techniques to show that LTP, with a presynaptic component of expression, can be induced in full glutamate receptor blockade, provided that presynaptic activation is paired with strong postsynaptic depolarization. My findings suggest that it may be possible for a presynaptic terminal to become potentiated without needing to release any glutamate, provided that its activity coincides with strong postsynaptic depolarization, which in a physiological setting would be driven by glutamate release at other co-active inputs. My findings also suggest that the mechanisms underlying the induction of LTP at the presynaptic locus clearly differ from the GluN receptor-dependent mechanisms that underlie the induction of LTP at the postsynaptic locus. In the next chapter, I explore the signalling pathways involved in presynaptic LTP, as induced under full glutamate receptor blockade.

4. MECHANISMS UNDERLYING THE LONG-TERM POTENTIATION OF PRESYNAPTIC FUNCTION

4.1 Introduction

The mechanisms underlying the induction of presynaptic plasticity have remained controversial. Although postsynaptic NMDA receptors (GluN) have long been implicated in the induction of presynaptic long-term potentiation (LTP), several studies have now demonstrated that increases in transmitter release probability (Pr) can in fact be induced in the presence of GluN receptor antagonists (Ryan et al., 1996; Stricker et al., 1999; Bayazitov et al., 2007; Ratnayaka et al., 2012). In the previous chapter I extended these findings and demonstrated that presynaptic potentiation could in fact be obtained in full glutamate-receptor blockade. Synapses releasing little or no glutamate may, therefore, still be able to become potentiated provided that their activity coincides with strong postsynaptic depolarization. In this chapter, I now examine the mechanisms underlying the induction of presynaptic LTP.

Presynaptic LTP, even in GluN receptor or glutamate receptor blockade, is still characteristically Hebbian/Konorskian in nature as it requires presynaptic stimulation to coincide with strong postsynaptic depolarization (Grover, 1998; Grover and Yan, 1999b). Several studies have demonstrated that GluN receptor-independent LTP requires the activation of L-type voltage-gated Ca^{2+} channels (L-VGCCs) (Aniksztejn and Ben-Ari, 1991; Kullmann et al., 1992; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995; Morgan and Teyler, 2001; Bayazitov et al., 2007). L-VGCCs would act as an ideal detector for postsynaptic depolarization, especially since their activation does not rely directly on glutamatergic signalling, meaning that they could enable the induction of

presynaptic LTP for synapses releasing little or no glutamate. However, the induction of a Hebbian/Konorskian form of LTP at the presynaptic locus requires, in addition to a postsynaptic detector of depolarization, a retrograde signal, which is synthesized postsynaptically but acts presynaptically. Several putative messengers have been proposed (Williams et al., 1989; Fitzsimonds and Poo, 1998; Blundon and Zakharenko, 2008); however, the most commonly investigated candidate has been, and continues to be, nitric oxide (NO) (Padamsey and Emptage, 2013).

NO is a diffusible gas, whose synthesis in the hippocampus can be driven in an activity-dependent manner (Garthwaite et al., 1988; von Bohlen und Halbach et al., 2002). Moreover, both neuronal and endothelial variants of NO synthase have been localized postsynaptically in CA1 neurons (Dinerman et al., 1994; Wendland et al., 1994), and double knockouts of these variants produces deficits in LTP induction (O'Dell et al., 1994; Son et al., 1996; Wilson et al., 1999). Although findings are inconsistent across labs, the vast majority of studies demonstrate impairments in LTP and in presynaptic changes following pharmacological disruption of NO signalling, either by inhibition of NO synthase, or by the use of NO scavengers (Bohme et al., 1991; O'Dell et al., 1991b; Schuman and Madison, 1991; Nikonenko et al., 2003; Stanton et al., 2005; Johnstone and Raymond, 2011; Ratnayaka et al., 2012; Padamsey and Emptage, 2013). Moreover, exogenous NO, either directly applied as gaseous form, indirectly applied using a NO donor compound, or photoreleased from a caged precursor, can induce LTP when paired with presynaptic activity (O'Dell et al., 1991b; Bon et al., 1992; Zhuo et al., 1993; Zhuo et al., 1994; Arancio et al., 1996; Malen and Chapman, 1997; Zhuo et al., 1998; Nikonenko et al., 2003) [but see (Boulton et al., 1994; Murphy et al., 1994; Murphy and Bliss, 1999)]. Thus, despite some inconsistent findings

across laboratories NO can, at least under some experimental conditions, function as an effective retrograde signal for the potentiation of the presynaptic functions.

The role of NO in presynaptic LTP creates an apparent paradox since many studies have shown that NO signalling is in fact triggered by the activation of postsynaptic GluN receptors (Garthwaite et al., 1988; Garthwaite and Boulton, 1995) , a receptor which I and others have shown to be unnecessary for presynaptic potentiation (Stricker et al., 1999; Bayazitov et al., 2007). The paradox, however, is resolved when one considers that Ca^{2+} influx via L-VGCCs might be linked to NO production (Sattler et al., 1999; Stanika et al., 2012); if such a mechanism is present in neuronal dendrites, then NO-driven potentiation of presynaptic terminals could occur in a manner dependent on postsynaptic depolarization, but independent of glutamatergic signalling.

Here, I use an optical approach to explore the mechanisms underlying presynaptic LTP. I demonstrate that activation of L-VGCCs by postsynaptic depolarization drives the production of NO synthesis in neuronal dendrites, and that NO, when paired with presynaptic activity, can drive presynaptic potentiation. This mechanism is therefore sensitive to paired pre- and postsynaptic activity and can induce presynaptic LTP in the absence of glutamatergic signalling.

4.2 Results

4.2.1 Long-term increases in Pr requires L-VGCC activation and NO signalling

In the previous chapter, I assessed presynaptic changes across a population of synapses by using CV^{-2} and PPR analysis. My first objective in this chapter was to study presynaptic changes more directly, and with single-synapse resolution. For this purpose, I used spine

Ca^{2+} imaging to assay glutamate release at individual synapses (Emptage et al., 2003; Ward et al., 2006; Padamsey and Emptage, 2011). Briefly, CA1 pyramidal cells were preloaded with the Ca^{2+} sensitive dye, Oregon Green BAPTA-1 (OGB-1). A glass stimulating electrode was then brought close to an imaged dendrite, within 100µm from the soma. At most CA3-CA1 synapses, uniquantal glutamate release, through AMPA receptor (GluA)-mediated depolarization, generates sufficient Ca^{2+} influx from GluN receptors and VGCCs to be detected by Ca^{2+} sensitive dyes (Emptage et al., 1999; Grunditz et al., 2008). As a result, it was often possible to find dendritic spines that fluorescently responded to afferent stimulation. Once a responsive spine was found, afferent stimulation was delivered at a low frequency (0.05Hz), and the proportion of stimuli that elicited a fluorescent response in the imaged spine was taken as a measure of transmitter release probability (Pr) at the associated presynaptic terminal (Emptage et al., 2003; Ward et al., 2006; Padamsey and Emptage, 2011). As a result of mechanical drift, it is possible to lose the ability to stimulate the imaged synapse, which in turn would affect Pr estimates. To guard against this possibility, I used paired stimuli, delivered 70ms apart, to evoke spine Ca^{2+} transients instead of single pulses. Since paired pulse stimulation is far more likely to drive glutamate release at a synapse, any loss in the ability to stimulate the imaged synapse would be rapidly detected. Under these conditions, Pr was calculated as the likelihood that the first stimulus of the pair elicited a fluorescence response in the spine (Figure 1).

Using Ca^{2+} imaging, I confirmed that LTP induced in glutamate receptor blockade had a presynaptic component of expression. In the previous chapter, LTP induction in glutamate receptor blockade only led to EPSP potentiation in 7 of 12 experiments. I suspected that failure to induce LTP in the remaining 5 experiments was due to dialysis; consequently, in

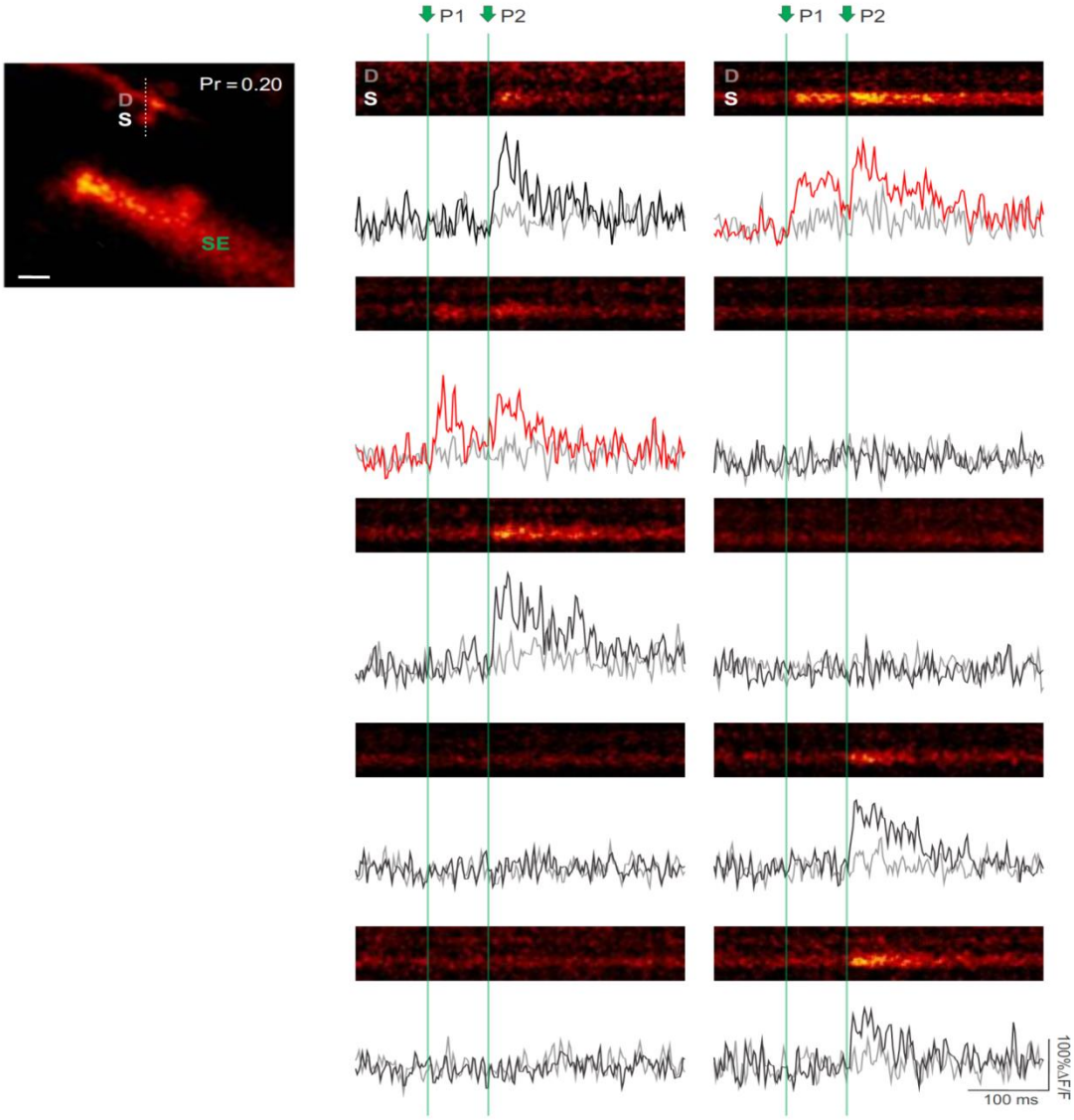


Figure 1. Assessing Pr at single synapses using Ca^{2+}-sensitive dyes. CA1 neurons were loaded with Ca^{2+}- sensitive dye (OGB-1). (**Left**) An image of a CA1 neuronal dendrite loaded with Oregon Green Bapta-1 Ca^{2+}-sensitive dye. A stimulating electrode (SE), labelled with Alexa488, was placed close to the dendrite in order to activate spines within the vicinity (scale bar: 2µm). Once a spine responsive to stimulation was located, evoked Ca^{2+} transients were rapidly imaged by restricting laser scanning to a line (broken line) across the spine head (S) and underlying dendrite (D). (**Right**) Samples of these linescans are shown. Traces quantifying the fluorescence change (%ΔF/F) for the spine (black trace; unfiltered) and dendrite (grey trace; unfiltered) are shown below each scan. Ca^{2+} responses were evoked using 2 stimuli delivered 70ms apart (P1 and P2, vertical green lines). Pr was calculated as the proportion of total stimulation trials in which the first of the two pulses elicited a Ca^{2+} transient (red traces). Based on 40 stimulation trials, 10 of which are shown, the Pr of this synapse was calculated to be 0.2.

order to minimize the effects of dialysis in these experiments, I minimized the amount of time the cell was patched for. To do so, I first loaded CA1 neurons with OGB-1 by transiently patching on to them for ~30-60s. Following this, I searched the cell for a spine responsive to afferent stimulation, and calculated its Pr using 15-40 paired pulses. I then transiently re-patched the cell for the purposes of delivering postsynaptic depolarization during LTP induction. As before, LTP was induced in a glutamate receptor blockade (50-100μM AP5, 10μM NBQX, 500μM MCPG, 100μM LY341495) by pairing each of 60 presynaptic stimuli, delivered at 5Hz, with a postsynaptic complex spike consisting of 3-6 postsynaptic action potentials. Glutamate receptor antagonists were then washed out for 25-30 minutes, and Pr was reassessed. Consistent with my electrophysiological findings, pairings produced robust and reliable increases in Pr (ΔPr: 0.33±0.06; n=13), which were observed in 12 of 13 imaged synapses (Figure 2). No such changes occurred with drug application alone (ΔPr: 0.04±0.03; n=8; antagonists vs. antagonists + paired depolarization; p=0.003), or when presynaptic stimulation was unaccompanied by postsynaptic spiking (ΔPr: -0.01±0.02; n=7; antagonists alone vs. antagonists + paired depolarization; p=0.02) (Figure 2B), suggesting that, as shown in chapter 3, induction of presynaptic LTP required paired pre- and post-synaptic depolarization.

Next, I examined whether presynaptic LTP was dependent on L-VGCC and NO signalling using a pharmacological approach. I found that the L-VGCC inhibitor, nitrendipine (20μM), abolished pairing-induced changes in Pr, (ΔPr: 0.04±0.04; n=6; antagonists + nitrendipine vs. antagonists: p=0.016) as did the NO scavenger, carboxy PTIO (cPTIO; 100μM), when applied either extracellularly (ΔPr: -0.02±0.04; n=7; antagonists + cPTIO vs. antagonists: p=0.0013), or injected in the postsynaptic neuron (ΔPr: -0.02±0.07; n=6; antagonists +

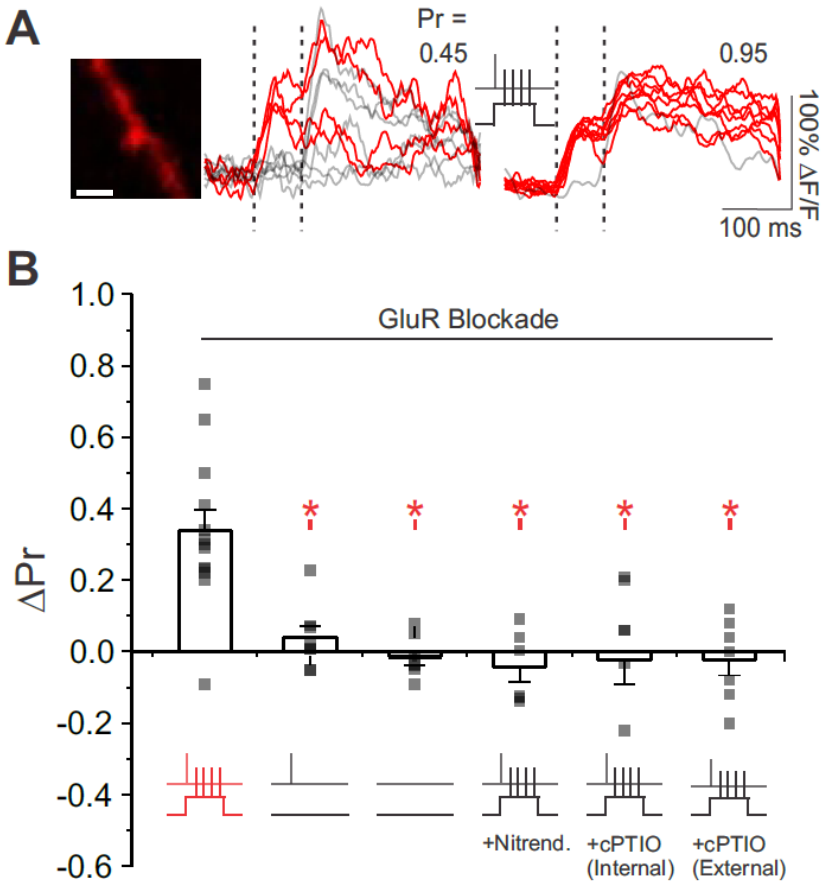


Figure 2. Presynaptic LTP requires L-VGCC activation and NO signalling. (**A**) Sample LTP induction experiment. 10 superimposed Ca^{2+} traces evoked in an imaged spine (white scale bar: 2µm) by paired pulse stimulation (2 stimuli delivered 70ms apart; represented by vertical broken lines); red traces depict successful release events in response to the first of the two pulses. Above the sample Ca^{2+} traces are values for Pr, which have been calculated as the proportion of total stimulation trials that resulted in a successful release event. For each spine, sample Ca^{2+} traces are shown during baseline and 25-30 minutes following LTP induction in glutamate receptor blockade (50µM AP5, 100µM NBQX, 500µM MCPG and 100µM LY341495). LTP induction consisted of 60 presynaptic pulses, delivered at 5Hz, each of which was paired with a postsynaptic complex spike consisting of 3-6 action potentials. (**B**) Group data depicting ΔPr across experimental conditions. Increases in Pr were only evoked by paired pre- and post- synaptic depolarization, and were abolished by the L-VGCC blocker nitrendipine, and by both extracellular and intracellular application of the NO scavenger cPTIO. Red asterisks denote significant differences from the first group (labelled in red) (Mann-Whitney test; $p<0.05$; n=5-13/condition). Error bars represent S.E.M.

intracellular cPTIO vs. antagonists: p=0.0029) (Figure 2B). Consistent with past studies, these findings suggest that L-VGCC activation and NO signalling are both essential for presynaptic potentiation (Aniksztejn and Ben-Ari, 1991; Kullmann et al., 1992; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995; Morgan and Teyler, 2001; Nikonenko et al., 2003; Stanton et al., 2005; Johnstone and Raymond, 2011; Ratnayaka et al., 2012).

4.2.2 L-VGCC activation promotes the dendritic synthesis of NO

I next examined whether L-VGCC activity promotes the release of NO from neuronal dendrites in the hippocampus, as hypothesized. CA1 pyramidal neurons were loaded with the NO-sensitive dye DAF-FM. Initially, postsynaptic current injections were used to drive postsynaptic spiking, while neuronal dendrites were imaged. However, no fluorescence changes were observed, which likely reflected the poor sensitivity of the dye. I then attempted to drive stronger and more prolonged depolarization by a 60s bath application of a high K^+ solution (90mM), in the presence of glutamate receptor antagonists (50-100μM AP5, 10μM NBQX, 500μM MCPG, 100μM LY341495). Under these conditions, depolarization drove robust increases in DAF-FM fluorescence (%ΔF/F: 37.70±4.17; n=5) (Figure 3). These increases were dependent on NO synthesis as they could be prevented either by postsynaptic injection of cPTIO (ΔF/F: -3.00±5.14; n=5; cPTIO vs. control: p=0.0090) or by bath application of the NO synthase inhibitor L-NAME (ΔF/F: -0.18±5.06; n=5; L-NAME vs. control: p=0.0090). These increases were also dependent on L-VGCC activation as they were reliably abolished by nitrendipine (ΔF/F: -2.28±5.54; n=5; nitrendipine vs. control: p=0.0090) (Figure 3), suggesting that NO synthesis required L-VGCC activation.

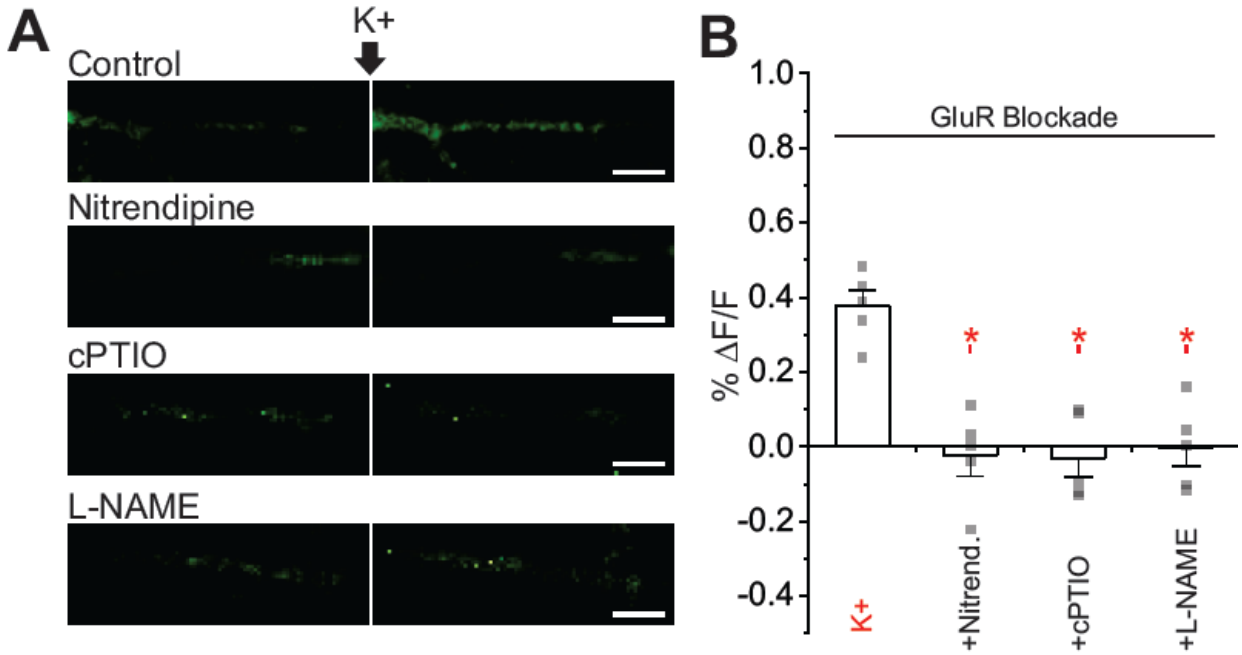


Figure 3. Postsynaptic depolarization results in L-VGCC-dependent synthesis of NO. (**A**) Sample images of CA1 apical dendrites loaded with NO-sensitive fluorescent dye (DAF-FM), prior to and following K+ (90mM) mediated depolarization (scale bar: 5µm) in control conditions, or in the presence of the L-VGCC inhibitor nitrendipine, the NO scavenger cPTIO, or the NO synthesis inhibitor L-NAME. (**B**) Average K+ induced fluorescence change (%ΔF/F) of DAF-FM in apical dendrites. Red asterisks denote significant difference from the first group (labelled in red) (Mann-Whitney test; $p<0.05$; n=5/condition). Errors bars represent S.E.M.

Collectively, my findings suggest that postsynaptic depolarization, via the activation of L-VGCCs, drives the synthesis and release of NO from neuronal dendrites.

4.2.3 NO release is sufficient for long-term increases in Pr at active presynaptic terminals

I next investigated whether an extracellular increase in NO was sufficient to drive increases in Pr at single synapses. For this purpose, I made use of a caged NO compound ($RuNOCl_3$; 0.5-1mM) that could be photoreleased with a 405nm laser; this method enabled precise spatiotemporal control of NO signalling. Following basal measurement of Pr, caged NO was bath applied with glutamate receptor antagonists, and each of 30-60 single presynaptic stimuli, delivered at 5Hz, were paired with light exposure directed in a ~1.2μm diameter region above the spine head. Photolysis was triggered 7-10ms after each presynaptic stimulus and consisted of a 20ms duration laser pulse, with sufficient power to release 7-10 nmol of NO, a concentration which has been shown to induce LTP in hippocampal cultures (Arancio et al., 1996). Remarkably, pairing presynaptic activity with NO photolysis generated significant increases in Pr, when assessed 30 minutes following drug washout (ΔPr: 0.29±0.06; n=10) (Figure 4). Notably, increases in Pr were abolished by extracellular application of the NO scavenger, cPTIO (ΔPr: 0.02±0.07; n=6; cPTIO vs. +10ms pairing: p=0.023), suggesting that presynaptic enhancements resulted from release of NO, and not any non-specific effects of the caged compound or the uncaging procedure (Figure 4). Moreover, photolysis 7-10ms before each presynaptic stimuli failed to induce changes in Pr, suggesting that the potentiating effects of NO are restricted to presynaptic terminals whose activity precede, rather than follow, NO release (ΔPr: -0.01±0.04; n=8; +10ms vs. -10ms pairing: p=0.0045) (Figure 4A-C).

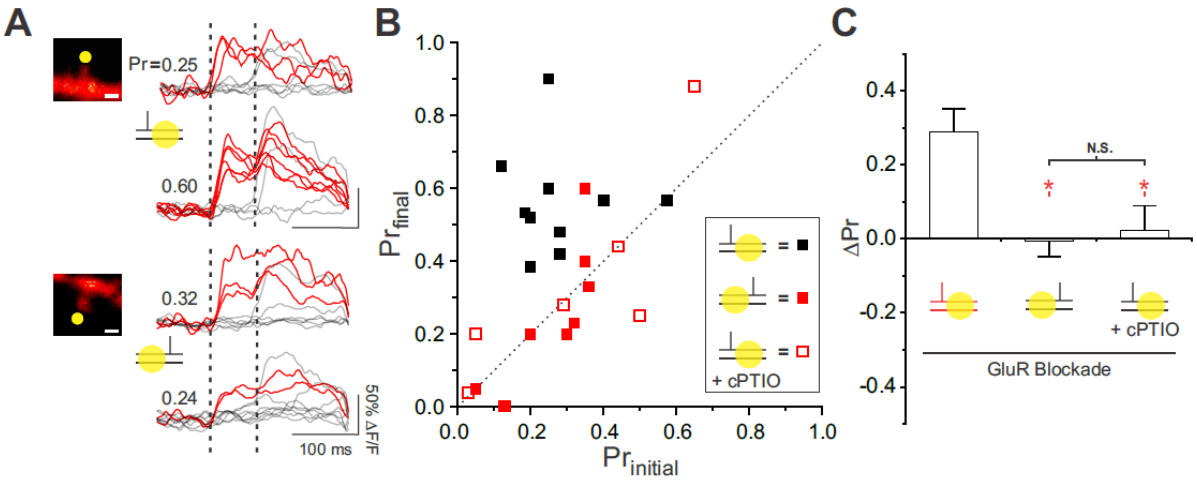


Figure 4. NO is sufficient to potentiate active presynaptic terminals (**A**) Samples of 10 superimposed Ca^{2+} traces evoked in imaged spines (white scale bar: 1µm) by paired pulse stimulation (broken vertical lines show 2 stimuli delivered 70ms apart); red traces depict successful release events to the first of the two pulses Above the sample Ca^{2+} traces are values for Pr, which have been calculated as the proportion of total stimulation trials that resulted in successful release events. Sample Ca^{2+} traces are shown during baseline and 25-30 minutes following a stimulation paradigm. The paradigm consisted of 30-60 presynaptic stimuli, delivered at 5Hz, that occurred either (top) 7-10ms before or (bottom) 7-10ms after NO photolysis at the synapse (yellow circle); photolysis occurred in glutamate receptor blockade and in the absence of postsynaptic depolarization. In some experiments photolysis was conducted in the presence of the NO scavenger cPTIO. (**B**) For each experiment conducted, the final Pr at the synapse measured 25-30 minutes following the stimulation paradigm is plotted against the initial Pr measured during baseline. The broken diagonal line represents the expected trend if Pr was unchanged. (**C**) Average change in Pr across experimental conditions. Only synapses whose activity preceded NO photolysis were potentiated. Red asterisks denote significant differences from the first group (labelled in red) (Mann-Whitney test; $p<0.05$; n=6-12 per condition). N.S. denotes non-significance. Error bars represent S.E.M.

4.2.4 Mechanisms of long-term presynaptic potentiation in acute hippocampal slices

The experiments thus far have been conducted in organotypic slices, which afford excellent optical access when compared to their acute slice counterparts. However, that development of these slices continues for 1 week in an incubator prior to use raises the possibility that some of my findings may not apply to more physiologically-relevant preparations. Therefore, I asked whether a similar, L-VGCC and NO dependent mechanism drove presynaptic potentiation in acute slices, in the absence of glutamatergic signalling. Given the poor optical access of confocal microscopy in acute slices, I conducted these experiments using standard electrophysiological techniques. Given incomplete recovery of synaptic responses following drug washout, changes in EPSPs recorded in the tetanized pathway were compared to changes in EPSPs recorded in a second control pathway. As in organotypic slices, I found that paired pre- and post- synaptic stimulation, conducted in glutamate receptor blockade, resulted in LTP induction in acute slices, measured 30 minutes post-drug washout (normalized fold $\Delta EPSP_{slope}$ tetanized/control: 1.78±0.23; fold $\Delta EPSP_{slope}$ tetanized vs. control: 0.67±0.17 vs. 0.43±0.14; n=5; p=0.03) (Figure 5A,B). Potentiation was also presynaptic in origin as it was accompanied by an increase in CV^{-2} (normalized fold ΔCV^{-2} tetanized/control: 2.65±0.48; fold $\Delta EPSP_{slope}$ tetanized vs. control: 1.68±0.23 vs. 0.78±0.17; n=5; p=0.03) and a decrease in PPR (ΔPPR: -0.13±0.04; n=5; p=0.02) (Figure 5C,D). Notably, such increases were abolished when paired pre- and post-synaptic stimulation occurred in the presence of either nitrendipine or cPTIO (nitrendipine normalized fold $\Delta EPSP_{slope}$ tetanized/control: 0.99±0.09; nitrendipine + blockade vs. blockade: n=5; p <0.01) (nitrendipine normalized fold ΔCV^{-2} tetanized/control: 1.05±0.05;

nitrendipine + blockade vs. blockade: n=5; p<0.01) (nitrendipine ΔPPR: 0.08±0.05; nitrendipine + blockade vs. blockade: n=5; p=0.02) (cPTIO normalized fold $\Delta EPSP_{slope}$ tetanized/control: 1.00±0.02; cPTIO + blockade vs. blockade: n=5; p<0.01) (cPTIO normalized fold ΔCV^{-2} tetanized/control: 0.98±0.11; cPTIO + blockade vs. blockade: n=5; p<0.01) (cPTIO ΔPPR: 0.04±0.03; cPTIO + blockade vs. blockade: n=5; p=0.01) (Figure 5A-D). These results suggest that, as in organotypic slices, presynaptic potentiation in acute slices can occur in the absence of glutamate receptor activation, in a manner dependent on L-VGCC and NO signalling.

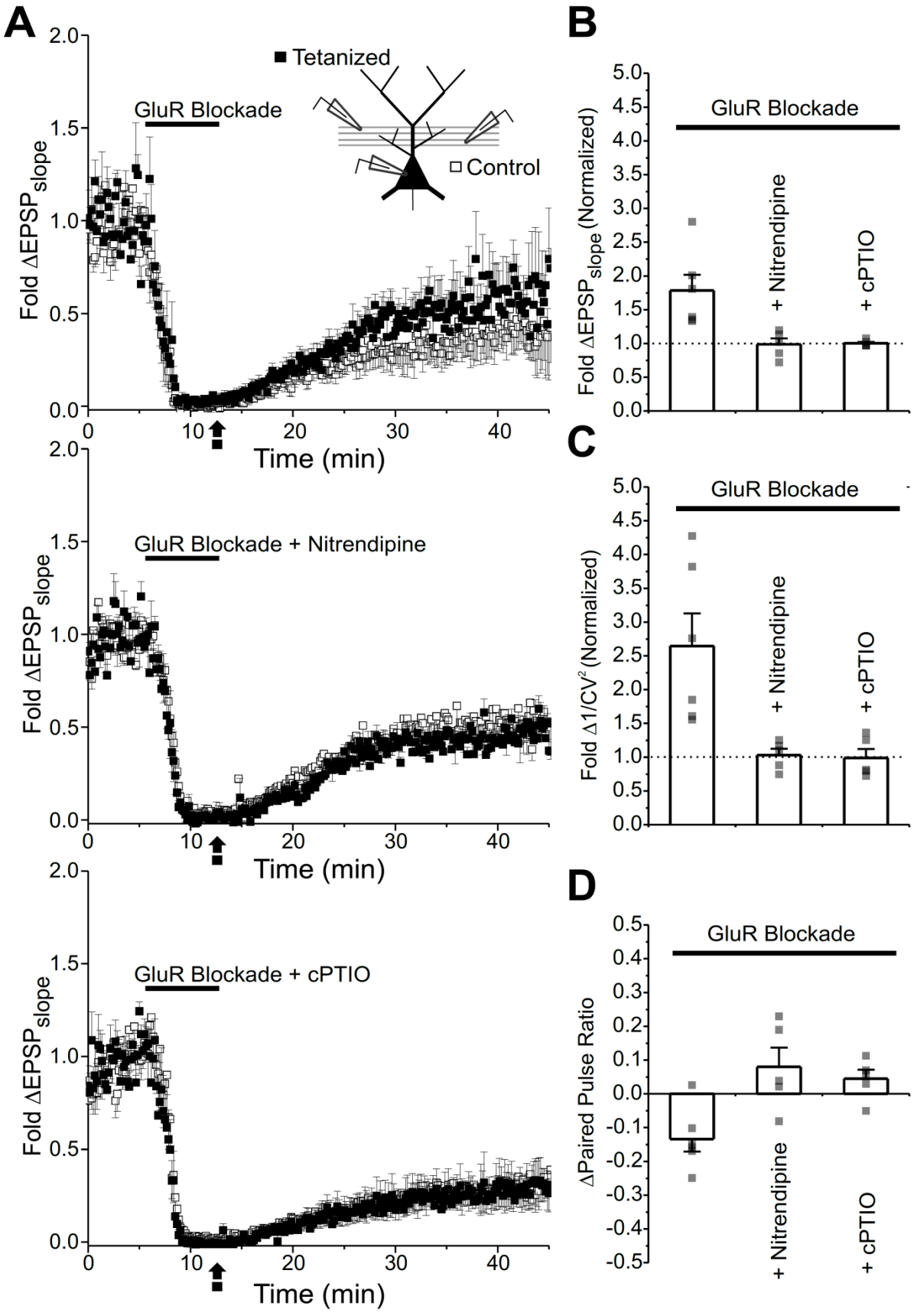

A
B
C
D
Tetanized
Control
GluR Blockade
GluR Blockade + Nitrendipine
GluR Blockade + cPTIO
Fold ΔEPSP slope
Time (min)
Fold ΔEPSP slope (Normalized)
Fold Δ1/CV² (Normalized)
ΔPaired Pulse Ratio
+ Nitrendipine
+ cPTIO

Figure 5: Induction of presynaptic LTP in acute slices is independent of glutamatergic signalling, but requires L-VGCC and NO signalling. CA1 pyramidal neurons in 3-4 week acute hippocampal slices were recorded from using low-resistance patch electrodes (4-8MΩ). EPSPs were recorded from two independent Schaffer-collateral pathways. LTP was induced in glutamate receptor blockade (50-100µM AP5, 10µM NBQX, 500µM MCPG, and 100µM LY341495) by pairing each of 60 presynaptic stimuli, delivered at 5Hz to the tetanized pathway, with a postsynaptic complex spike consisting of 3-6 action potentials. In some experimetns LTP was induced in the presence of the L-VGCC blocker nitrendipine or the NO scavenger cPTIO. **A**) Fold change in the EPSP slope is plotted against time for both control and tetanized pathways. (**B**) Average fold change in EPSP slope in the tetanized pathway 30-35 minutes following drug washout; values are normalized to the EPSP slope recorded in the control pathway. (**C**) Average fold change in CV^{-2} in the tetanized pathway; values are normalized to the EPSP slope recorded in the control pathway. (**D**) Average change in PPR in the tetanized pathway. Red asterisks denote significant differences from the first group (labelled in red) (Mann-Whitney test; $p<0.05$; n=5-6/condition). Error bars represent S.E.M.

4.3 Discussion

Here, I have demonstrated that postsynaptic depolarization triggers the activation of L-VGCCs, which in turn results in the synthesis and release of NO from neuronal dendrites, and that NO alone is sufficient to potentiate active presynaptic terminals. Such a mechanism could enable LTP induction at a given presynaptic terminal that releases little or no glutamate, provided that the activity of the terminal is coincident with postsynaptic depolarization.

In this study, I assume that the locus of L-VGCC function is postsynaptic, though it is important to mention that bath application of cell-permeable antagonists, such as nitrendipine, do not distinguish between pre- and post- synaptic channels. Like me, others who have implicated L-VGCC in LTP also parsimoniously assume that such L-VGCCs reside postsynaptically, in part, because selective blockade of postsynaptic L-VGCCs can currently only be achieved by tissue-specific genetic manipulations, but also because there is no evidence suggesting a major role of L-VGCCs in presynaptic function (Aniksztejn and Ben-Ari, 1991; Kullmann et al., 1992; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995; Morgan and Teyler, 2001)

L-VGCCs have been previously implicated in presynaptic plasticity, and in mediating GluN receptor-independent plasticity (Aniksztejn and Ben-Ari, 1991; Kullmann et al., 1992; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995; Morgan and Teyler, 2001). Although, how L-VGCC activation actually influences presynaptic changes has not been previously explored. Here, I demonstrate that NO can actually be driven by L-VGCC activation. Although NO can also be produced by postsynaptic GluN receptors (Garthwaite

and Boulton, 1995; Sattler et al., 1999; Stanika et al., 2012), in my study, only blockade of L-VGCCs, and not GluN receptor, abolished increases in Pr produced by paired pre- and post-synaptic stimulation. The differential importance of NO signalling by GluN receptors and L-VGCCs in presynaptic LTP may have to do with the differential magnitude and kinetics of NO signalling associated with the activation of each receptor. Certainly, studies using NO imaging have shown that GluN receptor activation promotes large increases in NO production, which occurs over a time course of tens of seconds (Sattler et al., 1999; Stanika et al., 2012). In contrast, NO production by L-VGCC activation, although more modest, is much more rapid (Sattler et al., 1999; Stanika et al., 2012). Given that nanomolar, rather than micromolar levels of NO are thought to be required for presynaptic potentiation (Arancio et al., 1996), and that NO release needs to closely follow presynaptic activity, the rapid yet modest release of NO by L-VGCC activation might be more efficient at triggering presynaptic LTP.

NO has previously been implicated as a retrograde signal, though findings have been inconsistent across labs. The reason for these inconsistencies is discussed at length in Chapter 6. Briefly though, it is important to recognize that while not all groups report an NO-sensitive component of LTP (Boulton et al., 1994; Murphy et al., 1994; Murphy and Bliss, 1999), not all groups report a presynaptic component of LTP expression either (Padamsey and Emptage, 2013). Thus a failure to observe a role of NO in LTP may reflect a failure to induce a presynaptically-expressed form of LTP. In fact, studies that directly monitor presynaptic function consistently report that inhibition of NO signalling abolishes presynaptic LTP (Nikonenko et al., 2003; Stanton et al., 2005; Johnstone and Raymond, 2011; Ratnayaka et al., 2012; Johnstone and Raymond, 2013).

Several groups have demonstrated that exogenous NO application can drive synaptic potentiation (O'Dell et al., 1991b; Bon et al., 1992; Zhuo et al., 1993; Zhuo et al., 1994; Arancio et al., 1996; Malen and Chapman, 1997; Zhuo et al., 1998; Nikonenko et al., 2003) [but see (Boulton et al., 1994; Murphy et al., 1994; Murphy and Bliss, 1999)], and furthermore, that NO-mediated potentiation is restricted to active presynaptic terminals (Zhuo et al., 1993; Zhuo et al., 1994). Here, I extend these findings to show that induction of presynaptic LTP specifically requires presynaptic activity to precede NO signalling in a manner that is consistent with the temporal requirements of spike-timing dependent protocols of LTP induction (Dan and Poo, 2004). My findings suggests that the induction of presynaptic LTP necessarily requires a presynaptic Hebbian integrator whose activation is dependent, not only on postsynaptic NO signalling, but additionally on presynaptic activity, which could be signalled by action potential-triggered Ca^{2+} influx via presynaptic VGCCs. One candidate integrator for this purpose might be guanylate cyclase (Garthwaite and Boulton, 1995), which is a protein that has previously been implicated in the induction of presynaptic LTP and is also known to be modulated by both NO and intracellular Ca^{2+} (Zabel et al., 2002).. In fact, in platelets and in lung endothelial cells, Ca^{2+} influx appears to enhance the NO-sensitivity of guanylate cyclase by re-locating the protein to the lipid membrane, where NO is likely to be most concentrated (Zabel et al., 2002); if such a mechanism is present in presynaptic terminals, then it would explain why triggering presynaptic activity prior to NO release preferentially induces presynaptic LTP in my experiments.

4.4 Conclusion

In this chapter I have elucidated the mechanisms of presynaptic LTP and have demonstrated how these mechanisms essentially can operate in the absence of glutamatergic signalling at any given synapse. In the next chapter, given that glutamatergic signalling is not necessary at a presynaptic terminal for it to potentiate, I examine the role of glutamatergic signalling in presynaptic long-term depression (LTD).

5. THE ROLE OF GLUTAMATE IN THE LONG-TERM DEPRESSION OF PRESYNAPTIC FUNCTION

5.1 Introduction

In previous chapters I have demonstrated that glutamatergic signalling is not a strict requirement for the induction of presynaptic long-term potentiation (LTP), meaning that presynaptic terminals may not need to release glutamate to undergo potentiation. But what happens to plasticity induction at a presynaptic terminal if it does in fact release glutamate? In a physiological context, glutamate release is still necessary to drive the strong postsynaptic depolarization required for presynaptic potentiation. However, glutamate release at any one synapse will have a minimal influence on postsynaptic depolarization, making it unlikely that a bouton's release of glutamate would profoundly contribute to its likelihood of being potentiated. In fact, that transmitter release probability (Pr) appears to be inversely related to the likelihood that a presynaptic terminal will potentiate (Larkman et al., 1992; Ryan et al., 1996; Slutsky et al., 2004; Hardingham et al., 2007; Saez and Friedlander, 2009), and that high Pr synapses are more likely to show presynaptic long-term depression (LTD) in response to a stimulation protocol that otherwise would potentiate low Pr synapses (Hardingham et al., 2007; Saez and Friedlander, 2009), would suggest that glutamate release by a presynaptic terminal mainly drives presynaptic depression. In fact, at neocortical synapses it has recently been shown that glutamate release is sufficient to promote presynaptic LTD by activating presynaptic NMDA receptors (GluN) (Rodriguez-Moreno et al., 2013). Given that presynaptic GluN receptors are also present on CA3 boutons (McGuinness et al., 2010), a similar mechanism of glutamate-driven depression may also operate at Schaffer-collateral synapses.

In this chapter, I examine how activity-dependent changes in Pr at a given presynaptic terminal are influenced by glutamatergic signalling at the terminal. I used Ca^{2+} imaging to monitor Pr at individual presynaptic terminals, and used photolysis and pharmacology to manipulate synaptic glutamate levels during activity. I found that glutamate actually drives decreases in Pr by activating presynaptic GluN receptors, and that this depression can operate independently and simultaneously with LTP-related processes. As a consequence, glutamate release at a synapse reduces the magnitude of presynaptic LTP driven by paired pre- and post- synaptic stimulation, and augments the magnitude of presynaptic LTD driven by presynaptic stimulation alone.

5.2 Results

5.2.1 Glutamate inhibits long-term increases in Pr

To examine how plasticity at a given presynaptic terminal might be affected by glutamate release, I augmented glutamatergic signalling at single synapses and asked what effect this had on activity-dependent changes in Pr. To assay Pr, I used postsynaptic Ca^{2+} imaging to monitor presynaptic glutamate release (see Chapter 3 Figure 1). For this purpose, CA3 or CA1 hippocampal pyramidal cells were impaled with sharp microelectrodes (70-100MΩ) containing the Ca^{2+} sensitive dye, Oregon Green BAPTA-1 (OGB-1). The use of a sharp microelectrode minimized intracellular dialysis, which affects synaptic plasticity, and allowed me to conduct electrophysiological recordings simultaneously with Ca^{2+} imaging. Following cell impalement, a glass stimulating electrode was then brought close to an imaged dendrite, in order to drive glutamate release at one or more imaged spines. At most hippocampal synapses, uniquantal glutamate release drives an increase in spine-Ca^{2+},

which is mediated by the activation of GluN receptor and voltage-gated Ca^{2+} channels (VGCCs) (Emptage et al., 1999; Grunditz et al., 2008). At such synapses, Pr can be calculated as the proportion of single presynaptic stimuli that has elicited a fluorescent response (Emptage et al., 2003).

Then, to manipulate levels of synaptic glutamate during activity, I used glutamate photolysis. Briefly, the slice was exposed to a caged variant of glutamate (MNI-glutamate), that is inert until activated by near-UV light. I then used a rapidly-shuttered 405nm laser to uncage glutamate in a ~1μm area above the spine head of interest. By adjusting the laser power, it was possible to mimic spine-Ca^{2+} transients that naturally occurred in response to single quanta of glutamate, as released by electrical stimulation (ΔF/F photolysis vs. stimulation: 0.44=0.08 vs. 0.58=0.09; n=16; p=0.72) (Figure 1A,B). Laser photolysis also generated synaptic potentials that, when of sufficient magnitude, could be recorded at the soma.

The average size of these events was 0.79mV=0.13mV (n=13), which is comparable to the unitary potentials generated at spines that are usually selected for Ca^{2+} imaging (Enoki et al., 2009) (Figure 1C). Under these conditions then, glutamate photolysis was an effective technique for emulating uniquantal glutamate release at single synapses.

I first examined the effect of augmenting glutamatergic signalling during paired pre- and post- synaptic stimulation, designed to induce LTP. Stimulation consisted of 60 presynaptic stimuli, delivered at 5Hz, each of which was coupled with postsynaptic depolarization of sufficient magnitude to generate 3-6 spikes. During LTP induction, each presynaptic stimulus was also coupled with glutamate photolysis above the spine head, so as to emulate

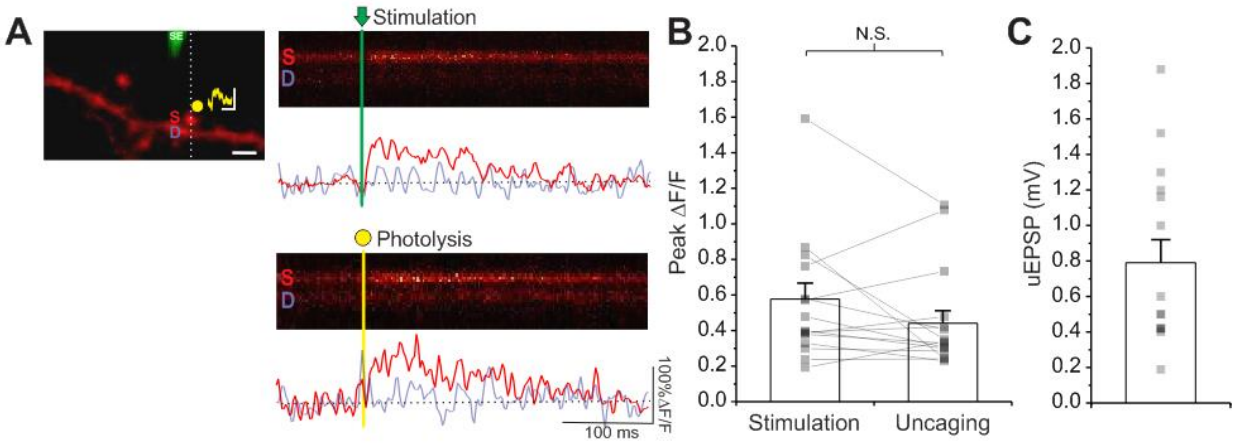


Figure 1. Glutamate photolysis at single synapses effectively emulates uniquantal glutamate release evoked by electrical stimulation. (**A**) Left. Image of a CA1 dendrite loaded with OGB-1 Ca^{2+}-sensitive dye (white scale bar: 2µm). Both a stimulating electrode (SE), and glutamate photolysis (yellow spot) was used to evoke Ca^{2+} responses in an imaged spine. The synaptic potential evoked by glutamate photolysis is shown above the spine head (yellow trace; scale bar: 1mV by 100ms). Laser scanning was restricted to a line across the imaged spine (S) and underlying dendrite (D) to allow high frequency imaging of Ca^{2+} dynamics. Right. Sample line scans during stimulation and glutamate photolysis. Fluorescent changes in the spine (red trace) and dendrite (purple trace) are quantified as %ΔF/F in the traces below each line scan. Ca^{2+} transients evoked by stimulation and by photolysis were similar. (**B**) Summary of group data showing no significant difference between peak ΔF/F fluorescence evoked by stimulation and photolysis (Wilcoxon matched-pairs test; $p>0.05$; $n = 16$). (**C**) Group data of photolysis-evoked EPSP amplitudes ($n = 13$). Error bars represent S.E.M.

reliable release at the synapse (Pr=1), whether glutamate was released or not. Whereas glutamate photolysis had little effect on global LTP induction, as recorded by the EPSP (fold ΔEPSP slope: control vs. photolysis; 1.97±0.13 vs. 2.06±0.20; n=11 and 12; p=0.48) (Figure 3), it completely abolished changes in Pr specifically produced at the imaged synapse (ΔPr: control vs. photolysis; 0.19±0.03 vs. -0.02±0.03; n= 14 and 12; photolysis vs. control: $p < 0.001$) (Figure 2). The failure to induce LTP at synapses receiving photolysis did not result from any non-specific effects of uncaging since, for 5 synapses, when a subsequent round of paired stimulation was delivered, in the presence of laser exposure, but in the absence of caged-glutamate, the expected increase in Pr was observed (ΔPr: 0.18±0.02; post-photolysis control vs. control; n=5 and 14; p=0.75) (post-photolysis control; Figure 2B,C). Increases in Pr were also observed when paired stimulation occurred in the presence of caged glutamate, but in the absence of laser exposure (ΔPr: 0.21±0.04; n=3). Glutamatergic signalling, therefore, appeared to have an inhibitory effect on presynaptic LTP.

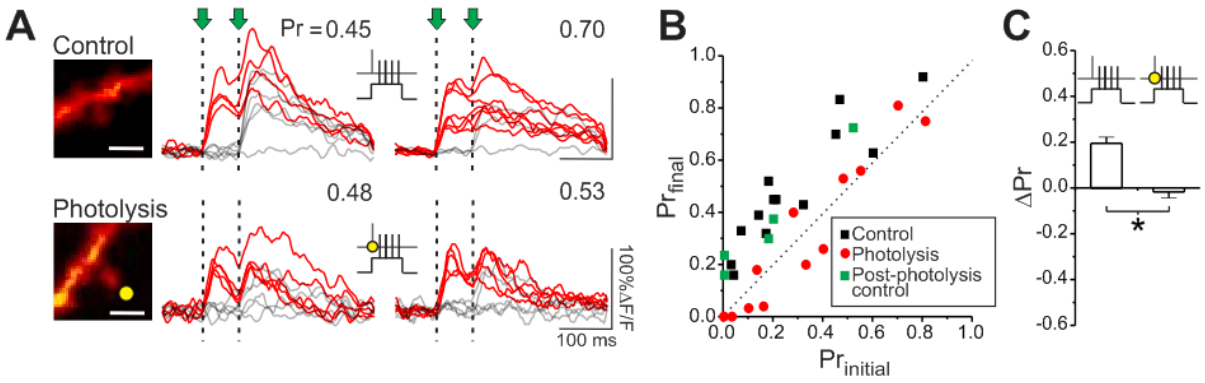


Figure 2. Glutamate photolysis inhibits activity-dependent increases in Pr (**A**) Samples of 10 superimposed Ca^{2+} traces evoked in imaged spines (white scale bar: 2µm) by paired pulse stimulation (broken vertical lines show 2 stimuli delivered 70ms apart); red traces depict successful release events to the first of the two pulses Above the sample Ca^{2+} traces are values for Pr, which have been calculated as the proportion of total stimulation trials that resulted in successful release events. Sample Ca^{2+} traces are shown during baseline and 25-30 minutes following paired pre- and post- synaptic stimulation either in the presence or absence of glutamate photolysis. Paired stimulation consisted of 60 presynaptic, delivered at 5Hz, each of which were paired with postsynaptic depolarization of sufficient magnitude to evoke 3-6 action potentials. (**B**) For each experiment conducted, the final Pr at the synapse measured 25-30 minutes following the stimulation paradigm is plotted against the initial Pr measured during baseline. The broken diagonal line represents the expected trend if Pr was unchanged. Increases in Pr observed under control conditions are prevented by the presence of glutamate photolysis during paired stimulation. The post-photolysis control experiments represent a proportion of photolysis experiments in which a second round of paired stimulation was delivered but in the absence of caged glutamate. (**C**) Average change in Pr across experimental conditions. No significant increases in Pr were observed when photolysis accompanied paired stimulation. Asterisks denote significant differences between groups (Mann-Whitney test; $p<0.05$; n=12-14 per condition). Error bars represent S.E.M.

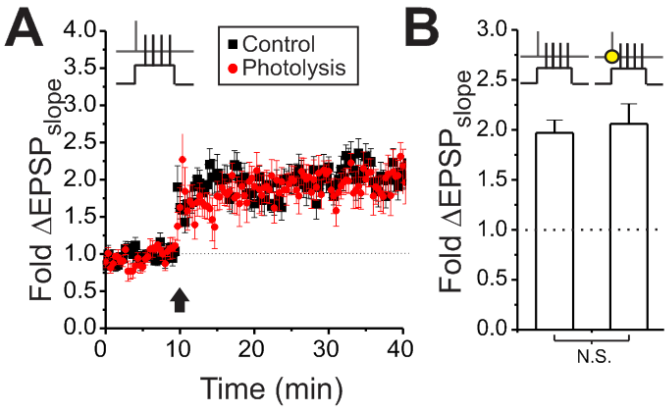


Figure 3. Long-term potentiation across a population of synapses is unchanged by glutamate photolysis (**A**) Group averages of EPSP slope in control and photolysis experiments, recorded in a portion of single spine Ca^{2+} imaging experiments shown in Figure 2. Paired pre- and post- synaptic stimulation (black arrow) produces long-lasting potentiation of similar magnitude in both control and photolysis experiments. (**B**) Average change in EPSP slope across experimental conditions. N.S. denotes no significant differences between groups (Mann-Whitney test; $p>0.05$; $n=11$-12 per condition). Error bars represent S.E.M.

5.2.2 Glutamate promotes long-term decreases in Pr

Given these results, I then asked whether glutamate release alone, in the absence of postsynaptic depolarization, could drive decreases in Pr. For this purpose, I repeated the experiments, but this time tetanic stimulation consisted of 60 presynaptic stimuli, either paired or unpaired with glutamate photolysis, delivered at 5Hz in the absence of postsynaptic current injection.

Under control conditions, presynaptic stimulation alone generally had little influence on global synaptic strength, as assessed by the recorded EPSP (fold $\Delta EPSP_{slope}$ control 1.03±0.10; n=9) (Figure 5), and had no effect on Pr at the majority of imaged synapses (Figure 4). However, synapses with initially high release probabilities (Pr>0.5), did show a modest decrease in Pr following unpaired stimulation (Figure 4B,C). Given that high Pr synapses (Pr>0.5) comprise an estimated <10% of synapses in our preparation (Ward et al., 2006), it is not likely that this modest depression was evident in electrophysiological recordings. However, when presynaptic stimulation was paired with glutamate photolysis a profound depression was observed at all imaged synapses, regardless of their initial Pr (ΔPr photolysis vs. control: -0.33±0.08 vs. -0.12±0.06; n=9,10 p=0.037)(Figure 4). This depression was specifically localized to the imaged synapse since photolysis induced no measurable effects on the recorded EPSP (ΔEPSP slope: 0.90=0.05; n=8; photolysis vs. control; p=0.22) (Figure 5).

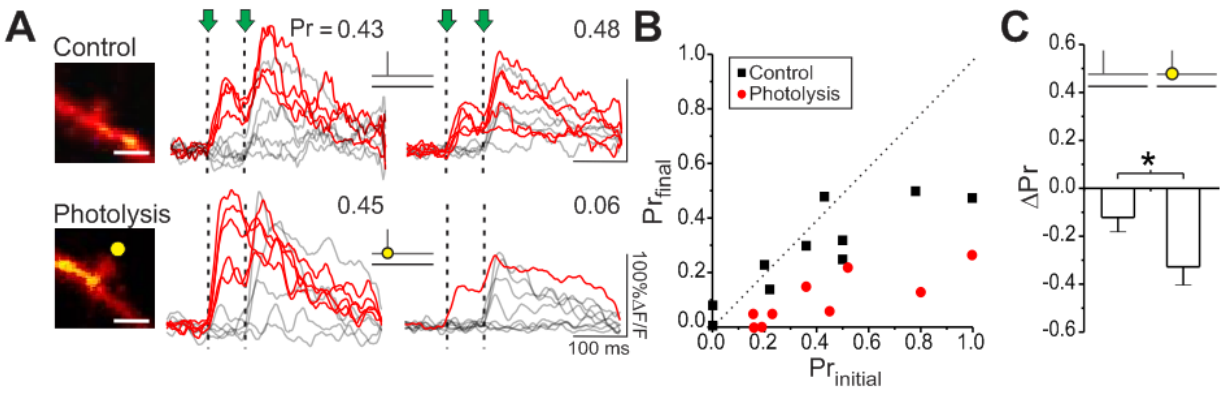


Figure 4. Glutamate photolysis augments activity-dependent decreases in Pr (**A**) Samples of 10 superimposed Ca^{2+} traces evoked in imaged spines (white scale bar: 2µm) by paired pulse stimulation (broken vertical lines show 2 stimuli delivered 70ms apart); red traces depict successful release events to the first of the two pulses Above the sample Ca^{2+} traces are values for Pr, which have been calculated as the proportion of total stimulation trials that resulted in successful release events. Sample Ca^{2+} traces are shown during baseline and 25-30 minutes following delivery of 60 presynaptic stimuli at 5Hz, either in the presence or absence of glutamate photolysis. (**B**) For each experiment conducted, the final Pr at the synapse measured 25-30 minutes following the stimulation paradigm is plotted against the initial Pr measured during baseline. The broken diagonal line represents the expected trend if Pr was unchanged. Decreases in Pr were restricted to high Pr synapses ($Pr \geq 0.5$) under control conditions, but occurred at all synapses when glutamate photolysis was present during presynaptic stimulation. (**C**) Average change in Pr across experimental conditions. Glutamate photolysis augmented decreases in Pr driven by presynaptic stimulation. Asterisks denote significant differences between groups (Mann-Whitney test; $p<0.05$; n=9-10 per condition). Error bars represent S.E.M.

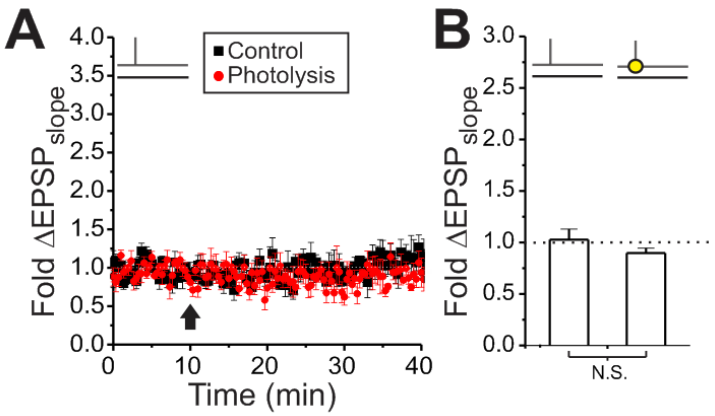


Figure 5. Synaptic efficacy across a population of synapses is unchanged by glutamate photolysis (**A**) Group averages of EPSP slope in control and photolysis experiments, recorded in a portion of single spine Ca^{2+} imaging experiments shown in Figure 4. Presynaptic stimulation (60 pulses at 5Hz; black arrow) has no consistent effect on EPSP slope in either control or photolysis experiments. (**B**) Average change in EPSP slope across experimental conditions. N.S. denotes no significant differences between groups (Mann-Whitney test; $p>0.05$; n=11-12 per condition). Error bars represent S.E.M.

5.2.3 Endogenous glutamatergic signalling promotes long-term decreases in Pr in a manner that is independent of postsynaptic activity

Collectively, my results thus far suggest that glutamate release is inhibitory to presynaptic LTP during paired pre- and post- synaptic depolarization and, during presynaptic stimulation alone, drives presynaptic LTD. A more parsimonious conclusion is that glutamate release simply drives decreases in Pr, regardless of postsynaptic activity. As a consequence, during paired pre- and post- synaptic stimulation, the processes of LTP, driven by postsynaptic depolarization, is occurring simultaneously with the process of LTD, driven by glutamate release. If this is true, then the inhibition of endogenous glutamatergic signalling during paired pre- and post- synaptic depolarization should augment increases in Pr.

To examine this possibility, I induced LTP in control conditions and under full glutamate receptor blockade (50-100μM AP5, 10μM NBQX, 500μM MCPG, 100μM LY341495), by pairing 60 presynaptic stimuli, delivered at 5Hz, with postsynaptic complex spikes, emulated by current injection. Consistent with my previous findings, this pairing protocol reliably produced increases in Pr when delivered in glutamate receptor blockade (Figure 6). Remarkably, these increases were significantly greater than those elicited by the same pairing protocol, but under control conditions, in the absence of glutamate receptor antagonists (ΔPr: blockade vs. control; 0.33=0.06 vs. 0.18=0.02; n=13 and 10; p=0.013). Glutamate receptor blockade also prevented presynaptic depression that normally occurred at high Pr sites when presynaptic stimulation was delivered alone (ΔPr blockade vs. control: 0.00=0.03 vs. -0.21=0.05; n=10 and 9; p=0.0048)(Figure 7). These findings

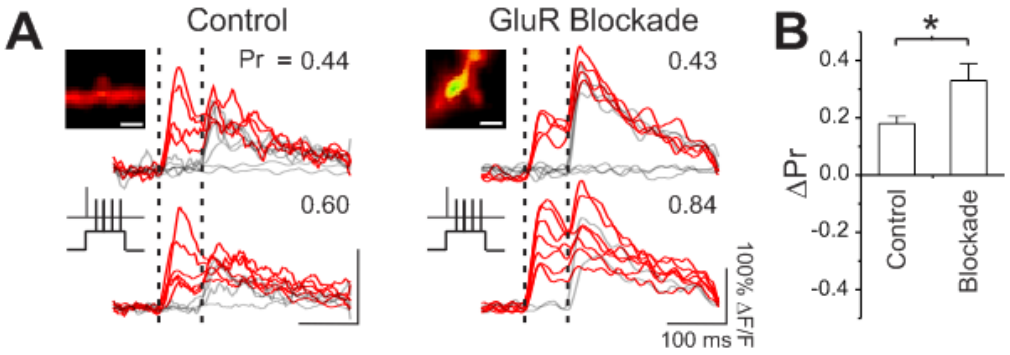


Figure 6. Endogenous glutamatergic signalling reduces activity-dependent increases in Pr. (**A**) Samples of 10 superimposed Ca^{2+} traces evoked in imaged spines (white scale bar: 2µm) by paired pulse stimulation (broken vertical lines show 2 stimuli delivered 70ms apart); red traces depict successful release events to the first of the two pulses Above the sample Ca^{2+} traces are values for Pr, which have been calculated as the proportion of total stimulation trials that resulted in successful release events. Sample Ca^{2+} traces are shown during baseline and 25-30 minutes following paired stimulation under control conditions or under full glutamate receptor blockade (50-100µM AP5, 10µM NBQX, 500µM MCGP, 100µM LY341495). Paired stimulation consisted of 60 presynaptic stimuli, delivered at 5Hz, paired with a postsynaptic complex spike triggered by current injection. (**B**) Average change in Pr across experimental conditions. Asterisks denote significant differences between groups (Mann-Whitney test; $p<0.05$; n=9-10 per condition). Error bars represent S.E.M.

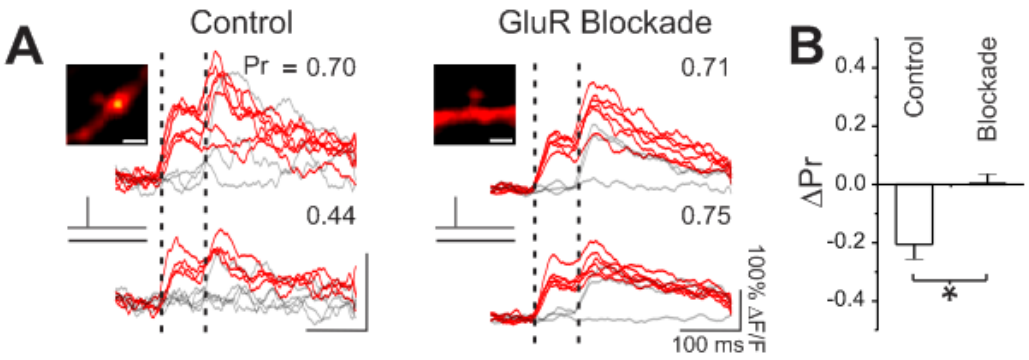


Figure 7. Endogenous glutamatergic signalling promotes net decreases in Pr when unaccompanied by postsynaptic spiking. (**A**) Samples of 10 superimposed Ca^{2+} traces evoked in imaged spines (white scale bar: 2µm) by paired pulse stimulation (broken vertical lines show 2 stimuli delivered 70ms apart); red traces depict successful release events to the first of the two pulses. Above the sample Ca^{2+} traces are values for Pr, which have been calculated as the proportion of total stimulation trials that resulted in successful release events. Sample Ca^{2+} traces are shown during baseline and 25-30 minutes following delivery of 60 presynaptic stimuli at 5Hz under control conditions or under full glutamate receptor blockade (50-100µM AP5, 10µM NBQX, 500µM MCGP, 100µM LY341495). (**B**) Average change in Pr across experimental conditions. Asterisks denote significant differences between groups (Mann-Whitney test; $p<0.05$; n=9-10 per condition). Error bars represent S.E.M.

suggest that endogenous glutamate release acts to decrease Pr, regardless of postsynaptic activity. I next examined the mechanism by which glutamatergic signalling promoted decreases in Pr. In the neocortex, activity-dependent presynaptic depression is known to be driven by the activation of presynaptic GluN receptors (Rodriguez-Moreno et al., 2013). These receptors have recently been reported at Schaffer-collateral synapses (McGuinness et al., 2010), suggesting that they might also be mediating the inhibitory effects of glutamate on Pr. Given the difficulties associated with selectively blocking pre-, as opposed to post-, synaptic GluN receptors, several groups have investigated the role of presynaptic GluN receptors in plasticity by comparing the effects of bath application of AP5 or MK801, which blocks both pre- and post- synaptic GluN receptors, with that of intracellular MK801 application, which selectively blocks postsynaptic GluN receptors (Cormier and Kelly, 1996; Nevian and Sakmann, 2006; Corlew et al., 2007; Corlew et al., 2008).

I sought to use a similar approach to examine the role of presynaptic GluN receptors in presynaptic plasticity. However, because intracellular application of MK801 does not readily washout, and since postsynaptic GluN receptors greatly contribute to spine Ca^{2+} influx (Emptage et al., 1999; Grunditz et al., 2008; Holbro et al., 2010), the permanent loss of postsynaptic GluN receptor signalling could potentially affect my ability to measure Pr using postsynaptic Ca^{2+} imaging. I investigated this possibility by calculating Pr before and during blockade of GluN receptors by bath application of AP5. I found that at 11 of 20 synapses, AP5 effectively abolished the Ca^{2+} transient; however, at the remaining 9 of 20 synapses, a residual GluN receptor-insensitive Ca^{2+} transient was clearly observed (Figure 8A,B). At these sites, estimates of Pr before and during bath application of AP5 did not differ (ΔPr: control vs. +AP5: 0.47=0.11 vs. 0.46=0.11; p=0.58) (Figure 8C); moreover, the average

Pr of these synapses did not significantly differ from that of synapses lacking an AP5-insenstive Ca^{2+}-transient (ΔPr: AP5-sensitive vs. AP5-insensitive: 0.42=0.07 vs. 0.47=0.11; p=0.67), suggesting that presynaptic efficacy was comparable between both groups. These findings suggest that, in GluN receptor blockade, AP5-insensitive spine-Ca^{2+} influx can be used as a means of calculating Pr at a sizeable and representative proportion of presynaptic terminals.

Residual, AP5-insensitive spine Ca^{2+} signals have previously been shown to result from the activation of VGCCs within the spine head, as driven by AMPA receptor (GluA)-mediated depolarization (Bloodgood and Sabatini, 2007; Grunditz et al., 2008). To confirm that the residual spine Ca^{2+} transients under my experimental conditions were also mediated by VGCCs, I first identified synapses exhibiting an GluN receptor-insensitive Ca^{2+} transient, and then emulated this transient using glutamate uncaging (ΔF/F: stimulation-evoked transient vs. uncaging-evoked transient photolysis; 0.29±0.01 vs.0.33±0.03; n=7; p=0.61) (Figure 9). Glutamate uncaging enabled me to examine the effect of VGCC antagonists on postsynaptic Ca^{2+} signalling independent of their effects on presynaptic transmitter release. Under these conditions, I found that bath application of the VGCC blockers Ni^{2+} (100μM) and Cd^{2+} (100μM) reliably abolished the GluN receptor-insensitive Ca^{2+} transient (ΔF/F: AP5 vs. AP5 + Ni^{2+} Cd^{2+}: 0.33±0.04 vs.0.05±0.02; n=7; $p < 0.01$).

Using Ca^{2+} imaging of VGCC-mediated spine Ca^{2+} influx, I then examined the effects of AP5 and MK801 on activity-driven changes in Pr. I found that blockade of both pre- and post-synaptic GluN receptors by bath application of either AP5 or MK801 augmented increases in Pr induced by paired pre- and post- synaptic stimulation in a manner similar to that seen

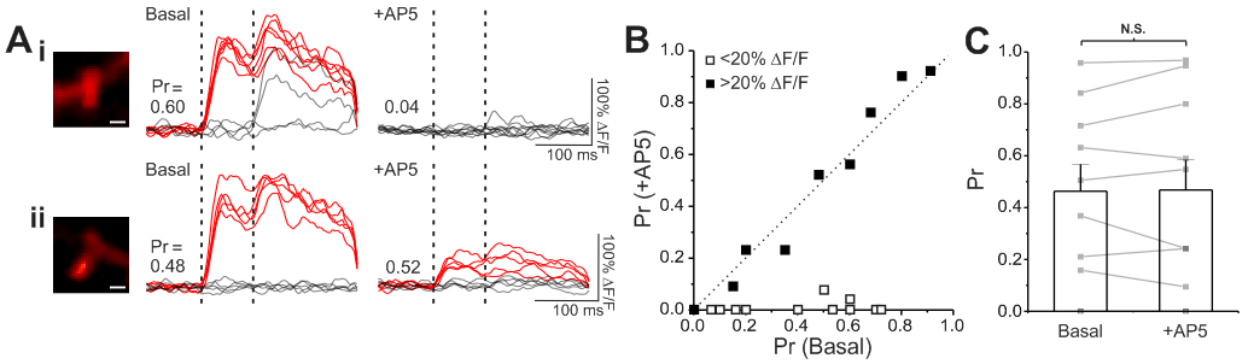


Figure 8: Ca^{2+} transients are detectable in NMDA-R blockade at a proportion of synapses, and can be used to accurately measure Pr. (**A**) Samples of 10 superimposed Ca^{2+} traces evoked in imaged spines (white scale bar: 1µm) by paired pulse stimulation (broken vertical lines show 2 stimuli delivered 70ms apart); red traces depict successful release events to the first of the two pulses. Pr is shown above the Ca^{2+} traces. Pr was calculated as the proportion of total stimulation trials that resulted in a Ca^{2+} response in the dendritic spine. (i,ii) For two imaged spines, sample Ca^{2+} traces are shown prior to and following bath application of AP5. AP5 abolished Ca^{2+} transients (%ΔF/F < 20%) in only one of the two spines. (**B**) For each synapse imaged, the Pr measured under basal conditions is plotted against the Pr measured during bath application of AP5. The broken diagonal line represents the expected trend if Pr is unchanged. For about 50% of synapses, a significant Ca^{2+} transient remained in AP5 (black boxes; %ΔF/F > 20%); at these sites, Pr measured under basal conditions was unchanged by AP5. (**C**) Group data depicted no significant difference between Pr measured under basal conditions and in AP5 for synapses for which an AP5-insensitive Ca^{2+} transient could be detected (%ΔF/F $\geq$ 20%). N.S. denotes no significant differences between groups (Wilcoxon match-paired test; p>0.05; n=9 per condition). Error bars represent S.E.M.

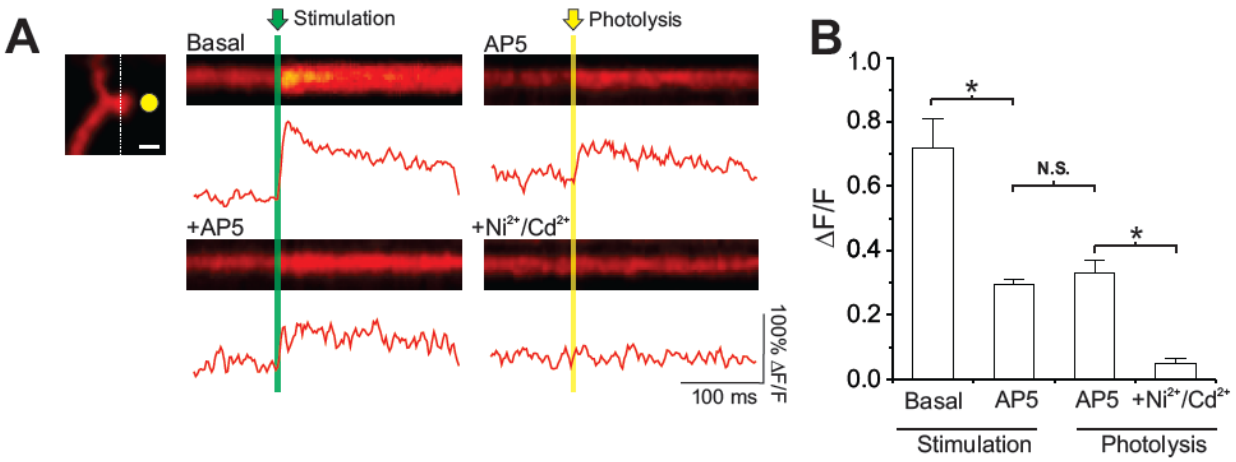


Figure 9: AP5-insensitive spine Ca^{2+} transients are mediated by VGCCs. (**A**) Images of sample Ca^{2+} responses evoked by stimulation or glutamate photolysis at a single spine (white scale bar: 1 µm) under various pharmacological manipulations. Below each image are traces quantifying the change in fluorescence intensity of the Ca^{2+} response (%ΔF/F). At the spine shown, AP5 reduced, but did not abolish, Ca^{2+} responses evoked by a single pulse of electrical stimulation. Inhibition of voltage-gated calcium channels by Ni^{2+} (100µM) and Cd^{2+}(100µM) abolished the AP5-insensitive component of the Ca^{2+} transient; since Ni^{2+} and Cd^{2+} inhibit transmitter release, glutamate release was simulated via photolysis under these conditions. (**B**) Average peak amplitude of evoked Ca^{2+} responses (ΔF/F) across experimental conditions (n=5-12/condition). Ca^{2+} responses evoked by glutamate photolysis did not significantly differ from those evoked by electrical stimulation, and was significantly reduced by Ni^{2+} and Cd^{2+} application. Asterisks denote significant differences ($p<0.05$; Wilcoxon-matched pairs test; n=7 per condition). N.S. denotes no significant difference. Error bars represent S.E.M.

under full glutamate receptor blockade (ΔPr with AP5: 0.32±0.03; n=9; blockade vs. AP5: p=0.84; control vs. AP5: p=0.0025) (ΔPr with bath MK801: 0.44±0.05; n=7; blockade vs. bath MK801: p=0.18; control vs. bath MK801: p=0.0024) (Figure 10). Bath application of AP5 or MK801 also similarly prevented decreases in Pr driven by presynaptic stimulation alone (ΔPr with AP5: -0.04±0.03; n=9; blockade vs. AP5: p=0.33; control vs. AP5: p=0.027) (ΔPr with bath MK801: 0.00±0.04; n=7; blockade vs. bath MK801: p=0.99; control vs. bath MK801: p=0.013)(Figure 11). In contrast, when postsynaptic GluN receptors were selectively blocked with intracellular application of MK801, increases in Pr (ΔPr: 0.16±0.04; n=9; intracellular MK-801 vs control: p=0.84; intracellular MK-801 vs blockade: p=0.01) induced by paired pre- and post- synaptic stimulation, and decreases in Pr driven by presynaptic stimulation alone (ΔPr: -0.25±0.07; n=9; intracellular MK-801 vs control: p=0.90; intracellular MK-801 vs. blockade: p=0.01) were no different than changes in Pr observed under control conditions, and significantly different from changes observed in full glutamate receptor blockade (Figure 10,11). These results were not attributable to an incomplete blockade of postsynaptic GluN receptors by intracellular MK801 since the amplitude of spine Ca^{2+} transients under these conditions were not significantly different from those recorded in AP5 (ΔF/F: intracellular MK-801 vs. AP5: 0.32±0.05 vs.0.26±0.03 n=10 and 12; p=0.65), but were significantly smaller than those recorded in control conditions (ΔF/F: intracellular MK-801 vs. control: 0.32±0.05 vs.0.72±0.01; n=10 and 9; $p < 0.01$) (Figure 12). These findings suggest that pre-, and not post-, synaptic GluN receptors mediate the inhibitory effects of glutamate on Pr, in a manner that is independent of postsynaptic depolarization.

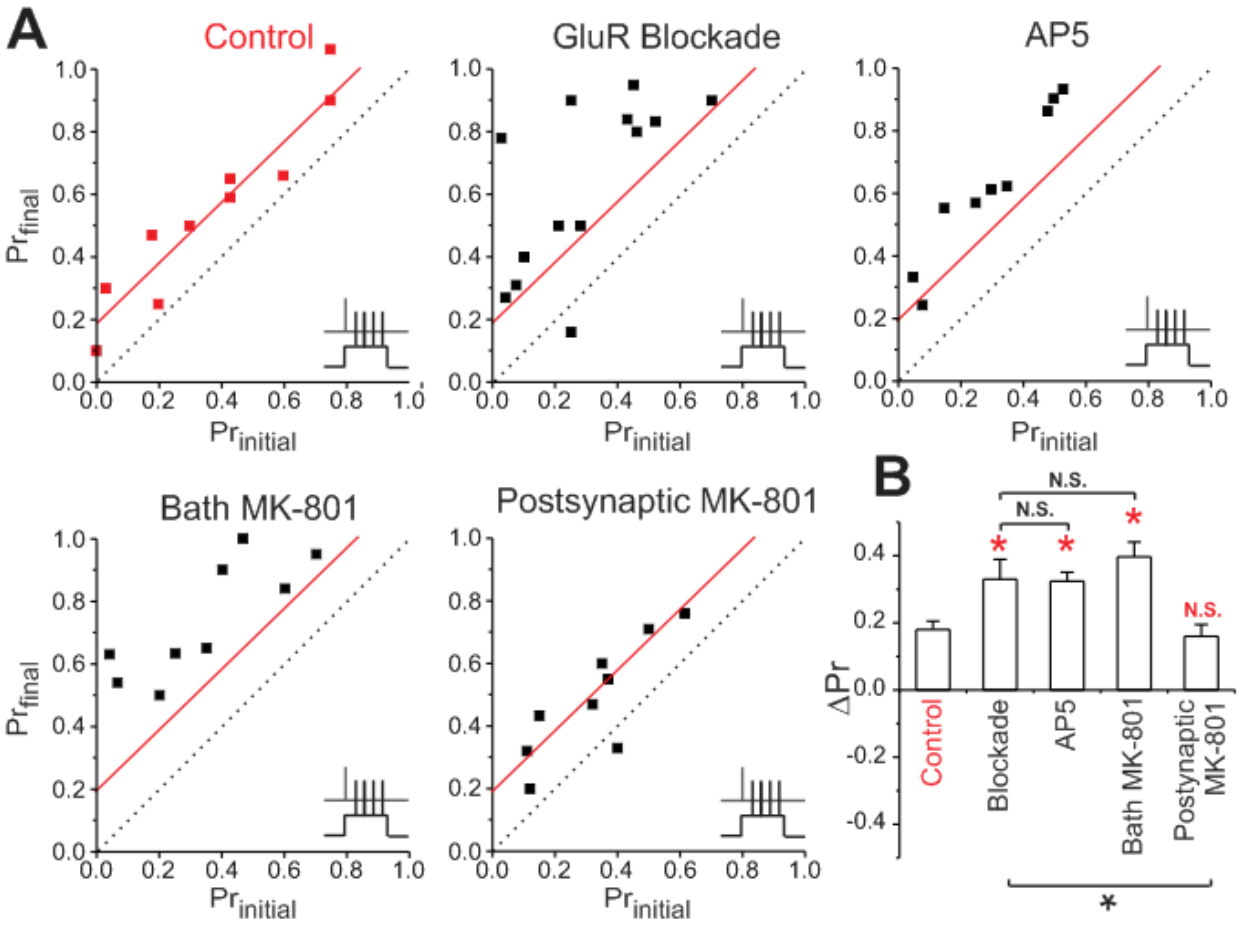


Figure 10. Presynaptic GluN receptors mediate the inhibitory effect of glutamate release on activity-dependent increases in Pr. (**A**) Summary of Pr changes evoked by paired pre- and post- synaptic stimulation (60 pairings at 5Hz) across various experimental conditions. For each experiment, the final Pr of the imaged synapse measured 25-30 minutes following paired stimulation is plotted against the initial Pr measured during baseline. The broken diagonal line represents the expected trend if Pr was unchanged. Red trend line has been fitted to the control dataset. Paired stimulation results in larger increases in Pr under conditions in which presynaptic GluN receptors are blocked (i.e. GluR blockade, APV, and bath MK801) than under control conditions. (**B**) Average change in Pr across experimental conditions. Black asterisk denotes significant differences between groups. Red asterisks denote significant differences specifically from the control group. (Mann-Whitney test; $p<0.05$; n=9-13 per condition). N.S. denotes no significant differences between groups. Error bars represent S.E.M.

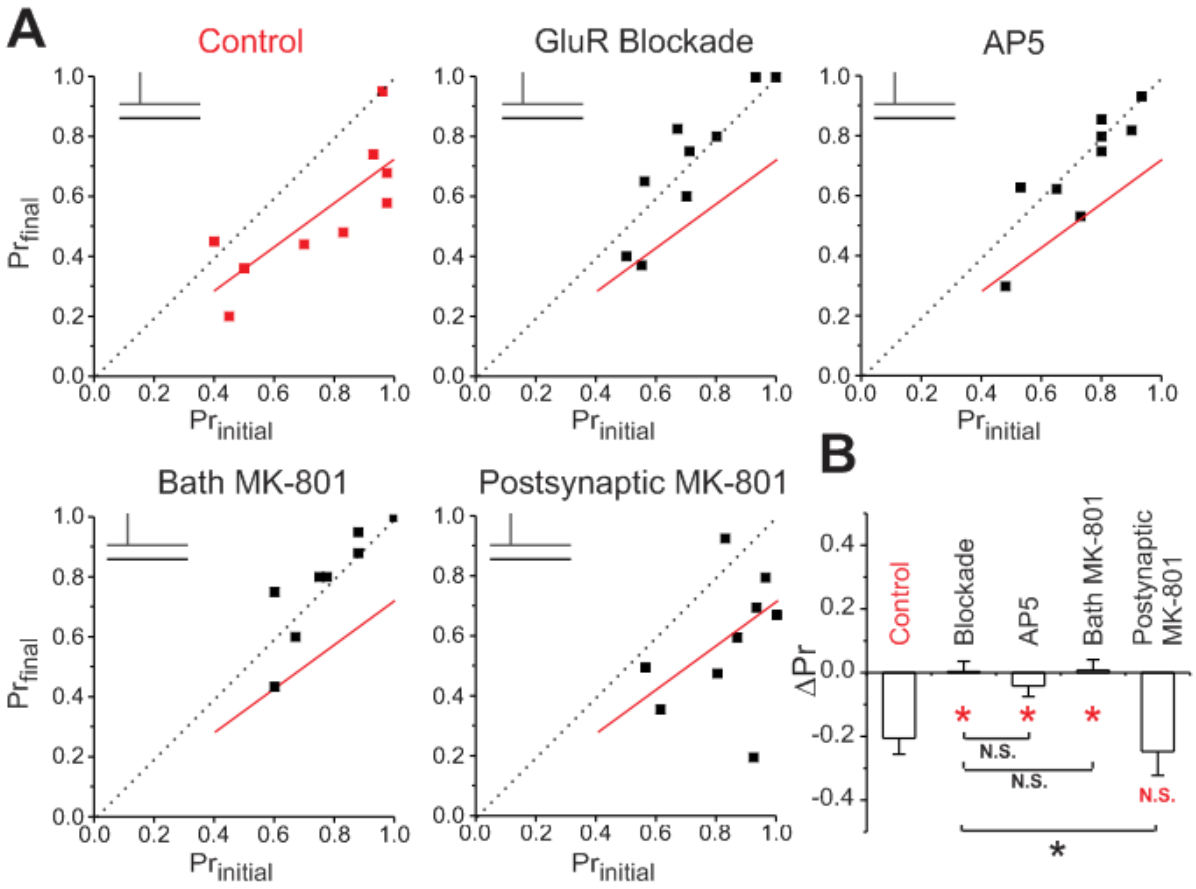


Figure 11. Presynaptic GluN receptors mediate decreases in Pr driven by glutamate release in the absence of postsynaptic spiking (**A**) Summary of Pr changes evoked by presynaptic stimulation (60 pulses at 5Hz) across various experimental conditions. For each experiment, the final Pr of the imaged synapse measured 25-30 minutes following paired stimulation is plotted against the initial Pr measured during baseline. The broken diagonal line represents the expected trend if Pr was unchanged. Red trend line has been fitted to control dataset. Decreases in Pr elicited by presynaptic stimulation in control experiments are abolished under conditions in which presynaptic GluN receptor are blocked (i.e. GluR blockade, APV, and bath MK801) (**B**) Average change in Pr across experimental conditions. Black asterisk denotes significant differences between groups. Red asterisks denote significant differences specifically from the control group. (Mann-Whitney test; $p<0.05$; n=9-13 per condition). N.S. denotes no significant differences between groups. Error bars represent S.E.M.

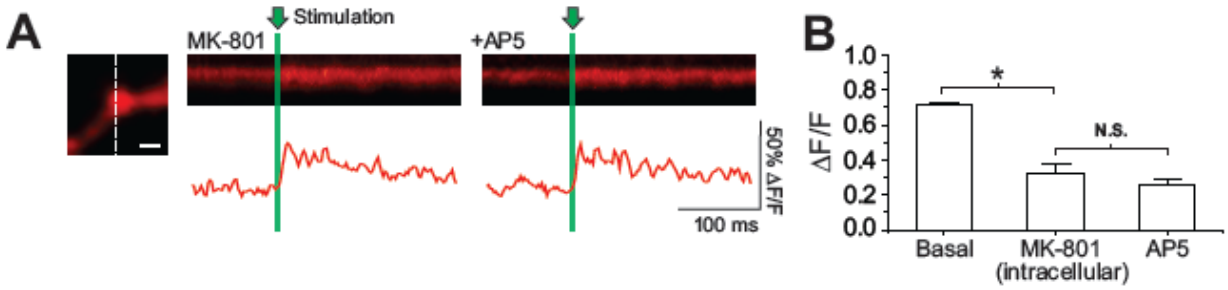


Figure 12: Intracellular MK-801 application effectively blocks postsynaptic GluN receptors. (**A**) Images of sample Ca^{2+} responses evoked at a single spine (scale bar: 1µm) by single presynaptic stimuli with intracellular application of MK-801, and following the addition of AP5. Below each image are traces quantifying the change in fluorescence intensity of the Ca^{2+} response (%ΔF/F). (**B**) Group data showing average peak Ca^{2+} fluorescence amplitude (ΔF/F) under basal conditions, with intracellular MK-801, and following addition of AP5. Response amplitudes recorded in AP5 and MK801 do not differ, and are significantly lower than those recorded in control conditions, in which GluN receptors are not blocked. Asterisks denote significant differences ($p<0.05$; Mann-Whitney or Wilcoxon-matched pairs tests; n = 9-12 per condition). N.S. denotes no significant difference. Error bars represent S.E.M.

5.3 Discussion

Here I demonstrate that glutamate release at the presynaptic terminal drives decreases in Pr by activating presynaptic GluN receptors, independent of postsynaptic activity. As such, I found that blocking presynaptic GluN receptors actually augmented activity-driven increases in Pr following LTP induction, which suggests that the potentiating effects of paired pre- and post - synaptic depolarization, and the depressing effects of glutamate release, occur simultaneously during synaptic activity. Thus, LTP and LTD are not temporally distinct mechanisms, but operate jointly to influence presynaptic function.

In order to implicate presynaptic GluN receptors in presynaptic depression, I examined how intracellular and extracellular application of GluN receptor antagonists differentially affected Pr. Since I was using postsynaptic Ca^{2+} imaging to assess Pr, I was restricted to using synapses that showed a VGCC-dependent component of spine-Ca^{2+} influx, one which was not blocked by APV or MK801. There is the possibility that such sites represent a unique subpopulation of synapses, for example, those that have sufficiently high GluA receptor number to generate the strong depolarization necessary to locally activate VGCCs. However, this possibility is unlikely since about 50% of all imaged synapses had an observable VGCC-dependent Ca^{2+} influx, and because the Pr distribution of these synapses was not significantly different from synapses imaged in the absence of GluN receptor antagonists. Moreover, in the case of postsynaptic GluN receptor blockade using MK801, the change in Pr at synapses was no different than under control conditions, suggesting that presynaptic plasticity operated similarly regardless of whether or not synapses showed a VGCC-dependent component of spine-Ca^{2+} signalling.

My results suggest that one reason high Pr synapses are less likely to undergo LTP and more likely to undergo LTD (Larkman et al., 1992; Ryan et al., 1996; Slutsky et al., 2004; Hardingham et al., 2007; Saez and Friedlander, 2009) is possibly because they release more glutamate, and therefore experience a stronger tendency to depress. This may also explain why the same pairing protocol can produce LTP at low Pr synapses, but LTD at high Pr synapses; at high Pr synapses, the magnitude of LTD driven by glutamate release is presumably greater than the magnitude of LTP driven by paired pre- and post- synaptic stimulation (Hardingham et al., 2007; Saez and Friedlander, 2009).

In this study, I found that blockade of pre-, but not post-, synaptic GluN receptors not only abolished presynaptic LTD, but also augmented presynaptic LTP. At neocortical synapses, presynaptic GluN receptors have similarly been implicated in the induction of presynaptic LTD; however, several studies have demonstrated that pharmacological inhibition of presynaptic GluN receptors does not appear to augment or effect LTP, contrary to my own findings (Rodriguez-Moreno and Paulsen, 2008; Rodriguez-Moreno et al., 2013). That my results differ from these studies may simply reflect the possibility that plasticity at neocortical and hippocampal synapses are different. Alternatively, it is possible that the low frequency (0.2Hz) of presynaptic stimulation used during LTP induction in these studies did not elicit sufficient levels of glutamate release to elicit presynaptic depression via presynaptic GluN receptor activation. By contrast, in my study, LTP induction involved presynaptic stimulation at a theta frequency, which is effective at promoting glutamate release at the synapse (McGuinness et al., 2010). As such, the inhibitory effects of presynaptic GluN receptors on LTP may only be evident at higher stimulation frequencies.

It has been previously demonstrated that presynaptic GluN receptors at hippocampal synapses facilitate transmitter release during theta stimulation (McGuinness et al., 2010). Thus, presynaptic GluN receptors appear to be important for presynaptic facilitation in the short-term, but, given my findings, also for presynaptic depression in the long-term. Presynaptic GluN receptors in the neocortex also appear to both facilitate evoked and spontaneous glutamate release, and yet are similarly implicated in presynaptic LTD (Corlew et al., 2008). It may appear peculiar for a single protein to subserve seemingly disparate functions; however, another way to view the presynaptic GluN receptor is as a dynamic regulator of presynaptic activity, appropriately tuning glutamate release depending on the patterns of pre- and postsynaptic activity. As such, the receptor may aid glutamate release during theta-related activity, but, triggers presynaptic LTD when this release fails to elicit sufficiently strong levels of postsynaptic depolarization.

In this study, I found that selective blockade of postsynaptic GluN receptors made no difference to increases in Pr during paired pre- and post- synaptic stimulation or to decreases in Pr during presynaptic stimulation alone. These findings confirm that activation of postsynaptic GluN receptors is not strictly required for presynaptic plasticity. That some studies do find a reduction or abolition of presynaptic changes during GluN receptor blockade may simply reflect the reduced levels of postsynaptic depolarization that come from inhibiting the receptor (Grover and Yan, 1999b; Schiller et al., 2000; Grover et al., 2009). In my experiment, presynaptic stimulation was paired with strong postsynaptic spiking so as to circumvent the need for glutamate-driven postsynaptic depolarization.

5.4 Conclusion

In this chapter I have demonstrated that glutamate release drives decreases in Pr via the activation of presynaptic GluN receptors, and that this decrease occurs independently of postsynaptic activity. This means that both an LTP process, as driven by L-type voltage-gated Ca^{2+} channels and nitric oxide signalling (NO), and an LTD processes, as driven by glutamate release and presynaptic GluN receptor activation, can operate independently and simultaneously to determine net changes in Pr.

My findings further underscore the fundamentally different roles glutamate and glutamate receptors play in pre- and post- synaptic plasticity. Whereas Ca^{2+} signalling via postsynaptic GluN receptor appears to be crucial for both postsynaptic potentiation and depression, it plays no role in presynaptic plasticity. Rather, Ca^{2+} signalling via postsynaptic L-VGCCs drives presynaptic potentiation via NO signalling, and presynaptic GluN receptor activation drives presynaptic depression.

The locus of LTP expression has been a decades long debate and several studies have cast doubts as to whether the presynaptic terminal is a major locus of LTP expression. In the next chapter, I argue that the mechanistic differences underlying pre- and post- synaptic plasticity can help to explain 1) the inconsistences with which presynaptic LTP is induced across laboratories, and 2) the inconsistencies in the degree to which retrograde signalling is implicated in synaptic plasticity.

6. RECONCILING DISCREPANCIES IN THE LITERATURE PERTAINING TO THE INDUCTION OF A NITRIC OXIDE SENSITIVE AND PRESYNAPTICALLY EXPRESSED FORM OF LONG-TERM POTENTIATION

6.1 Introduction

Long-term potentiation (LTP) can be expressed either presynaptically, by increases in transmitter release probability or postsynaptically, by increase in AMPA receptor (GluA) number or conductance. For decades the locus of NMDA receptor (GluN)-dependent LTP expression in the hippocampus has been debated. Postsynaptic changes are generally reliably induced across studies and have been causally linked with GluN receptor-mediated Ca^{2+} influx. In contrast, presynaptic changes are not consistently induced across groups, and its link to GluN receptor activation and retrograde signalling remains both tentative and controversial. As such, whether the presynaptic locus represents a major locus of LTP expression has been questioned (Luscher and Malenka, 2012; Bliss and Collingridge, 2013).

6.2 Meta-analysis I – Examining the inconsistent induction of presynaptic LTP in the literature

There has been a long-held assumption in the field that both pre- and post- synaptic forms of plasticity require the activation of GluN receptors (Kullmann and Siegelbaum, 1995; Larkman and Jack, 1995; Lisman, 2003; Lisman and Raghavachari, 2006; Bliss and Collingridge, 2013). However, since the 1990s there have been reports of a second form of LTP, whose induction is independent of GluN receptors but requires activation of L-type voltage gated Ca^{2+} channels (L-VGCCs) (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Kullmann et al., 1992; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995; Morgan and Teyler, 2001), and whose expression is likely to be presynaptic

(Stricker et al., 1999; Bayazitov et al., 2007) [but see (Grover, 1998)]. However, owing to the aggressive stimulation protocols required for its induction, such as the use of high frequency tetanus, or the addition of voltage-gated K^+ channel blockers during presynaptic stimulation, GluN receptor-independent LTP has not been considered to represent a major form LTP (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Kullmann et al., 1992; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Petrozzino and Connor, 1994; Hanse and Gustafsson, 1995; Huber et al., 1995; Platt et al., 1995; Stricker et al., 1999; Zakharenko et al., 2001; Zakharenko et al., 2003). However, GluN receptor-independent LTP has been generated by theta-burst stimulation protocols, which are thought to reflect in vivo patterns of hippocampal activity (Thomas et al., 1998; Morgan and Teyler, 2001; Bayazitov et al., 2007; Grover et al., 2009). Moreover, the finding that inhibition of L-VGCCs augments the impairment to spatial memory caused by GluN receptor antagonists, suggests that L-VGCCs support some aspects of learning and memory *in vivo,* independent of GluN receptors (Borroni et al., 2000; Woodside et al., 2004; Moosmang et al., 2005).

In Chapters 3 and 4 of this thesis, I extend these findings further by showing that a presynaptically-expressed form of LTP, that is similarly dependent on L-VGCC activation, can be induced by pairing presynaptic activity with strong postsynaptic depolarization in the form of complex spikes, which represent a physiological hippocampal output (Grienberger et al., 2013). This form of LTP is not only induced in the absence of GluN receptor activity, but in the absence of any glutamatergic signalling. My findings suggest that a presynaptic terminal may not need to release glutamate in order to be potentiated, provided that its activity coincides with strong postsynaptic depolarization, as driven by glutamate release at neighbouring synapses. According to this view, the predominant role

of glutamate release in the induction of presynaptic LTP is to generate the postsynaptic depolarization needed to drive Ca^{2+} influx through L-VGCCs; in contrast, the role of glutamate in the induction of postsynaptic LTP is to trigger Ca^{2+} influx through postsynaptic GluN receptors. This notion is certainly consistent with the findings of Bayazitov et al. (2007), who demonstrate that GluN receptor activation is indispensable for postsynaptic enhancements, while L-VGCC activation is indispensable for presynaptic enhancements (Bayazitov et al., 2007).

The differential requirement for L-VGCC activation in the induction of pre-, but not post-, synaptic plasticity may help to explain the inconsistences with which LTP is induced across laboratories. L-VGCC activate at high voltages and desensitize during periods of prolonged depolarization (>100ms). As such, the likelihood of inducing LTP with a presynaptic component of expression will depend on the magnitude and duration of postsynaptic depolarization during presynaptic stimulation. I tested this hypothesis by conducting a meta-analysis on past studies examining the locus of LTP expression, to see if the induction of presynaptic LTP depended on the type of stimulation used during LTP induction. In this analysis, I examined studies cited in past reviews on LTP expression, including those that predominantly favored a pre- or post- synaptic component of expression. These studies used an assortment of methods to assess changes in presynaptic function, including measuring the GluN receptor-component of synaptic responses, using paired pulse ratio or short trains of stimuli to assess changes in short-term plasticity, recording glutamate transport currents in glial cells, estimating release probability with use-dependent receptor blockers and optically monitoring presynaptic function with either FM stryl dyes or Ca^{2+}-sensitive indicators. I, however, excluded studies using co-efficient of variation analysis,

minimal stimulation, or paired recordings. With these techniques, the unmasking of postsynaptically silent synapses can masquerade as presynaptic changes. Postsynaptic unmasking is thought to contribute substantially to LTP expression, especially during the initial weeks of postnatal development, when synaptic plasticity is most often studied (Abrahamsson et al., 2008). As such, it is difficult to assess whether changes in the co-efficient of variation or in the synaptic failure rate, following the induction of LTP in young tissue, are due to increases in pre- or post- synaptic function.

I examined a total of 38 studies, which assessed LTP expression across 53 experimental conditions (Table I). Presynaptic changes were reported in 23 of the 38 studies and in 23 of the 53 experimental conditions. LTP was generally induced either by brief, high-frequency tetanic stimulation (50-200Hz), or by a pairing protocol, in which lower frequency stimulation (generally <2Hz but ranging between 0.2-100Hz) was delivered while voltage-clamping the postsynaptic neuron between -10 and 10mV, often for tens of seconds. From the meta-analysis, I found that LTP is significantly more likely to have a presynaptic component of expression when induced by tetanic stimulation (20 of 35 conditions) rather than by pairing (3 of 18 conditions) ($X^2 = 7.92$; $p=0.005$). LTP induced by pairing, rather than tetanic stimulation, also appeared to be insensitive to L-VGCC blockers (Kullmann et al., 1992; Morgan and Teyler, 2001; Zakharenko et al., 2001; Zakharenko et al., 2003; Bayazitov et al., 2007). Perhaps one reason for these findings is that prolonged periods of depolarization that are involved in pairing protocols, although effective at relieving the Mg^{2+} block of GluN receptors, may desensitize L-VGCCs; the resulting LTP is therefore insensitive to L-VGCC antagonists and lacks a presynaptic component of expression. That said, pairing protocols can elicit L-VGCC dependent LTP when postsynaptic depolarization

consists of several brief, rather than one long, voltage step; this protocol may more effectively activate L-VGCCs without triggering channel desensitization (Kullmann et al., 1992).

Tetanic stimulation, however, did not always produce presynaptic changes. Although, given the high voltage activation threshold for L-VGCCs, the likelihood of generating presynaptic potentiation will depend on the ability of the tetanus to produce sufficiently strong postsynaptic depolarization. Consistent with this, Zakharenko et al. (2001,2003) and Bayazitov et al. (2007) demonstrated, using optical techniques, that theta-burst or 200Hz stimulation generated a L-VGCC sensitive form of LTP involving robust presynaptic enhancements, whereas no such L-VGCC sensitive enhancements were induced by 50Hz or 100Hz stimulation (Zakharenko et al., 2001; Zakharenko et al., 2003; Bayazitov et al., 2007; Blundon and Zakharenko, 2008). The enhanced probability of obtaining presynaptic changes under high frequency stimulation likely reflects the requirement for strong postsynaptic activity rather than for high frequency presynaptic activity *per se*, since I and others have shown that presynaptic LTP can be induced by low frequency pairing of individual presynaptic stimuli with strong postsynaptic depolarzation (Aniksztejn and Ben-Ari, 1991; Kullmann et al., 1992; Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995; Stricker et al., 1999; Morgan and Teyler, 2001; Enoki et al., 2009). Other experimental conditions may also influence the level of postsynaptic depolarization achieved during tetanus including: the temperature of the preparation, the divalent cation concentration, the presence or absence of $GABA_A$ receptor antagonists, as well as the intensity and duration of presynaptic stimulation used during tetanus, all of which vary

considerably across studies. As such, tetanic stimulation might preferentially generate presynaptic enhancement under some experimental conditions, but not others.

I next examined whether the magnitude of LTP generated by tetanic stimulation reflects the likelihood that LTP is associated with presynaptic enhancement, regardless of the actual pattern of stimulation and the experimental conditions under which it is induced. I reasoned that stimulation achieving sufficiently strong depolarization would recruit both pre- and post- synaptic components of LTP, and would therefore generate larger enhancements in synaptic activity. Consistent with this notion, I found that the average amplitude of LTP was 194.59%±9.62% (n=17) when it was associated with presynaptic enhancement, but only 153.50%±7.77% (n=12) when it was not ($U = 34$; $p = 0.003$) (Figure 1). Moreover, presynaptic enhancement was reported in 91.67% of experiments (n=11/12) that produced LTP with a magnitude $\geq$ 180% (Figure 1; broken line), but only in 35.3% of experiments (n=6/17) producing LTP with a lower magnitude ($X^2 = 9.21$; $p=0.002$). Only experiments that induced LTP using tetanic stimulation under standard experimental conditions were included in my analysis (29 of 35 conditions); experiments in which LTP was induced in AMPA receptor (GluA) blockade or in GluA2 knockout animals were therefore excluded (6 of 35 conditions). Collectively, these findings demonstrate that LTP at the presynaptic terminal is not some enigmatic and sporadic process, but a predictable form of plasticity whose induction is likely to depend on the levels of postsynaptic depolarization achieved during tetanus.

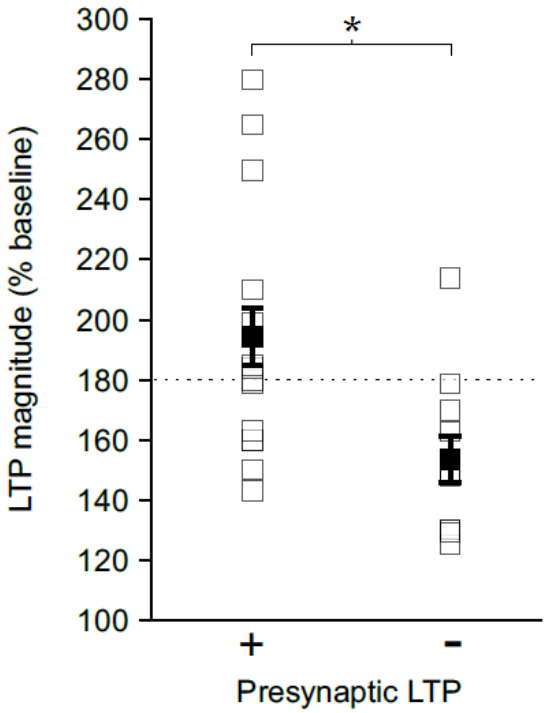


Figure 1. LTP magnitude predicts a presynaptic component of expression. LTP magnitude following tetanic stimulation is shown for 29 experimental conditions, 17 of which report a presynaptic component of expression (+). LTP with a magnitude $\geq$ 180% (broken line) had a higher probability of being associated with a presynaptic component of expression (91.67%) than LTP with a lower magnitude (35.3%). Asterisk denotes significant difference between groups (Mann-Whitney test; $p<0.05$; n = 12-17 per condition). Error bars represent S.E.M.

Table I. Studies examining the presynaptic expression of LTP

Citation[1]	Method	Protocol	%LTP[2]	ΔPre?[3]	Additional Notes
Tetanus-induced LTP					
(Muller and Lynch, 1988)	GluN receptor[4]	(4@100Hz)x10@5Hz	163%	Yes (119%)	LTP induced in low Mg^{2+}
(Muller et al., 1988a; Muller et al., 1992)	GluN receptor	(4@100Hz)x10@5Hz	130%	No	LTP induced in low Mg^{2+}
	GluN receptor	(4@100Hz)x10@5Hz	120%	Yes (120%)	LTP induced in low Mg^{2+} & DNQX
(Muller et al., 1989)	GluN receptor	(4@100Hz)x10@5Hz	148%	No	GluN receptor assessed using bursts (4@100Hz)
(Bashir et al., 1991)	GluN receptor	25@100Hz	143%	Yes (143%)	LTP induced in CNQX
(Asztely et al., 1992)	GluN receptor	10@50Hz	114%	Yes (114%)	LTP induced in low Mg^{2+} & CNQX
(Clark and Collingridge, 1995)	GluN receptor	100@100Hz	160%	Yes (160%)	
(Kullmann et al., 1996)	GluN receptor	(5@100Hz)x50@5Hz	150%	Yes (120%)	
(Mainen et al., 1998)	GluN receptor	(50-100@100Hz)x1-3	265%	No	GluA2 knockout mouse
	Polyamine use-dependent block	(50-100@100Hz)x1-3	254%	No	GluA2 knockout mouse
(Muller and Lynch, 1989)	Paired pulse ratio (PPR)	(4@100Hz)x10@5Hz	150%	No	
(Zalutsky and Nicoll, 1990)	PPR	(100@100Hz)x4@0.1Hz	163%	No	
(Foster and McNaughton, 1991)	PPR	(8@400Hz)x4	125%	No	
(Schulz et al., 1994)	PPR	(50@100Hz)x10@5Hzx1-6	179%	Yes	
(Schulz, 1997)	PPR + other occlusion/rescue experiments	(50@100Hz)x10@5Hzx1-6	250%	Yes	See reference for more details on occlusion/rescue exps.
(Kleschevnikov et al., 1997)	PPR	100@100Hz (strong)	280%	Yes	
	PPR	100@100Hz (weak)	150%	No	
(Volianskis and Jensen, 2003)	PPR	(4@100Hz)x10@5Hz	160%	Yes	
(Pananceau et al., 1998)	Short-term plasticity (STP) & PPR	(25@200Hz)x5	214%	No	STP assessed w/ 5@20Hz and 50Hz

(Yasui et al., 2005)	STP	50-100@100Hz	144%	Yes	STP assessed w/ 10@10Hz
(Volianskis et al., 2013)	STP	(4@100Hz)x10@5Hz	180%	Yes	STP assessed w/ 7@12.5Hz; decayed 2hr post- tetanus
(Luscher et al., 1998)	Glial transport currents (GTC)	(50@50H)zx4@0.05Hz OR (100@50Hz) x4@0.05Hz	159%	No	LTP induced in CNQX
	GTC	(100@50Hz)x4@0.05Hz	179%	No	
(Diamond et al., 1998)	GTC	(100@100Hz)x3	170%	No	
(Johnstone and Raymond)	FM-dye	((5@100Hz)x10@5Hz)x1	130%	No	
	FM-dye & PPR	((5@100Hz)x10@5Hz)x4@0.0033Hz	180%	Yes	
	FM-dye &PPR	((5@100Hz)x10@5Hz)x8@0.0033Hz	184%	Yes	
(Zakharenko et al., 2001; Zakharenko et al., 2003)	FM-dye	50@50Hz	129%	No	
	FM-dye	(100@100Hz)x4@0.05Hz	154%	No	
	FM-dye	(40@200Hz)x10@0.2Hz	185%	Yes	
	FM-dye	((4@100Hz)x5@5Hz)x3@0.0033Hz	210%	Yes	
(Bayazitov et al., 2007)	pHlourin	(40@200Hz)x10@0.2Hz	200%	Yes	pHlourin response assessed w/ 50@10Hz
	pHlourin	((4@100Hz)x5@5Hz)x3@0.0033Hz	220%	Yes	pHlourin response assessed w/ 50@10Hz
(Emptage et al., 2003; Ward et al., 2006)	Ca^{2+} imaging & PPR	(20@100Hz)x3@0.75Hz	265%	Yes	
(Enoki et al., 2009)	Ca^{2+} imaging	(20@100Hz)x3@0.75Hz OR 100@0.003Hz, each paired with 3 postsynaptic spikes	199%	Yes	
Pairing-induced LTP					
(Perkel and Nicoll, 1993)	GluN receptor[4]	(100@100Hz)x2@0.1Hz	175%	No	LTP induced in CNQX
	GluN receptor	40@2Hz; 0mV	190%	No	LTP induced in CNQX
	GluN receptor	50@0.5-0.7Hz; 30mV	165%	No	
(Kauer et al., 1988)	GluN receptor	19@2Hz; 0mV	150%	No	LTP induced in CNQX
	GluN receptor	(100@100Hz)x2@0.05Hz; 0mV	180%	No	LTP induced in CNQX
	GluN receptor	(100@100Hz)x2@0.05Hz; 0mV	152%	No	
(Plant et al., 2006)	GluN receptor	50-100@0.5-2Hz;-10 to 0 mV	155%	No	
(Kullmann et al., 1996)	GluN receptor	120@2Hz; 0mV	162%	Yes (110%)	

	MK801	100@100Hzx2; 0mV	162%	Yes	Use-dependent block
(Manabe and Nicoll, 1994)	MK801	40@2Hz; 0mV	158%	No	Use-dependent block
(Manabe et al., 1993)	PPR	80@2Hz; 0mV	178%	No	
	PPR	100@100Hz; 0mV	178%	No	
(Palmer et al., 2004)	PPR	40@0.5Hz; 0mV	234%	Yes	P6 rodent slices
	PPR	40@0.5Hz; 0mV	223%	No	P12 rodent slices
(Hjelmstad et al., 1997)	Synaptic refractory period (SRP)	100@1Hz; 0mV	200%	No	SRP probed w/ 2@250Hz
	SRP	(100@100Hz)x2@0.67Hz;0mV	200%	No	SRP period probed w/ 2@250Hz
	4-AP occlusion	((100@100Hz)x2@0.67Hz)x5;0mV	260%	No	
(Selig et al., 1999)	Short-term plasticity	120@1Hz;-10mV	286%	No	Short-term plasticity assessed w/ 7@25Hz

[1]Blank spaces represent additional experiments conducted by same study cited in the row above. Studies with very similar experimental conditions have been combined, and are represented in one row.

[2] % LTP is expressed as % of baseline measured 30 minutes post-tetanus. %LTP was estimated from graphs in studies where LTP magnitude was not mentioned in the text. In instances where a study conducted multiple experiments under similar conditions, %LTP was taken as the average across experiments.

[3]quantitative changes in presynaptic efficacy were reported in some studies and are shown in brackets where appropriate

[4]GluN receptor = NMDA-receptor mediated component of synaptic response

6.3 Meta-analysis II – Examining the inconsistent role of nitric oxide as a retrograde signal in the literature

In addition to the inconsistencies in which presynaptic enhancements are induced, the failure to link presynaptic changes with postsynaptic GluN receptor activation has cast further doubt as to whether or not the presynaptic terminal is a major locus of LTP expression. Postsynaptic GluN receptor activation has traditionally been thought to be signalled to the presynaptic terminal via the generation of a retrograde signal. Although several putative signals have been proposed (Williams et al., 1989; Fitzsimonds and Poo, 1998; Blundon and Zakharenko, 2008), the most commonly investigated candidate has been, and continues to be, nitric oxide (NO).

During my studies, I have found that although NO can be produced by GluN receptor activation, it is the NO synthesis triggered by L-VGCCs that is necessary for presynaptic potentiation; in this way, NO is a signal for strong postsynaptic depolarization, rather than a signal for postsynaptic GluN receptor activation. The role of NO in LTP, however, remains controversial, principally because some studies fail to find LTP impairments following the inhibition of NO signalling. Much like the presynaptic expression of LTP, the importance of NO looks to be dependent on the stimulus paradigm used to induce LTP. For example, Johnston and Raymond (2013) demonstrated that NO inhibitors only affected LTP induced by multiple trains of theta-burst stimulation, as opposed to a single train, which in their hands failed to enhance presynaptic strength (Johnstone and Raymond, 2013). Consistent with these findings, other studies which also confirm presynaptic enhancements following LTP induction, reliably demonstrate that these changes are abolished by inhibition of NO signalling. Based on these findings, I reasoned that the likelihood that LTP has a NO-

sensitive component of expression will depend on whether it also has a presynaptic component of expression. To test this idea, I looked at studies investigating the effects of NO inhibitors on LTP at Schaffer-collateral synapses; all relevant studies searched on PubMed (search terms: LTP and nitric oxide) were included. Since most of these studies did not specifically monitor presynaptic strength, I looked to see whether, across studies, the sensitivity of LTP to NO inhibitors was correlated with the magnitude of LTP, which I have already shown reflects the likelihood that an enhancement in presynaptic function has occurred post-tetanus (see Figure 1).

I examined a total of 36 experiments across 21 studies (Table II); experiments were divided into NO-sensitive and NO-insensitive, depending on whether NO blockade reduced the expression of LTP. I found that the magnitude of control LTP was 162%±5.5% in NO-sensitive experiments (25/36), but only 136%±8.0% in NO-insensitive experiments (11/36) ($p=0.02$). I also divided experiments based on those reporting 1) strong LTP, as defined as having a magnitude $\geq$180%, which have a high probability (91.67%) of being associated with presynaptic changes (see Figure 1), and 2) those reporting weak LTP (<180%), which are less likely (35.3%) to be associated with presynaptic changes. Although the age and temperature of the preparation, as well as the type and concentration of NO inhibitors varied greatly across experiments (Table II), I found that NO inhibition reduced LTP in 10 of 10 experiments that yielded strong LTP but in only 16 of 26 experiments that yielded weak LTP ($X^2 = 11.08$; $p= 0.0009$). Such findings suggest that the degree to which plasticity is dependent on NO signalling depends on the magnitude, and potentially the locus, of LTP expression. It should be mentioned, however, that independent of its role as a retrograde signal, NO has known effects on postsynaptic signalling. As a result, inhibition of

NO synthesis may have additionally affected postsynaptic plasticity under certain experimental conditions (Williams et al., 1993; Malen and Chapman, 1997; Ko and Kelly, 1999; Wang et al., 2005; Taqatqeh et al., 2009).

There have also been disagreements regarding the effect of exogenous NO on synaptic function. Bohme et al. (1991) first demonstrated that NO donors persistently potentiated synaptic responses; similar effects were later confirmed using NO donors, free NO, and photo-activated NO (Bohme et al., 1991; O'Dell et al., 1991b; Bon et al., 1992; Zhuo et al., 1993; Zhuo et al., 1994; Arancio et al., 1996; Malen and Chapman, 1997; Zhuo et al., 1998; Nikonenko et al., 2003). In contrast, two groups have failed to elicit LTP with NO application (Boulton et al., 1994; Murphy et al., 1994; Murphy and Bliss, 1999). Exogenous NO, therefore, appears to have varied effects on synaptic responses across studies. However, it is important to recognize that, like any transmitter in the nervous system, NO has a diverse repertoire of effects on neuronal function (Garthwaite and Boulton, 1995). As with glutamate, the specific effect of NO at a synapse will very likely depend on 1) the spatiotemporal dynamics and concentration of signalling, 2) the current pattern of neuronal activity and 3) the state of the synapse. For NO, the parameters required for the induction of LTP remain largely unknown and may not always be emulated by the application of exogenous NO, in whatever form. The fact that the vast majority of studies manage to potentiate synaptic responses using exogenous NO (Garthwaite and Boulton, 1995), whilst having little knowledge of the dynamics of endogenous NO signalling, is remarkable in and of itself, and certainly a compelling demonstration that NO signalling has the potential to induce LTP; though, as with glutamate, this potential is likely to be realized only under certain conditions.

Table II. Studies examining the involvement of NO in LTP

Citation[1]	Protocol	%LTP[2]	ΔLTP in NO blockade[3]	Age/Animal	Temp (ºC)	NO inhibitors
(Bohme et al., 1991)	(100@100Hzx2)@0.02Hz	146%	Decreased (9%)	5-6wks SD	32ºC	L-NoArg(0.1µM)
(Schuman and Madison, 1991)	(100@100Hz)x4-5@0.033-0.066Hz	143%	Decreased (0-10%)	2-3wks SD	22ºC	L-NoArg(100µM), L-MeArg(100µM), Hg(100µM)
(O'Dell et al., 1991b)	(100@100Hz)x2@0.05Hz	205%	Decreased (20%)	Age? Guinea pig	24ºC	L-NoArg(50µM), L-MeArg(1000µM intracellular), Hg(20µM)
(Bon et al., 1992)	(100@100Hzx2)@0.02Hz	200%	Decreased (43%)	5-6wks SD	32ºC	L-NoArg (0.1-100nM), Hg (10-100nM)
(Gribkoff and Lum-Ragan, 1992)	(100@100Hz); 50% max intensity	135%	No change (40%)	4-12wks F-344 male rat	32ºC	L-NoArg(50-200µM), NMMA(100µM)
	100@100Hzx2)@0.017Hz; max intensity	190%	Decreased (25%)	4-12wks F-344 male rat	32ºC	L-NoArg(100µM), NMMA(100µM)
(Haley et al., 1992)	(4@100Hz)x10@5Hz	137%	Decreased (2%)	4-6wks SD	31ºC	L-NoArg (10nM-10µM), Hg(100µM)
(Haley et al., 1993)	(25@100Hz)x2@0.2Hz	119%	Decreased (6%)	4-6wks SD	31ºC	L-NoArg (10-1000µM)
	(50@100Hz)x2@0.1Hz	115%	No change (17%)	4-6wks SD	31ºC	L-NoArg (10-1000µM)
	(25@100Hz)x2@0.2Hz; 2x intensity	118%	No Change (15%)	4-6wks SD	31ºC	L-NoArg (10-100µM)
(Kato and Zorumski, 1993)	30@100Hz	109%	Increased (33%)	3-4wks male albino rat	30ºC	L-NoArg (5-100µM); Hg(10µM)
(Chetkovich et al., 1993)	100@100Hzx3@l0.02Hz; 50% max intensity	150%	Decrease (13%)	~4wks SD	32ºC	L-NoArg(100µM)
	100@100Hzx3@l0.02Hz; max intensity	175%	No change (75%)	~4wks SD	32ºC	L-NoArg(100µM)
(Musleh et al., 1993)	(4@100Hz)x10@5Hz	142%	Decrease (-8%)	4-6wks SD	35ºC	L-NoArg(20µM), L-MeArg(100µM), Hg(50µM)
(Williams et al., 1993)	(20@100Hz)x6@0.33Hz	130%	Decrease (0%)	5-7wks SD	24ºC	L-NoArg(100µM) L-NAME(0.1mM), Hg(20µM)
	(20@100Hz)x6@0.33Hz	180%	Decrease (22%)	5-7wks SD	24ºC	L-NoArg(100µM)
	(20@100Hz)x6@0.33Hz + Bicuculline	156%	No change (60%)	5-7wks SD	29ºC	L-NoArg(100µM)
	(20@100Hz)x6@0.33Hz	159%	No change (56%)	16-24wks SD	29ºC	L-NoArg(0.1-1mM), L-NAME(0.1mM), Hg(20µM)

(Nicolarakis et al., 1994)	(50@100Hz)x2@0.1Hz	192%	Decreased (32%)	3-5Wks Wistar	21-23°C	L-NAME(100-300µM)
(Cummings et al., 1994)	(100@100Hz)x4@0.033Hz	150%	Decreased (56%)	2-3wks SD	25-29°C	L-NoArg(100µM)
(O'Dell et al., 1994)	(4@100Hz)x25@5Hz; baseline intensity	125%	Decreased (5%)	Age? Mouse	30°C	L-NoArg(50µM)
	(4@100Hz)x25@5Hz; 50% max intensity	190%	Decreased (60%)		30°C	L-NoArg(50µM),
(Boulton et al., 1995)	100@100Hz	168%	Decreased (32%)	4-6wks? Wistar	24°C	L-NoArg(100µM)
	100@100Hz	180%	Decreased (34%)	4-6wks? Wistar	30°C	L-NoArg(100µM)
(Malen and Chapman, 1997)	900@30Hz	123%	Decreased (3%)	2-20wks SD	32°C	L-NAME(100µM)
	50@100Hz	115%	No change (17%)	2-20wks SD	32°C	L-NAME(100µM)
(Zhuo et al., 1998, 1999)	100@100Hz	163%	Decrease (-6%)	4-6wks SD	28-30°C	L-NoArg(100µM)
	100@100Hzx; 2x baseline duration	210%	Decrease (81%)	4-6wks SD	28-30°C	L-NoArg(100µM)
(Wilson et al., 1999)	10@100Hzx3	138%	Decrease (8%)	8-12wks mouse	29-31°C	L-NoArg(200µM)
	10@100Hzx3; 2x baseline duration	150%	Decrease (40%)	8-12wks mouse	29-31°C	L-NoArg(200µM)
(Ko and Kelly, 1999)	(25@100Hz)x5@0.2Hz	180%	Decrease (25%)	5-8wks SD	32°C	L-NAME(100µM), C-PTIO(30µM), MGD-Fe(75/150µM)
(Bon and Garthwaite, 2003)	100@100Hz	150%	Decrease (25%)	6-8wks SD	30°C	L-NoArg(100µM), L-NIO(100µM)
(Johnstone and Raymond)	((4@100Hz)x10@5Hz)x1	130%	No change (35%)	6-8wks Wistar	~22°C	L-NAME(100µM), cPTIO(40µM)
	((4@100Hz)x10@5Hz)x4@0.003Hz	150%	No change (60%)	6-8wks Wistar	~22°C	
	((4@100Hz)x10@5Hz)x8@0.0033Hz	180%	Decrease (40%)	6-8wks Wistar	~22°C	

[1]Blank spaces represent additional experiments conducted by same study cited in the row above. Studies with very similar experimental conditions have been combined, and are represented in one row.

[2]%LTP is expressed as % of baseline measured 30 minutes post-tetanus. %LTP was estimated from graphs in studies where LTP magnitude was not mentioned in the text. In instances where a study conducted multiple experiments under similar conditions, %LTP was taken as the average across experiments.

[3]%LTP obtained with NO inhibition is included in brackets

SD = Sprague-Dawley

6.4 Conclusion

Discrepancies in the literature have raised doubts over a presynaptic locus of LTP. In this chapter, I argue these discrepancies actually reflect the presence of two mechanistically distinct forms of LTP: one, which is expressed postsynaptically and dependent on Ca^{2+} influx from GluN receptors, and the other, which is expressed presynaptically and dependent on Ca^{2+} influx from L-VGCCs. Experimental protocols that successfully activate L-VGCCs are most likely to recruit a presynaptic component of LTP expression and are also most likely to involve a retrograde signal, such as NO. As research continues to elucidate the mechanistic basis of presynaptic plasticity, one thing is becoming clear: the current, postsynaptic-dependent dogma of LTP needs to change in order to reflect the more comprehensive understanding of synaptic plasticity that is supported by a growing body of literature. There are two sides to the synapse, and both can change.

7. CONCLUSIONS AND IMPLICATIONS OF A NOVEL MECHANISTIC MODEL OF PRESYNAPTIC PLASTICITY

In my thesis, I provide evidence in support of a new model of presynaptic plasticity, one in which changes in transmitter release probability (ΔPr) at active presynaptic terminals are controlled by two dynamically opposed and independent processes that can operate simultaneously (Figure 1). One process is driven by postsynaptic depolarization (ΔV_{post}), which increases Pr via L-type voltage-gated Ca^{2+} channel (L-VGCC) - dependent release of nitric oxide (NO) from neuronal dendrites. That NO is only effective at active presynaptic terminals suggests the requirement of some presynaptic Hebbian detector, whose activation, in addition to requiring the presence of NO, also depends on presynaptic activity, which might be signalled by action potential-triggered Ca^{2+} influx from presynaptic VGCCs. The second process is driven by glutamate release ([glu]), which decreases Pr via activation of presynaptic NMDA receptors (GluN). Net changes in Pr therefore depend on the relative strength of each process, and thus, on the levels of ΔV_{post} and [glu] release that occur during synaptic activity. In this chapter, I discuss the potential theoretical implications of the proposed model. For the sole purpose of aiding discussion, in the sections that follow, I conceptually represent the plasticity process at an active presynaptic terminal with the equation: $\Delta Pr = k_1 \Delta V_{post} - k_2[glu]$, where: 1) ΔV_{post} is the change in the postsynaptic membrane potential following activation of the presynaptic terminal, 2) [glu] is the level of glutamate released following activation of the presynaptic terminal, and 3) k_1 and k_2 are constants of proportionality with units chosen to make the solution of the equation dimensionless (e.g. $k_1 = mV^{-1}$ and $k_2 = mM^{-1}$).

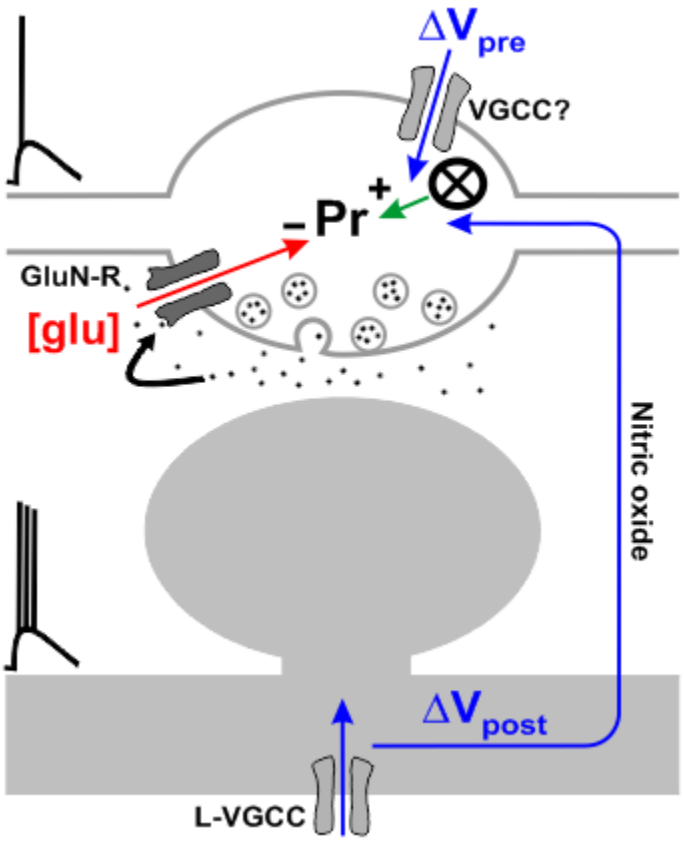


Figure 1. Mechanistic model of presynaptic plasticity. Changes in transmitter release probability (Pr) are driven by two opponent processes that can operate simultaneously. 1) Increases in Pr are driven by a Hebbian detector (represented by a circled cross), activation of which requires presynaptic activity (ΔV_{pre}) to precede postsynaptic depolarization (ΔV_{post}). ΔV_{post} is signalled by retrograde nitric oxide (NO) signalling, the generation of which requires Ca^{2+} influx from postsynaptic L-type voltage-gated Ca^{2+} channels (L-VGCCs). ΔV_{pre} is potentially signalled by Ca^{2+} influx from presynaptic VGCCs. 2) Decreases in Pr are driven by glutamate release ([glu]), via presynaptic GluN receptor (GluN-R) signalling. Net changes in Pr depend on the relative strength of each process.

7.1 Glutamate release as a predictor of postsynaptic depolarization

A model of presynaptic plasticity based on $\Delta Pr = k_1\Delta V_{post} - k_2[glu]$ predicts that, in response to a constant and sustained pattern of synaptic activity, Pr will tend to an equilibrium value (i.e. $\Delta Pr=0$). Consider a scenario in which presynaptic activity is repeatedly paired with a given magnitude of ΔV_{post} at a synapse. In such an instance, Pr will tend to an equilibrium value, in which the depression driven by the [glu] released during presynaptic activity is balanced by potentiation driven by ΔV_{post}, such that further pairings will no longer elicit a change in Pr (i.e. $\Delta Pr=0$ when $k_1\Delta V_{post} = k_2[glu]$). If, for example, presynaptic activity at synapses with initially low release probabilities is paired with high values of ΔV_{post}, then Pr will increase until the [glu] released during presynaptic activity is sufficiently high so as to offset the potentiating effects of a large ΔV_{post}. Similarly, activity at synapses with initially high release probabilities, if paired with low values of ΔV_{post}, will cause decreases in Pr until the [glu] released is sufficiently low so as to offset the small potentiating effects of a small ΔV_{post}.

At equilibrium then, when there is no net change in Pr (i.e. $\Delta Pr = 0$), the levels of [glu] released at a synapse could be used to accurately predict the levels of ΔV_{post} that would accompany presynaptic activity (i.e. $k_2[glu] = k_1\Delta V_{post}$). A given pattern of presynaptic activity that elicits a high level of [glu] release at equilibrium will therefore be very likely to coincide with a large ΔV_{post}. This prediction does not simply reflect the fact that [glu] release at a synapse will elicit greater levels of ΔV_{post}, since the activation of a single synapse alone has minimal influence on postsynaptic activity, but rather reflects how well presynaptic activity has been consistently associated with postsynaptic firing in the past, which in a

physiological context, would require it to be associated with the co-activation of several synapses impinging on the same postsynaptic cell.

7.2 A prediction-error framework of presynaptic plasticity

[Glu] release would only be an accurate predictor of the levels of ΔV_{post} at equilibrium. However, given that network activity is constantly changing with the external environment, the pattern of activity at synapses will inevitably change. Thus, for example, it is possible that activity at a high Pr synapse will no longer be associated with a large ΔV_{post}. However, as soon as there is a discrepancy between the levels of ΔV_{post}, as predicted by [glu] release, and the actual levels of ΔV_{post}, Pr would change in an attempt to minimize future discrepancies; the actual magnitude and direction of this change (ΔPr) would be determined by $\Delta Pr = k_1 \Delta V_{post} - k_2[glu]$. As a result, even in dynamically changing environments, the levels of [glu] release during presynaptic activity would be constantly updated so that it would continue to be a useful predictor of the levels of ΔV_{post} that accompany presynaptic activity.

Such a model of presynaptic plasticity appears to adhere to the principles of prediction error. Prediction error is a highly influential learning theory that has been applied widely in systems-level neuroscience (Schultz and Dickinson, 2000). According to this theory, animals use an internal model of the surroundings to make useful predictions about events in their environments. Learning is driven by a mismatch between predicted and actual outcomes, and serves to update the internal model of the environment so as to improve the accuracy of future predictions. It is therefore interesting to consider that prediction error, which drives learning on the behavioral level, might also drive plasticity at the synaptic

level. Accordingly, the levels of [glu] released by a presynaptic terminal during activity reflects a prediction of the average levels of postsynaptic depolarization to follow. Any mismatch between the predicted ([glu]) and actual (ΔV_{post}) levels of postsynaptic depolarization results in a change in Pr ($\Delta Pr = k_1 \Delta V_{post} - k_2[glu]$), which by altering [glu] release improves the accuracy of future prediction by minimizing synaptic errors. Such a mechanism may provide a synapse-level explanation as to why learning at the behavioral level preferentially occurs in novel and unexpected situations.

7.3 Implications for associative learning

Understanding presynaptic plasticity through a prediction-error framework might help to elucidate the underlying logic of synaptic changes. Long-term potentiation (LTP) is an associative phenomenon since co-active synapses that drive postsynaptic spiking become potentiated. As a result of potentiation, re-activation of even a subset of these synapses can effectively drive spiking in the postsynaptic neuron, and thus can be used to predict the presence of the original input pattern. Such a process is thought to underlie associative learning in animals, in which a given stimulus comes to predict an outcome that it has previously been paired with. The accuracy of prediction, however, depends on the ability for synaptic strength to be constantly adjusted so as to reflect the likelihood that its activation signals the original input pattern, and thus postsynaptic spiking. I argue, based on a prediction-error framework of presynaptic plasticity, that synaptic strength, at least at the presynaptic locus, is constantly adjusted to reflect the likelihood that its activation will be associated with postsynaptic activity. In this way, synapses whose activity becomes associated with postsynaptic firing are potentiated to enhance the sensitivity of pattern

detection. Similarly, synapses whose activity is no longer associated with postsynaptic firing are depressed so as to minimize the likelihood of false detections. As a consequence, since a neuron's activity reflects a summation of activity across its synapses, the likelihood of detecting a pattern of associated input will be accurately reflected by neuronal spiking activity. Moreover, in dynamically changing environments, presynaptic plasticity, by constantly updating presynaptic strength, would ensure that the accuracy of pattern detection is preserved.

7.4 Presynaptic plasticity optimally tunes presynaptic function

It is important to recognize that the total [glu] released by presynaptic activity not only depends on Pr, but also on the pattern of presynaptic spiking. In general, for a given value of Pr, [glu] release will increase with the frequency and number of presynaptic spikes (Dobrunz et al., 1997; Dobrunz and Stevens, 1997; McGuinness et al., 2010). As a consequence, if high frequency bursts of presynaptic stimulation are repeatedly paired with large values of ΔV_{post}, at equilibrium, Pr would be lower than if single presynaptic spikes were used in the pairing instead. This is because presynaptic bursting drives more [glu] release than single spikes (Dobrunz et al., 1997; Dobrunz and Stevens, 1997), which in turn would limit increases in Pr, since: $\Delta Pr = k_1 \Delta V_{post} - k_2[glu]$. There is, however, an advantage of setting Pr low at a synapse in which presynaptic bursts predict strong postsynaptic activity, and setting Pr high at a synapse in which single presynaptic spikes predict strong postsynaptic activity. In both cases, the value of Pr is set to optimize the amount of [glu] released by the very pattern of presynaptic spiking that was actually paired with strong postsynaptic activity; whereas presynaptic bursts are efficient at promoting [glu] release at

low Pr synapses, single spikes are efficient at promoting [glu] release at high Pr synapses (Dobrunz et al., 1997; Dobrunz and Stevens, 1997). Consequently, under the proposed model of presynaptic plasticity ($\Delta Pr = k_1 \Delta V_{post} - k_2[glu]$), Pr is optimally tuned such that the pattern of presynaptic activity that previously coincided with high values of ΔV_{post} is the pattern of activity that comes to most efficiently drive [glu] release. Put another way, presynaptic plasticity, by adjusting Pr, enables [glu] release to be selectively driven by patterns of presynaptic activity that are useful predictors of postsynaptic spiking.

7.5 Both pre- and post- plasticity are required to optimally tune postsynaptic response properties

That [glu] release is both stochastic and dependent on the pattern of presynaptic activity suggests that presynaptic terminals act as dynamic filters of presynaptic information. Low Pr synapses, for example, act as narrowly tuned filters that preferentially transmit information conveyed by bursts of action potentials over that conveyed by single spikes; high Pr synapses, in contrast, are more broadly tuned filters that transmit information conveyed by a variety of presynaptic spiking patterns. The concept of presynaptic filtering becomes considerably useful when one considers that the pattern of presynaptic spiking in and of itself holds useful information about stimuli in the environment. In general, neurons tend to burst at a higher frequency in response to a preferred stimulus, and at a lower frequency in response to less preferred stimuli. For example, neurons in the visual cortex show maximal rates of activity when presented with a bar of one orientation, but then progressively reduce their rate of spiking as the bar is rotated away from the preferred orientation.

That Pr can be used to selectively filter the information content of presynaptic spiking at presynaptic terminals suggests that presynaptic plasticity might play a fundamentally distinct role in synaptic function than postsynaptic plasticity, particularly with respect to controlling the response properties of the postsynaptic neuron. Decreasing Pr at a given synapse would make the postsynaptic neuron responsive only to a narrow bandwidth of presynaptic stimuli that are capable of eliciting high frequency firing in the presynaptic neuron. Increasing Pr, on the other hand, would make the postsynaptic cell responsive to the wide range of stimuli that are able drive any pattern of presynaptic spiking, whether it be single action potentials or high frequency bursts of activity. In contrast to presynaptic changes, postsynaptic changes, rather than acting as a filter of presynaptic activity, would act as a gain control, either amplifying or dampening the contribution of a given synapse to postsynaptic activity, regardless the pattern of presynaptic spiking,

Having a synapse with two modifiable components - a presynaptic filter and a postsynaptic gain controller – could, therefore, be an efficient way of modifying synapses to maximize postsynaptic pattern detection. Presynaptic changes could be used to selectively enable patterns of presynaptic activity that predict postsynaptic spiking to reliably release [glu]. That is, if only high frequency bursts of the presynaptic neuron have any predictive information of postsynaptic spiking, Pr would, according to the proposed model ($\Delta Pr = k_1\Delta V_{post} - k_2[glu]$), be set to low in order to filter out information conveyed by single presynaptic spikes. Postsynaptic changes could then be used to adjust the gain of the synapse in a manner that reflects the extent to which the now filtered presynaptic activity carries predictive information about postsynaptic firing. Synapses where the optimally filtered pattern of presynaptic activity is highly predictive of postsynaptic spiking should

have high postsynaptic efficacies. In contrast, synapses where the optimally filtered pattern of presynaptic activity is poorly predictive of postsynaptic spiking should have low postsynaptic efficacies.

The advantages of having plasticity at both pre- and post- synaptic loci are made apparent in the following example (Figure 2). Consider a synapse at which only high frequency bursts convey highly predictive information of postsynaptic spiking. Under such conditions, it would be optimal to set presynaptic efficacy (Pr) low and postsynaptic efficacy high in order to selectively amplify the transmission of information conveyed by high frequency bursts. If, however, it were only possible to modify the postsynaptic locus, selective enhancements in postsynaptic efficacy would mean that single presynaptic spikes, which at the synapse in question convey irrelevant information, would have a large and disruptive influence on postsynaptic processing by potentially causing spiking of the postsynaptic neuron in response to irrelevant patterns of presynaptic information. In contrast, if only presynaptic changes were possible, although Pr would be set low to filter out the irrelevant information conveyed by single presynaptic spikes, there would be no additional means to further alter synaptic efficacy (whether amplify or dampen) without altering the tuning properties of the synapse. In the case of the synapse in question, the highly predictive value of presynaptic bursts would not be appropriately amplified, which in turn would increase the likelihood of the postsynaptic neuron to fail to respond to relevant patterns of presynaptic information. Moreover, there would be no means of distinguishing potential differences in the predictive value of presynaptic information communicated by high frequency bursts at one synapse from that communicated by high frequency bursts at another synapse, especially if high frequency bursts at each synapse differ in their ability to

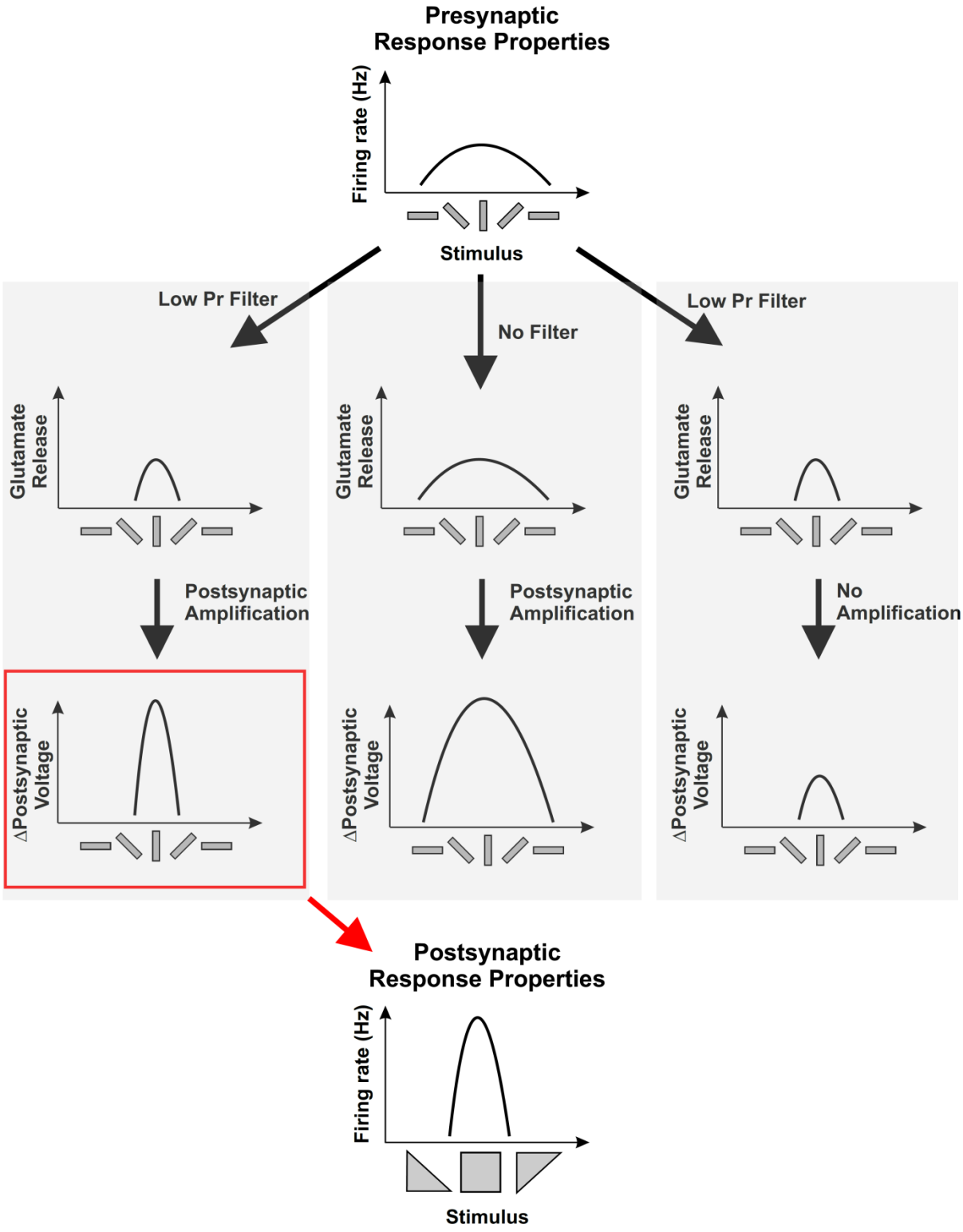

Presynaptic
Response Properties
Firing rate (Hz)
Stimulus
Low Pr Filter
No Filter
Low Pr Filter
Glutamate Release
Glutamate Release
Glutamate Release
Postsynaptic Amplification
Postsynaptic Amplification
No Amplification
ΔPostsynaptic Voltage
ΔPostsynaptic Voltage
ΔPostsynaptic Voltage
Postsynaptic
Response Properties
Firing rate (Hz)
Stimulus

Figure 2. Dual pre- and post- synaptic plasticity optimize postsynaptic pattern detection. Responses of a presynaptic neuron (top graph) and its postsynaptic target (bottom graph) to various stimuli presented in the visual field. Only the presence of the presynaptic neuron's preferred stimulus, a vertical bar, is highly predictive of the postsynaptic neuron's preferred stimulus, a box. Three states are depicted (grey columns) for the synapse mediating communication between the pre- and post- synaptic neuron. In the first grey column, the synapse is depicted in an optimal state. The synapse has a low transmitter release probability (Pr) in order to filter out the effects of low frequency presynaptic spiking generated by non-preferred presynaptic stimuli on postsynaptic processing. The synapse also has a strong postsynaptic efficacy to ensure that the presence of a vertical bar, which is highly predictive of the presence of a box, elicits a strong postsynaptic response. Together, the low presynaptic efficacy and the high postsynaptic efficacy produce a postsynaptic response (red box) that matches the sharp tuning response properties of the postsynaptic neuron. In the second grey column, the synapse is depicted in a suboptimal state, where only a strong postsynaptic amplification is present without a presynaptic filter. Under these conditions, the postsynaptic response mediated by the synapse is broadly tuned and would impede the selectivity of the postsynaptic neuron in responding to boxes in the visual field. In the third grey column, the synapse is depicted in another suboptimal state, where a presynaptic filter is present but without any postsynaptic amplification. Under these conditions, the postsynaptic response generated by the synapse, although appropriately tuned, is weak, and would impede the sensitivity of the postsynaptic neuron to detect boxes in the visual field.

predict postsynaptic spiking. Thus plasticity at only one locus, whether pre- or post-synaptic, greatly restricts the ability for postsynaptic pattern detection. Essentially, presynaptic changes are needed to filter out irrelevant presynaptic information and postsynaptic changes are needed to ensure that the remaining, relevant presynaptic information is appropriately weighted in accordance to how well the information predicts postsynaptic spiking.

7.6 Conclusion

Understanding synaptic plasticity from the same prediction-error framework that is used to describe learning in behaving animals is appealing, especially since it provides an intuitive and simple means of understanding how and when a system should change to optimize its performance, both at the level of the behaving animal, and, I would argue, at the level of the single synapse. Using this framework, in this chapter I have proposed that 1) that presynaptic plasticity optimally tunes Pr at a synapse such that the pattern of presynaptic activity that previously coincided with strong postsynaptic depolarization ($\Delta V_{post)}$ is the pattern of activity that comes to most efficiently drive glutamate release ([glu]) and 2) that this optimal tuning is achieved by minimizing prediction-errors that arise during synaptic activity; that is, the magnitude and direction of changes in Pr are determined by: $\Delta Pr = k_1 \Delta V_{post} - k_2[glu]$. Postsynaptic changes, then, provide an additional degree of freedom by which synaptic weight can be altered without changing the tuning characteristics of the presynaptic terminal. I would therefore argue both forms of plasticity are likely required for optimizing pattern detection in neuronal networks.

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